Structure Elucidation, Biological Activity, and Ecology of Terpene Isocyanides from Phyllidiid species (Nudibranchia) and Their Sponge-preys from The Thousand Islands National Park, Indonesia

(Strukturelle Identifizierung, Biologische Aktivität, und Ökologie von Terpen-Isocyaniden aus Phyllidien (Nudibranchia) und ihren Beuteschwämmen aus Die Thousand Island National Park, Indonesien)

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To my wife and my cute pseudotwin- sons

Erklärung

Hiermit erkläre ich ehrenwörtlich, daß ich die vorliegende Dissertation "**Strukturelle Identifizierung, Biologische Aktivität, und Ökologie von Terpen-Isocyaniden aus Phyllidien (Nudibranchia) und ihre Beuteschwämmen aus Die Thousand Island National Park, Indonesien**" selbständig angefertigt und keine anderen als die angegebenen Quellen und Hilfsmittel verwendet habe.

Diese Dissertation wurde weder in gleicher noch in ähnlicher Form in einem anderen Prüfungsverfahren vorgelegt. Außerdem erkläre ich, daß ich bisher noch keine weitere akademischen Grade erwoben oder zu erweben versucht habe.

Düsseldorf, den 8.11.2005

Yasman

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Yasman
Düsseldorf, Germany

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ZUSAMMENFASSUNG

Anhand von Fütterungsversuchen im Feld und Isolierung von Isocyaniden aus Phyllidien und deren Futterschwämmen wurde die Räuber-Beute Beziehung dieser Organismen untersucht. Das Sammeln von Proben und Beobachtungen im Feld fanden im Korallenriff vor Thousand Islands in Indonesien statt, während die chemischen Untersuchungen am Institut für Pharmazeutische Biologie und Technologie der Heinrich-Heine Universität Düsseldorf durchgeführt wurden.

In dieser Studie wurden Phyllidien ausschließlich auf Schwämmen der Ordnung Halichondria gefunden, insbesondere auf Arten, die kein ektosomales Skelett besitzen. Eine mögliche Erklärung für diese Präferenz ist der geringere Aufwand, der zum Vorverdau der Schwammepidermis erforderlich ist, wodurch der Kontakt des Räubers mit dem Mesohyl der Schwammes schneller erfolgt, d.h. die Nahrungsaufnahme optimiert wird. Interessanterweise gab es in dieser Studie keine Korrelation zwischen der Morphologie des Pharynx der Phyllidien und ihren jeweiligen spezifischen Beuteschwämmen. Von zehn gesammelten Phyllidien waren sechs Arten (*Phyllidia varicosa*, *P. zeylanica*, *P. pustulosa*, *P. krempfi, P. shireenae* und *P. menindie*) Nahrungsspezialisten, die sich von der Schwammart *Axinyssa* spp. (Halichondriidae) ernähren, während drei Arten (*P. lizae*, *P. rudmani* und *P. ocellata*) exklusiv von *Acanthella cavernosa* (Dictyonellidae) und eine Art (*P. elegans*) von *Dragmacidon* sp. (Axinellidae) fressen. Die in dieser Arbeit gesammelten Schwämme wurden vom Autor selbst oder unter Anleitung von Dr. Van Soest (Zoologisches Museum Amsterdam) identifiziert. Die Identifizierung basierte sowohl auf der Art und Größe der ausgewachsenen Spicula als auch auf morphologischen Daten.

Vier Thio- und Isothiocyanat-Sesquiterpene (**1**, **2**, **3**, **5**) und ein diterpenes Isocyanid, 8-isocyano-1(12)-cycloamphilectin (**4**), wurden isoliert. Die neuen Substanzen 9α-thiocyanatopupukeanan (**1**) und 9β-thiocyanatopupukeanan (**2**) haben ein Pupukeanan-Skelett, während der bekannte verwandte Stoff 2-thiocyanatoneopupukeanan (**3**) ein modifiziertes Rückgrat aus Pupukeanan und Epipolasin-A (**5**) ein Epimaalian-Skelett besitzt (Abbildung 1).

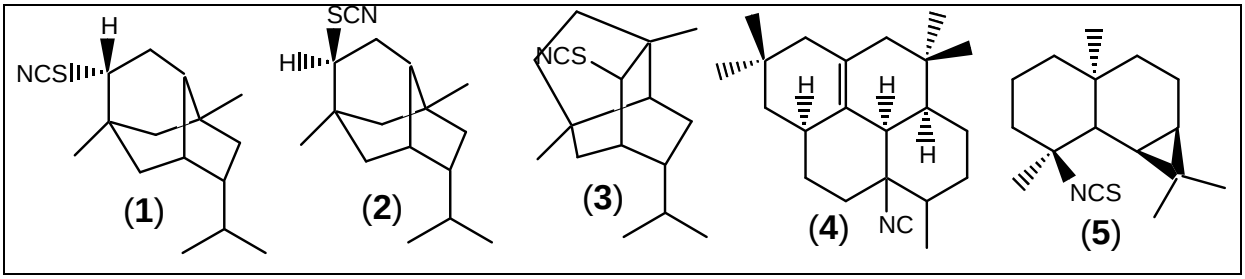


Abbildung 1. Aus Phyllidien und ihren Beuteschwämmen isolierte Terpen-Isocyanide, gesammelt in Indonesien, Thousand Islands.

Die Schwammetabolite in organischen Extrakten der gesammelten Schwammspezies bzw. Schwämme von unterschiedlichen Fundorten variierten sowohl in ihrer relativen Zusammensetzung als auch in der Konzentration der einzelnen Substanzen. Diese Variation könnte auf Unterschiede zwischen

Schwammzellen zurückgeführt werden. Es ist bekannt, dass marine Terpen-Isocyanide als strukturelle Komponenten von Schwamm-Zellmembranen fungieren können (Garson & Simpson, 2004). Die Analyse von Phyllidien und deren Beuteschwämmen zeigten, dass die Anwesenheit von Terpen-Isocyaniden in Räuber und Beute im Hinblick auf die Inhaltsstoff-Verhältnisse nicht immer vergleichbar waren. Dies spricht dafür, dass die Phyllidien die Metabolite ihrer Nahrung nicht nur aufgenommen, sondern selektiv akkumuliert haben.

Untersuchungen zu der organspezifischen Verteilung der isolierten Substanzen in den Phyllidien ergaben, dass die Konzentration der Terpen-Isocyanide in der Verdauungsdrüse im Vergleich zum Mantel und Fuß am höchsten war. Dieses Ergebnis befürwortet die Idee, dass die Verdauungsdrüse das hauptverantwortliche Organ zur Speicherung der über die Nahrung aufgenommenen Metabolite der Nudibranchien ist. Es wird angenommen, dass die Nudibranchien die Metabolite später in den leichter verletzbaren Mantel transportieren. Dies deckt sich mit der in dieser Studie gefundenen höheren Konzentration an aufgenommenen Terpen-Isocyaniden im Mantel im Vergleich zum Fuß.

In der vorliegenden Arbeit zeigten die Terpen-Isocyanide **1**, **2**, **3** und **4** keine signifikante fraßhemmende Aktivität. Die epimere Mischung von 9-thiocyanatopupukeananen (**1** und **2**) stellte sich als toxisch für Salinenkrebse heraus (LC_{50} bei 5 ppm). Bei einer Dosis von 20 µg waren diese Substanzen schwach bis mäßig aktiv gegen *Bacillus subtilis* bzw. *Candida albicans*. Für amphilectene Isocyanide wurde antimikrobielle Aktivität beschrieben, was auf alternative ökologische Funktionen der isolierten Terpen-Isocyanide hindeuten könnte.

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. (Aplysiniae: 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 phenomenons 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, isothiocyanato, and thiocyanato 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 isothiocyanate 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 sugested 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 phylllidiids and/or their sponge-preys.

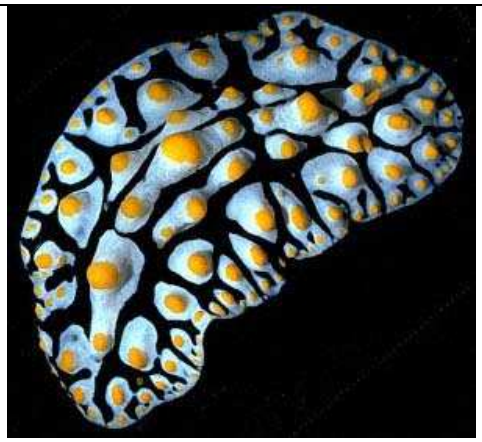
1.3 Short review of current knowledge of phyllidiid species

1.3.1 Taxonomy, biology, and ecology of the family Phyllidiidae

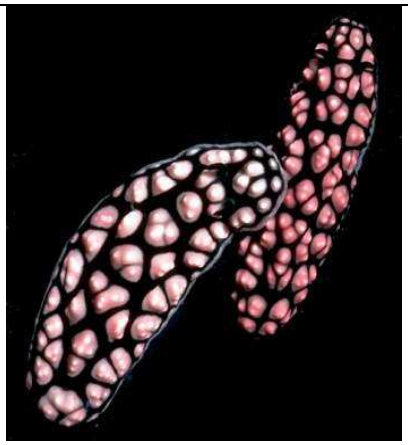
Phyllidiids are one of the most intensive studied nudibranch groups. The taxa have been intensively studied by Brunckhorst (1993), which has greatly influenced phyllidiids's taxonomy. This taxonomy seems to remain unchanged up to the present. Some recent findings were published as new phyllidiid species are still following the existing genera (Yonow, 1996; Valdes & Gosliner, 1999; Perrone, 2000; Fahrner & Schrödl, 2000a, b; Fahrner & Beck, 2000, Valdes & Behrens, 2002). However, some changes may occur in the current status of the taxonomy of the phyllidiid species. The species *P*. *multifaria* which was first described by Yonow (1988) was re-examined by Brunckhorst (1993) and was given the synomym name of *P*. *elegans*. But in 2002, Picton again re-investigated the same species and concluded that *P*. *multifaria* is a valid species. Picton also further commented that the proposed new species, *P*. *schupporum*, (Fahrner & Schroedl, 2000b) is an unnecessary synonym for *P*. *multifaria* [*see* www.seaslugforum.net]. Some specimens of phyllidiid species still remain to be identified [see www.seaslugfroum.net]. In addition, it is believed that there are still some other additional species which have not yet ever been explored. The representative species of each genus in the family of Phyllidiidae is shown in Figure 1.

Phylum: Mollusca
Class: Gastropoda
Subclass: Opisthobranchia
Order: Nudibranchia
Family: Phyllidiidae Rafinesque, 1814
Genus:
- *Phyllidia* Cuvier, 1797
- *Phyllidiella* Berg, 1869
- *Phyllidiopsis* Berg, 1875
- *Fryeria* Gray, 1853
- *Ceratophyllidia* Eliot, 1903
- *Reticuladia* Brunckhorst, 1990

[Source: Brunckhorst, 1993]



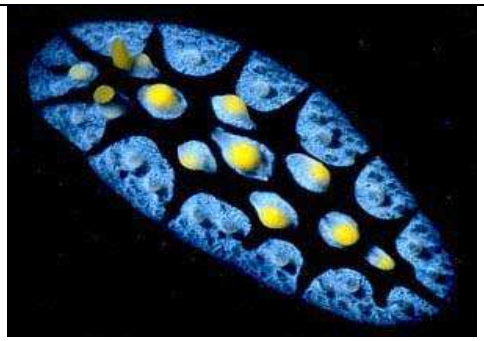
Phyllidia varicosa



Phyllidiella pustulosa

Phyllidiopsis krempfi

Ceratophyllidia papilligera



Fryeria menindie

Reticulia halgerda

Figure 1. Representative species of each genus in the Family of Phyllidiidae [Reproduced from www.seaslugforum.net]

In general, phyllidiid species are tough-bodied, oval, and dorsoventrally flattened nudibranchs. All of them have retractile, lamellate rhinophores. They also have ventrolateral gill leaflets which distinguish them from other dorids. The existing 6 genera of the family Phyllidiidae can be distinguished from each other through their external morphology: oral tentacle, coloration pattern of the mantle, the rhinophore coloration, location of anus, the presence of rhino tubercle, and notal (tubercles) ornament of the mantle. This external morphology, especially the pattern of the mantle and rhinophore, can be used to recognize a particular phyllidiid species. However, a combination with anatomical characteristics must be used for taxonomical studies (Brunckhorst, 1993).

Table 1. Features of external morphology used in distinguishing phyllidiid's genera

Genus Character	*Phyllidia*	*Phyllidiella*	*Phyllidiopsis*	*Fryeria*	*Ceratophyllidia*	*Reticulidia*
Location of anus	dorsal	dorsal	dorsal	Postero-ventral	dorsal	dorsal
Oral tentacle	separate	separate	fused	separate	partly fused	separate
Mantle coloration	Grey blue, yellow to orange, black	pink-sometimes green, black, white to cream	pink, black and blue, white to cream	grey blue, yellow to orange, black	Creamish yellow, cream , and black	orange, white, and black
Rhinophore coloration	cream to yellow	black	various color: bi- or multicolored	cream to yellow	white to cream	orange
Notal ornament	Tubercu-late (may form ridge)	Tubercu-late (may form ridge)	Tuberculate (may form ridge)	Tubercu-late	Stalked papillae (no tubercles)	Smooth ridges (no tubercles)
Rhino-tubercle	present	absent	absent	present	absent	absent

(Reproduced from Brunckhorst, 1993)

Phyllidiids are predators and specialized on sponges. They possess oral glands, pharyngeal retractor muscles, but lack radula or other buccal hard part (e.g. jaws). They can extend externally the pharyngeal bulb on- or into the sponge (Brunckhorst, 1993; personal observations). The phyllidiids's foregut has been proposed to be a functional device that sucks the predigestive food material from sponges. Brunckhorst (1993) assumed that the phyllidiids's oral glands can function as a predigesting tool for suctorial feeding. Therefore phyllidiid species are described as suctorial sponge feeders (Brunckhorst, 1993). While he did not check through the digestive tract of phyllidiids, he found that the feces do not contain any spicules from the sponge-prey.

Present knowledge of the ecology of phyllidiids species is still very limited. The lack of ecological informations does not merely reflect the rarity of observations of phyllidiid species which might be due to either the difficulties in making prolonged underwater observation or the difficulties in keeping phyllidiids in aquarium. From his long field observations, Brunckhorst reported that the rarity of finding large numbers of phyllidiids together also made the record of feeding, predation, or spawning of phyllidiids difficult. Karuso (1987) suggested that a close collaboration among biologists, pharmacists, and chemists may help to solve the limited information on the phyllidiids's ecology, feeding behaviour, food preference, mating, predation, larval biology, etc.

Generally, nudibranch defense mechanisms can be classified into three basic categories: behaviour, morphology, or chemistry (Todd, 1981). Even though nudibranchs are believed to produce an offensive compound when disturbed (Thompson, 1960a&b; Johannes, 1963), their primary defense seems to avoid detection by potential predators (Todd, 1981). Phyllidiid nudibranchs, however, make no attempt to conceal their being soft-bodied (Faulkner & Ghiselin, 1983; Karuso, 1987). Even species of *Phyllidia* tend to show aposomatic or warning coloration. Brunckhorst (1991) reported that phyllidiid species demonstrate consistent behaviour with their apparent warning coloration. None of the phyllidiids has been reported to have any known predators. It is assumed that some coloration patterns of phyllidiid species are mimicked by other nudibranch species or even by other marine organisms, e.g. marine flatworms and sea cucumber so as not to be attacked by predators [*see* www.seaslugforum.net].

The geographic distribution of phyllidiid species has been described by Brunckhorst (1993). Phyllidiids mainly occur in the tropical Indo-Pacific and some species can also be found in the tropical Atlantic region. Few phyllidiid species are reported to be endemic in the Red Sea, Mediterranean, and Indian Ocean. Some current records (www.seaslugfroum.net), however, extend the known range of phyllidiid species. Re-elucidation of biographic pattern of phyllidiids is apparently an interesting project to complete the current knowledge on the family Phyllidiidae.

1.3.2 Isolated compounds from phyllidiid species

The offensive mechanism of phyllidiid species were first recorded for *Phyllidia varicosa* where specimens of the species were placed together with lobsters in an aquarium and after 30 minutes, the death of the latter was observed (Johannes, 1963). It was later characterized that 9-isocyanopupukeanane, which was sequestered from its sponge-prey *Hymeniacidon* sp., was the metabolite responsible for this toxic response (Burreson *et al*., 1975a). Subsequently, this group again successfully isolated 2-isocyanopupukeanane from the same species (Hagadone *et al*., 1979). Since then phyllidiid species have received full attention from many chemists, biologists, as well as pharmacists.

To date, 36 isocyanide compounds have been isolated from phyllidiids by many scientists from different research groups (Manzo *et al*., 2003; *see* Garson *et al*, 2000, Garson & Simpson, 2004). So far, these studies coincidently concentrated on two phyllidiid species: *Phyllidia varicosa* and *Phyllidiella pustulosa* (Burreson *et al*., 1975a; Hagadone *et al*., 1979; Gulavita *et al*., 1986; Fusetani *et al*., 1991; Kassuehlke *et al*., 1991; Okino *et al*., 1996a; Dumdei *et al*., 1997; Simpson *et al*., 1997; Hirota *et al*., 1998; Yasman *et al*., 2003, Manzo *et al*., 2004). The total isolated terpene cyanides from phyllidiid species are shown in Table 2; whereas some structures of representative compounds are shown in Figure 2.

Table 2. Cyanide terpenes and related metabolites isolated from phyllidiids

Phyllidiids and metabolites	Sponge-prey	Literatures
Phyllidia sp. 3-isocyanotheonellin	not known	Gulavita *et al*., 1986
Phyllidia bourgini * 9-isocyanopupukeanane 9-epi-isocyanopupukeanane	not known	Fusetani *et al*., 1990
Phyllidiopsis krempfi 10-isocyano-4-cadinene 10α-isocyano-4-amorphene	not known	Okino *et al*., 1996a
Phyllidia ocellata Cavernothiocyanate 10α-isocyano-4-amorphene	*Acanthella* cf. *cavernosa*	Fusetani *et al*., 1992; Okino *et al*., 1996a
Phyllidia pustulosa ** 3-isocyanotheonellin 4α-isocyanogorgon-11-ene 4α-isothiocyanatogorgon-11-ene 4α-formamidogorgon-11-ene 11-isocyano-7β-*H*-eudesm-5-ene 11-isothicyanato-7β-*H*-eudesm-5-ene Axisonitrile-3 Axisothiocyanate 10-epi-axisonitrile-3 4-thiocyanatoneopupukeanane 2-thiocyanatoneopupukeanane 9-isocyanopupukeanane 9-epi-isocyanopupukeanane 2-isocyanoallopupukeanane 10-isocyano-4-cadinene 10-isocyano-5-cadinen-4-ol 3-isocyanobisabolene-9, 10-diene 7-isothiocyanato-7,8dihydro-α-bisabolene 4α-isocyano-9-amorphene Axisonitrile-2	not known	Fusetani *et al*., 1991; Kassuehlke *et al*., 1991; Okino *et al*., 1996a;
Axisonitrile-3 Axisothiocyanate	*A*. *cavernosa*	Dumdei *et al*., 1997

Phyllidiella pustulosa		
9-isothiocyanatopupukeanane	*Axinyssa* n.sp.	Simpson *et. al*., 1997
2-thiocyanatoneopupukeanane		
9-isocyanaopupukeanane		
Epipolasin-A		
10-isothiocyanato-4-cadinene	*Phakellia carduus*	Wright, 2003
Axisonitrile-3		
Kalihinene	not known	Manzo *et al*., 2004
Amphilectene		
Kalihinol-A		
Kalihinol-E		
3-isocyanotheonellin		
additional 3 new compounds		
Phyllidia pulitzeri ***	*Axinella cannabina*	Cimino *et al*., 1982
Axisonitrile-1		
Phyllidia varicosa	not known	Kassuehlke *et al*., 1991;
4α-isocyanogorgon-11-ene		Okino *et al*., 1996a
4α-formamidogorgon-11-ene		
10-isocyano-4-cadinene		
2-isocyanotrachyopsane		
2-isocyanopupukeanane	*Hymeniacidon* sp.	Burreson *et al*., 1975;
9-isocyanopupukeanane		Hagadone *et al*., 1979
9α-isothiocyanatopupukeanane	*Axinyssa* cf. *aculeata*	Yasman *et al*., 2003
9β-isothiocyanatopupukeanane		
Phyllidia coelestis	not known	Manzo *et al*., 2003
8,15-diisocyano-11,(20)-amphilectene		
7-epi-isocyanoamphilecta-11,(20),15-diene		
7-epi-isocyanoamphilecta-11,14-diene		
7-epi-isocyanoamphilecta-1,14-diene		
Reticulidia fungia	not known	Tanaka & Higa, 1999
Reticulidin A		
Reticulidin		
Reticulidin B		

Note: * re-identified as *Phyllidiella rosans*

** re-identified as *Phyllidiella pustulosa* as

*** re-identified as *Phyllidia flava* (source: Brunckhorst, 1993)

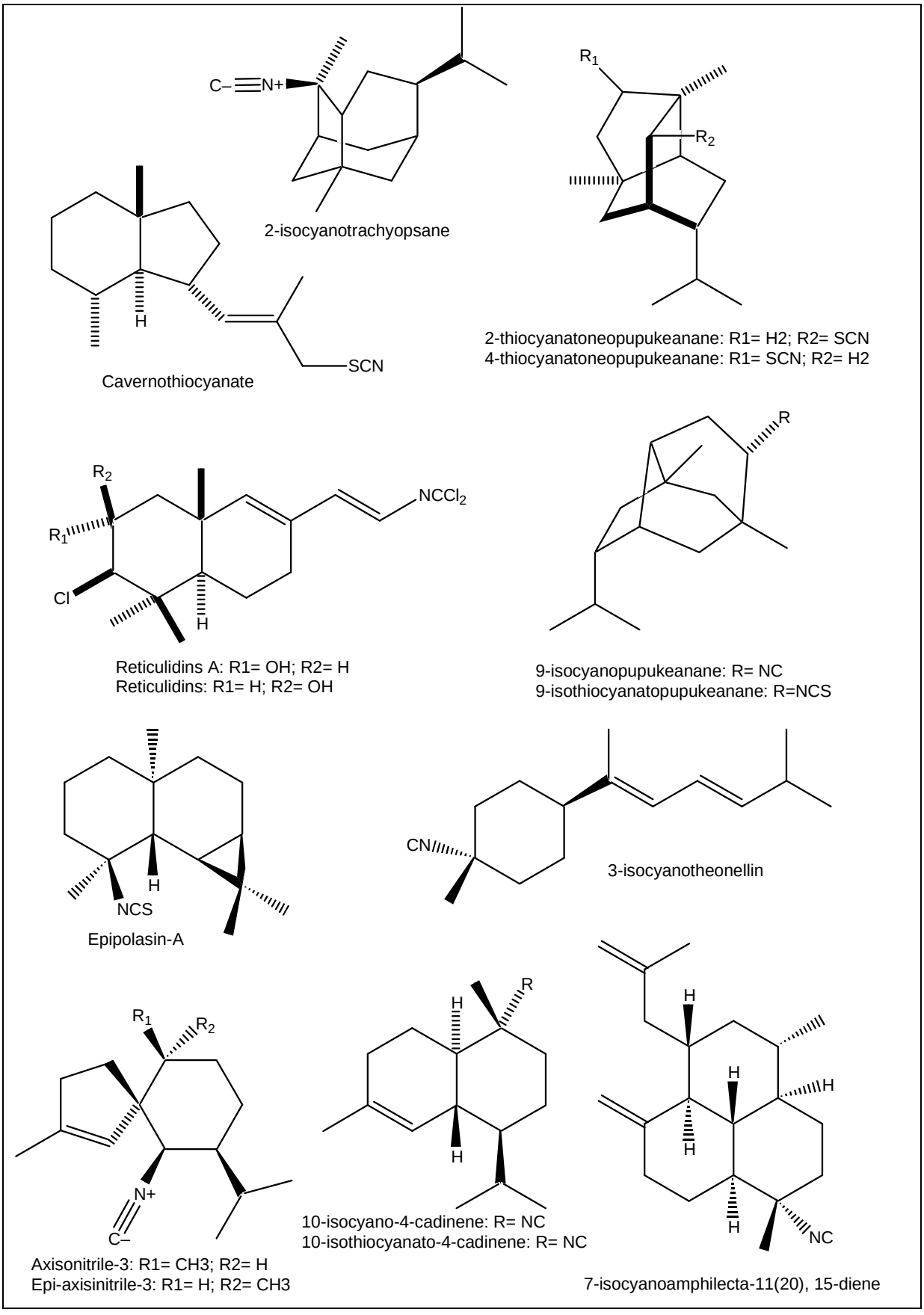


Figure 2. Some isolated isocyanide compounds from phyllidiid species.

1.3.3 Biosynthesis of isocyanide compounds in phyllidiid species

The selective accumulation of a chemical defense agent in the phyllidiid diet was first established by the group of Scheuer (Burreson *et al*., 1975a; Hagadone *et al*., 1979). Garson's group, however, was able to obtain definitive evidence for dietary transfer of sponge metabolites by carrying out laboratory experiments in which phyllidiid *Phyllidiella pustulosa* was allowed to feed on ^{14}C labeled sponge, *Acanthella cavernosa* (Dumdei *et al*., 1997). At the end of the experiments, they found out that phyllidiid metabolites were all radioactive. Further experiment of direct injection of either ^{14}C cyanide or ^{14}C thiocyanate into the digestive gland of phyllidiid *Phyllidiella pustulosa* implied that phyllidiid species was unable to use cyanide/thiocyanide directly in their biosynthetic processes.

1.4 Short review of the Phylum Porifera (sponges)

1.4.1 Definition and biology of sponges

Phylum Porifera (sponges) are metazoans that are united by the unique possesion of choanocytes chambers, a system of afferent and efferent canals with external pores, lacking a tissue grade of construction but having a highly mobile population of cells capable of totipotency, and possessing siliceous or calcitic spicules in many (but not all) species (Hooper *et al*., 2002). Some sponges are freshwater invertebrates but predominantly they are marine species living from the intertidal to the deepest seas (Hooper & Van Soest, 2002).

Sponges possess inhalant and exhalant pores connected by chambers lined by choanocytes. The external surface and the canals are lined by pinacocytes (exo- and endopinacocytes, respectively). Water is inhaled through small pores (10-100µm in diameter), tranvesing the afferent canals towards the choanocyte chamber, and is expelled through efferent canals and the larger exhalant osculum. Water currents are unidirectional, maintained by an active beating within chambers. Food particles and oxygen are removed from the water by various cells, including the choanocytes. Other cells, including archaecytes, are instrumental in transporting these respiratory and dietary products throughout the sponge body, in addition to other functions (see Figure 3). Cells are highly mobile, such as those that move freely within an extra

cellular matrix made of fibrils of collagen (the mesohyl): archaeocytes, collencytes, spiculocytes, spongocytes, gylcocytes, cell with inclusions, etc. Most of the cells, especially the archaeocytes, have an ability to continually evolve into several other cell types as required by the individual organism (totipotency), which provide the sponge with plasticity for its organization. Firmness of the sponge body is provided by collagen fibrils of the mesophyl, spongin fibres, and an inorganic skeleton consisting of various supporting mineral elements composed of either $CaCO_3$ or SiO_2 material. Articulated and hyper calcified skeletons are absent in most recent taxa, but were much more prevalent in fossil faunas. These organic and inorganic materials are manufactured or otherwise engineered by various types of cells. Sponge have free-swimming or creeping larvae, although most groups have considerable means of asexual propagation, and all have extensive regenerative power that appear to be vital for sustaining the local population (Hooper *et al*., 2002).

Four sponge classes are currently recognized. Recent sponge species belong to three classes: Hexactinellida (mostly deep water), Demonspongiae, and Calcarea. All the species belonging to the class Archaeocyatha is now presumed extinct (Hooper *et al*., 2002).

Class Demonspongiae includes about 85% of all described recent species with around 15 orders, 88 families, 500 valid genera, and about 6000 valid species. This estimate is probably conservative as it largely neglects the grossly under-sampled and under-studied encrusting, cryptic, sciaphilic and other small taxa that pervade the many crowded marine communities, such as the coral reefs. Within Demonspongiae three subclasses are recognized: Homoscleromorpha, Tetractinomorpha, and Ceractinomorpha. Orders included are: (1) subclass Homoscleromorpha: Homosclerophorida; (2) subclass Tetractinomorpha: Astrophorida, Chondrosida, Hadromerida, Lithistida, Spirophorida; (3) subclass Ceractinomorpha: Agelasida, Dendroceratida, Dictyoceratida, Halichondrida, Halisarcida, Haplosclerida, Poecilosclerida, Verongida, and Verticillitida (Hooper & Van Soest, 2002).

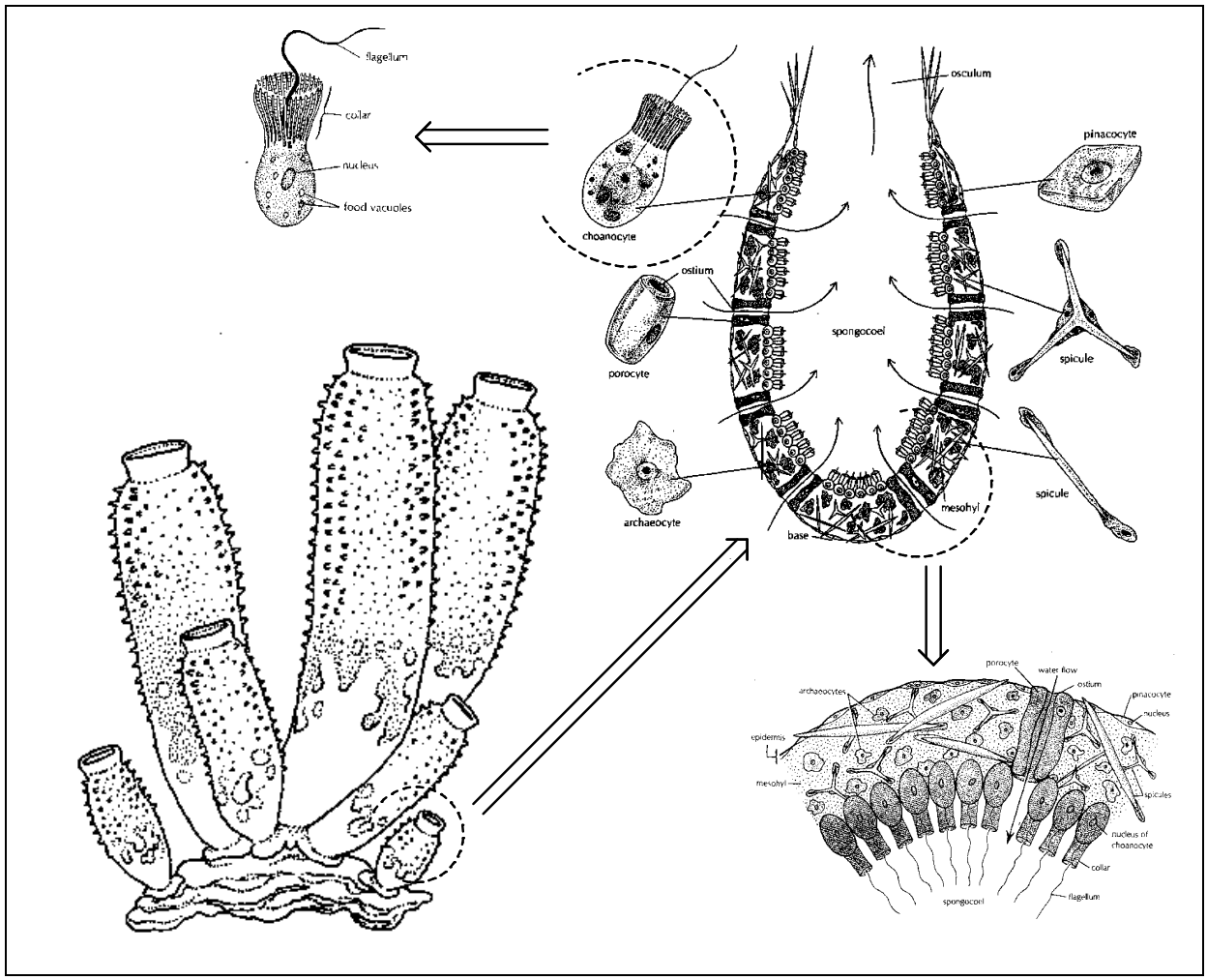
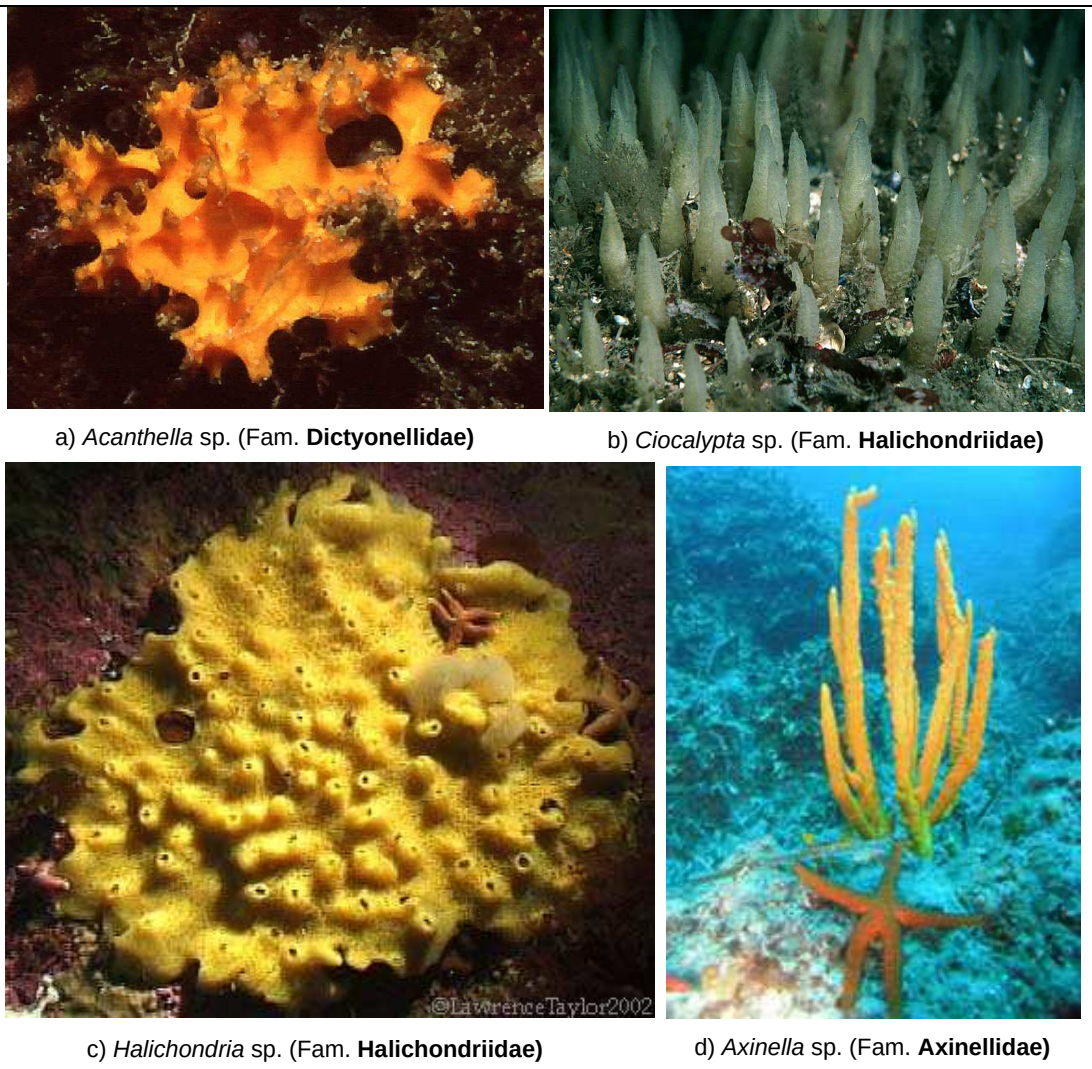


Figure 3. Detail illustration of the body wall of a sponge [Reproduced from Pechenik, 2000]

1.4.2 Sponges containing isocyanide metabolites and related compounds

The existence of sesquiterpene isocyanides was first reported from the Mediterranean sponge *Axinella cannabina* (Cafieri *et al*., 1973), closely followed by isocyanide metabolites from the Mediterranean sponge *Acanthella acuta* by Minale *et al*. (1974) and the Hawaiian sponge *Halichondria* sp. and *Hymeniacidon* sp. by Burreson *et al*. (1974; 1975a). All those sponges belong to the order Halichondrida which are very well known as producers of compounds containing the isocyanide functionality and related compounds from marine ecosystem (*see* Garson *et al*., 2000, Garson & Simpson, 2004). Representative sponge species in the order Halichondrida are shown in Figure 4.



a) *Acanthella* sp. (Fam. **Dictyonellidae)**

b) *Ciocalypta* sp. (Fam. **Halichondriidae)**

c) *Halichondria* sp. (Fam. **Halichondriidae)**

d) *Axinella* sp. (Fam. **Axinellidae)**

Figure 4. Representative sponge species and its life form in the order Halichondrida: a. bushy; b. fistulose; c. encrusting; d. arborescent.

1.4.2.1 Sponges of the family Axinellidae

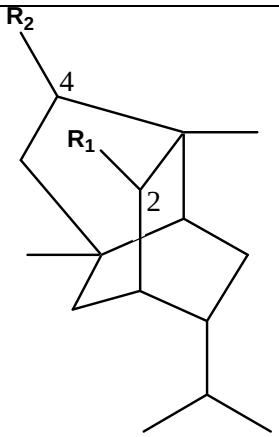
Phylum: Porifera
Class: Demospongiae
Subclass: Tetractinomorpha
Order: Halichondrida
Family: Axinellidae
[Source: Van Soest & Hooper, 2002]

The family Axinellidae was formerly classified under the order of Axinellida. In 1990, this family belongs to a redefined order Halichondrida. The synamorphy that

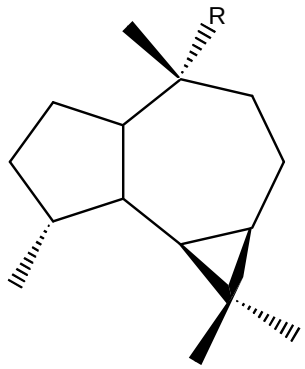
proposed the Axinellidae within order Halichondrida is the presence of an axially condensed and extra-axial plumoreticulate choanosomal skeleton (Belinda & Hooper, 2002).

The only sponge species of the genus *Axinella* which was very intensively studied with regard to the presence of isocyanide metabolites is *Axinella cannabina*. Cafieri *et al*. (1973) who first initiated a chemical study of the species successfully isolated axisonitrile-1 and axisothiocyanate. Subsequently, this group also reported axisonitrile-2, axisonitrile-3, axisothiocyanate-2, axisothiocyanate-3, axamide-1, axamide-2, axamide-3, and some minor sesquiterpene isocyanide from the same species (Fattorusso *et al*., 1974; 1975, Di Blasio *et al*., 1976, Adinolfi *et al*., 1977, Lengo *et al*., 1977; 1979). From a further investigation of the extracts from the same sponge species (*A*. *cannabina*), fourteen additional sesquiterpene isocyanide were also described (Ciminiello *et al*., 1984; 1985; 1986; 1987).

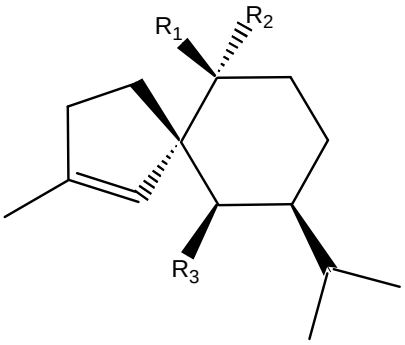
Another sponge species of the family Axinellidae which was known to produce isocyanide compounds is *Phycopsis terpnis*. From this Okinawan marine sponge, 2- and 4-thiocyanatoneopupukeanane were isolated (Pham *et al*., 1991). Subsequently fifteen diterpenes containing isocyanate, isothiocyanate, and isonitrile functionalities were isolated from the sponge *Cymbastela hooperi* (König *et al*., 1996); whereas 15 diterpene isonitrile were also isolated from the sponge *Phakellia pucherrima* (Wolf & Schmitz, 1998). More recently, 10-isothiocyanato-4-cadinene and axisonitrile-3 were isolated from the sponge-prey of phyllidiid *P*. *pustulosa*, *Phakellia carduus* (Wright, 2003). Some representative isolated compounds from the family Axinellidae are shown in Figure 5.



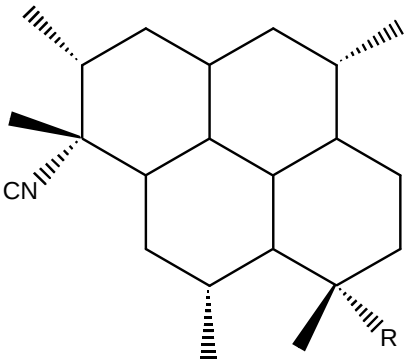
R1 = SCN, R2 = H: 2-thiocyanatoneopupukeanane
R1 = H, R2 = SCN: 4-thiocyanatoneopupukeanane



R = NC: 10α-isocyanoalloaromadendrane
R = NCS: 10α-isothiocyanoalloaromadendrane
R = NHCHO: 10α-formamidoalloaromadendrane



R1 = CH3, R2 = H, R3 = NC: Axisonitrile-3
R1 = H, R2 = CH3, R3 = NC: Epi-axisonitrile-3
R1 = CH3, R2 = H, R3 = NCS: Axisothiocyanate-3
R1 = CH3, R2 = H, R3 = NHCHO: Axamide-3



R = NC: 7,20-diisocyanoisocycloamphilectane
R = NCS: 20-isocyano-7-isothiocyanatoisocycloamphilectane
R = NHCHO: 20-isocyano-7-isocyanatoisocycloamphilectane

Figure 5. Representative isolated compounds from sponge of the family Axinellidae

1.4.2.2 Sponges of the family Dictyonellidae

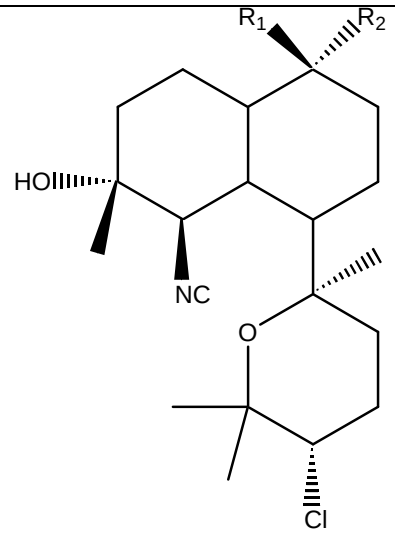
Phylum: Porifera
Class: Demospongiae
Subclass: Tetractinomorpha
Order: Halichondrida
Family: Dictyonellidae
[Source: Van Soest & Hooper, 2002]

The family Diytyonellidae was only recently erected in an attempt to rearrange the genera assigned to the former orders Axinellida and Halichondrida, which was necessary because of the increased awareness that these groups as then understood were clearly polyphyletic (Van Soest *et al*., 2002).

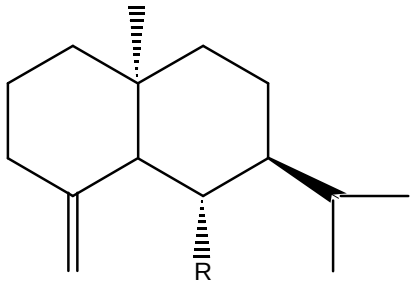
The presence of sesquiterpene isocyanides from the family Dictyonellida was first reported from the Mediterranean sponge *Acanthella acuta* by Minale *et al*. in 1974. Subsequently, three additional isocyanide-isothiocyanate pairs have been isolated from the same species by another group (Mayol *et al*., 1987; Ciminiello *et al*., 1987) and very closely followed by the finding of 1-isocyanoaromadendrane (Braekman, *et al*., 1987). In the same year, the group of Scheuer isolated eleven diterpene isocyanide from the sponge *Acanthella* spp. (Chang *et al*., 1984; 1987). One year later, Capon *et al*. (1988) have also isolated two new sesquiterpene isothiocyanates from *A*. *pulcherrima*, another sponge species of the genus *Acanthella*. In the early 1990's, Fusetani *et al*. (1990) isolated diterpene isonitriles, kalihinene and isokalihinol B, from the sponge *A*. *klethra*; whereas the group of Sticher isolated two new sesquiterpene isocyanides along with five known isocyanide metabolites from the same sponge species (Angerhofer *et al*., 1992; König *et al*., 1992). More recently, Burgoyne *et al*. (1993) isolated acanthenes A to C from unidentified species of the genus *Acanthella*.

The most intensively studied sponge in genus *Acanthella*, however, is *A*. *cavernosa*. This sponge species was first studied by Omar *et al*. (1988) who successfully isolated 4 diterpene isonitriles: kalihinol A, F, X, and Isokalihinol F. From the same species, Alvi *et al*. (1991) isolated kalihinol Y, J, and I in addition to kalihinol X. In 1994, Braekman *et al*. (1994) isolated the first two new kalihinenes: kalihinene A and B, while Crews's group isolated another type of diterpene

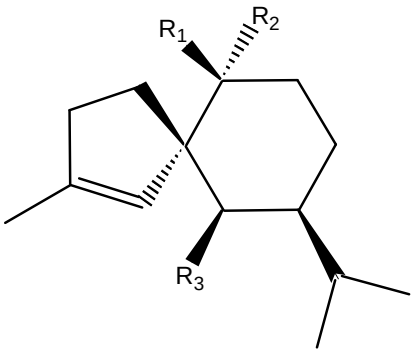
isocyanides from the sponge *A*. *cavernosa* collected from Fijian water. Eight new kalihinene types of metabolites were isolated along with the previously known kalihinol A and isokalihinol F (Rodriguez *et al*., 1994); Meanwhile Trimurtulu & Faulkner (1994) isolated another new type of diterpene isocyanide, kalihipyran, along with five other kalihinene compounds together with isokalihinol B. More recently, the group of Fusetani has isolated additional kalihinene diterpenes (kalihinene X, Y, and Z) and two new kalihipyran formamides from the Japanese sponge *A*. *cavernosa*. Of the isolated diterpene isonitriles, only six sesquiterpene containing isocyanide and thiocyanate functionalities were isolated from the sponge-prey of phyllidiid *P*. *ocellata*, *A*. *cavernosa*, collected off Hachijo-jima Island, Japan (Fusetani *et al*., 1992).



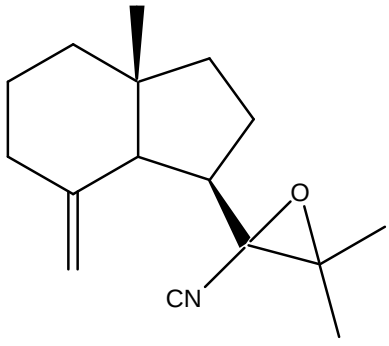
R1 = NC, R2 = Me: Kalihinol A
R1 = Me, R2 = NCS: Kalihinol X
R1 = Me, R2 = NC: Kalihinol Z



R = NC: 6α-isocyano-5α-H, 7α-H, eudesm-4(14)-ene
R = NCS: 6α-isothiocyano-5α-H, 7α-H, eudesm-4(14)-ene
R = NHCHO: 6α-formamido-5α-H, 7α-H, eudesm-4(14)-ene



R1 = CH3, R2 = H, R3 = NC: Axisonitrile-3
R1 = CH3, R2 = H, R3 = NCS: Axisothiocyanate-3



Cavernoisonitrile

Figure 6. Selected compounds from the family Dictyonellidae.

1.4.2.3 Sponges of the family Halichondriidae

Phylum: Porifera
Class: Demospongiae
Subclass: Tetractinomorpha
Order: Halichondrida
Family: Halichondriidae
[Source: Van Soest & Hooper, 2002]

Scheuer's group was the first to report he presence of sesquiterpene isocyanides from sponges of the family Halichondriidae. The Hawaiian marine sponge *Halichondria* sp. was described to contain amorphane isocyanide and formamide sesquiterpenes as well as diterpene isonitriles (Burreson & Scheuer, 1974; Burreson *et al*., 1974; 1975b). Subsequently, Scheuer also isolated 9- and 2-isocyanopupukeanane from the sponge-prey of phyllidiid *P*. *varicosa*, *Ciocalypta* sp. (Burreson *et al*., 1975a). Over a decade later, Faulkner also isolated terpene isocyanides and formamide from the Palauan sponge *Halichondria* sp. and *H*. cf. *lendenfeldi*, as well as from the Ponape sponge *Halichondria* sp. (Sullivan *et al*., 1986; Molinski *et al*., 1987; Kassuehlke *et al*., 1991). Subsequently, Alvi *et al*. (1991) isolated axisonitrile-3 from the sponge *Topsentia* sp.

Axinyssa is the most intensively studied sponge species of the family Halichondriidae and was reported to contain terpene isocyanides. This study was initially and intensively done by Faulkner who isolated various isocyanide terpenes from the sponge *Axinyssa* spp. collected in Guam, Palau, and Pohnpei (Marcus *et al*., 1989; He *et al*., 1989; 1992; Compagnone & Faulkner, 1995), which was closely followed by Alvi *et al*. (1991) who isolated 10-isothiocyanato-4-amorphene, 4-isothiocyanato-9-amorphene, 10-isothiocyanato-4,6-amorphadiene, and 10-isothiocyanato-5-amorphene-4-ol from the Fijian sponge *A*. *fenestratus*. Another intensive study was done by the group of Garson who isolated some isocyanide compounds and was able to propose the biological pathways of sesquiterpene isocyanides in the sponge *Axinyssa* n.sp. (Simpson *et al*., 1997; Simpson & Garson, 1998; Simpson & Garson, 2001). More recently, Li *et al*. (1998; 1999) have isolated 4α-isocyanogorgon-11-ene, 4α-isocyanatogorgon-11-ene, and 4α-formamidogorgon-11-ene as well as 3-formamidotheonellin and three other congeners from the

Micronesian marine sponge *A*. *terpnis* and *Axinyssa* sp., respectively; meanwhile Petrichtcheva *et al*. (2002) isolated three new nitrogenous eudasmane-type terpenes from the Caribbean sponge *A*. *ambrosia*. Some compounds from the family Halichondridae are shown in Figure 7.

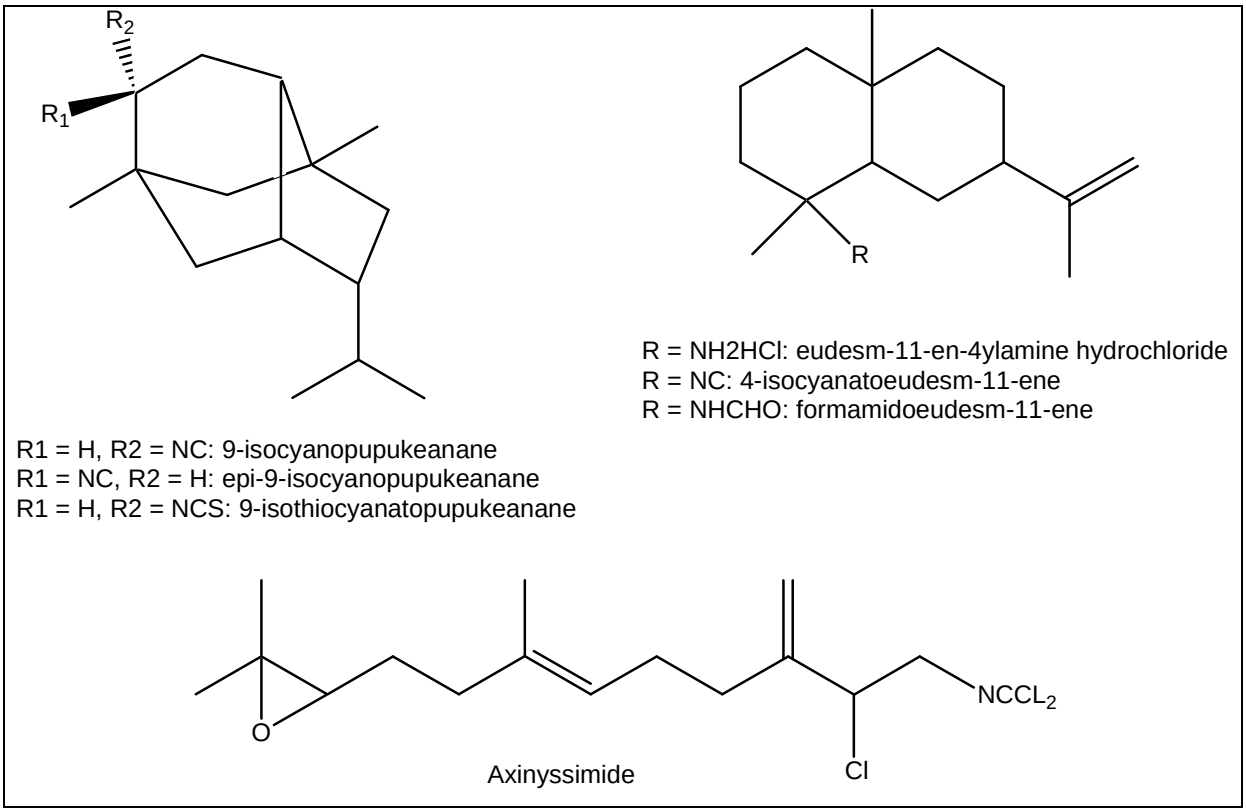


Figure 7. Some selected compounds from the family Halichondridae

1.4.2.4 Other sponges not belonging to the order Halichondrida

Only a few species of sponges that do not belong to the order Halichondrida have been reported to contain isocyanide compounds. It was first reported by Kazlauskas *et al*. (1980) who isolated 6 diterpene isocyanides from the marine sponge *Haliclona* sp. (as *Adocia* sp.) which belongs to the family Chalinidae (suborder Haplosclerina: order Haplosclerida). In 1984, Nakamura *et al*. isolated isocyanatotheonellin and formamidotheonellin from the Okinawan sponge *Theonella* cf. *swinhoei* (Order Lithistida: Fam. Theonellidae). A series of isocyanide and isothiocyanate amphilectenes was also isolated from the Caribbean sponge *Cribochalina* sp. (fam. Niphatidae, sub order Haplosclerina, order Haplosclerida) (Ciavatta *et al*., 1999);

whereas Hamann & Scheuer (1991) isolated cyanopuupehenol from a sponge of the order Verongida.

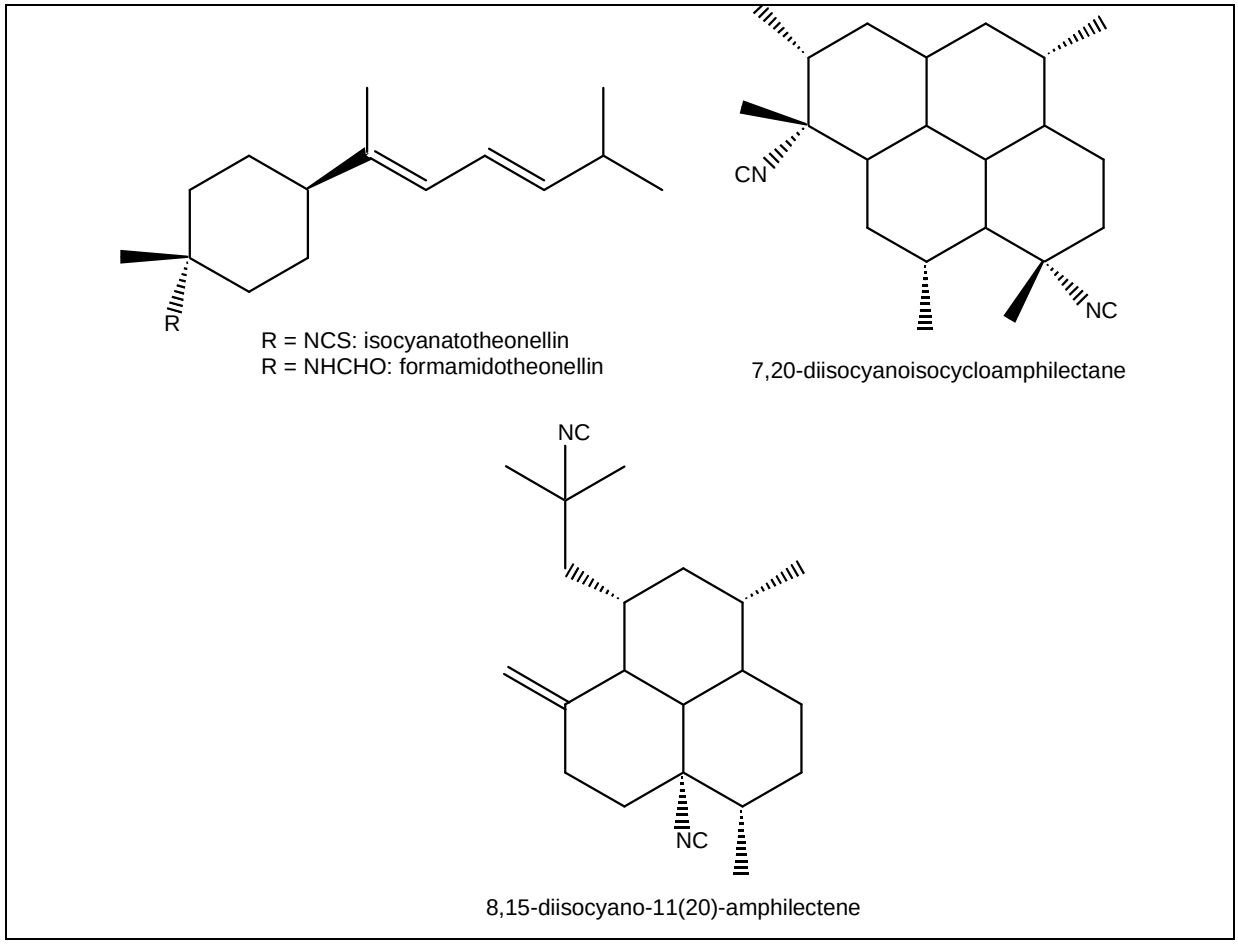


Figure 8. Isolated compounds from the sponge *Theonella swinhoei* and *Adocia* sp.

1.4.3 Biosynthesis of the isocyanide group in marine sponges

Garson's research was the first to show that the isocyanide groups of diisocyanoadociane are derived from inorganic cyanide through incorporation studies done on the Great Barrier Reef sponge *Amphimedon terpenensis*. The ^{14}C-label was proven to be specifically associated with the isocyanide carbons through stepwise hydrolytic degradation to the corresponding formamide, and then to its amine (Garson, 1986; Simpson and Garson, 1999). Subsequently, studies by Karuso and Scheuer (1989) confirmed that ^{14}C-cyanide is a precursor for 2-isocyanopupukeanane in a Hawaiian *Ciocalypta* sp. and the diterpene kalihinol F in *Acanthella* sp. from Guam.

Regarding the biosynthesis of marine isothiocyanates, the group of Garson has established that thiocyanate ion was a precursor of the isothiocyanate moiety in axisothiocyanate-3 isolated from Australian specimens of *A*. *cavernosa*. Inorganic cyanide was also a precursor to the isothiocyanate moeity in this sponge, suggesting a facile conversion of cyanide to thiocyanate had occurred (Dumdei *et al*., 1997). The enzyme rhodanase, which is widespread in nature, detoxifies cyanide through conversion to thiocyanate. Unfortunately, it is not yet known if this enzyme is present in marine sponges such as *A*. *cavernosa* (Garson & Simpson, 2004). The interconversion of isocyanides and isothiocyanates by sponges may represent a mechanism for these marine organisms to adjust the concentrations of these metabolites for ecological purposes. Biosynthetic pathways of isocyanide and isothiocyanate in the sponge *A*. *cavernosa* are shown in the Figure 9.

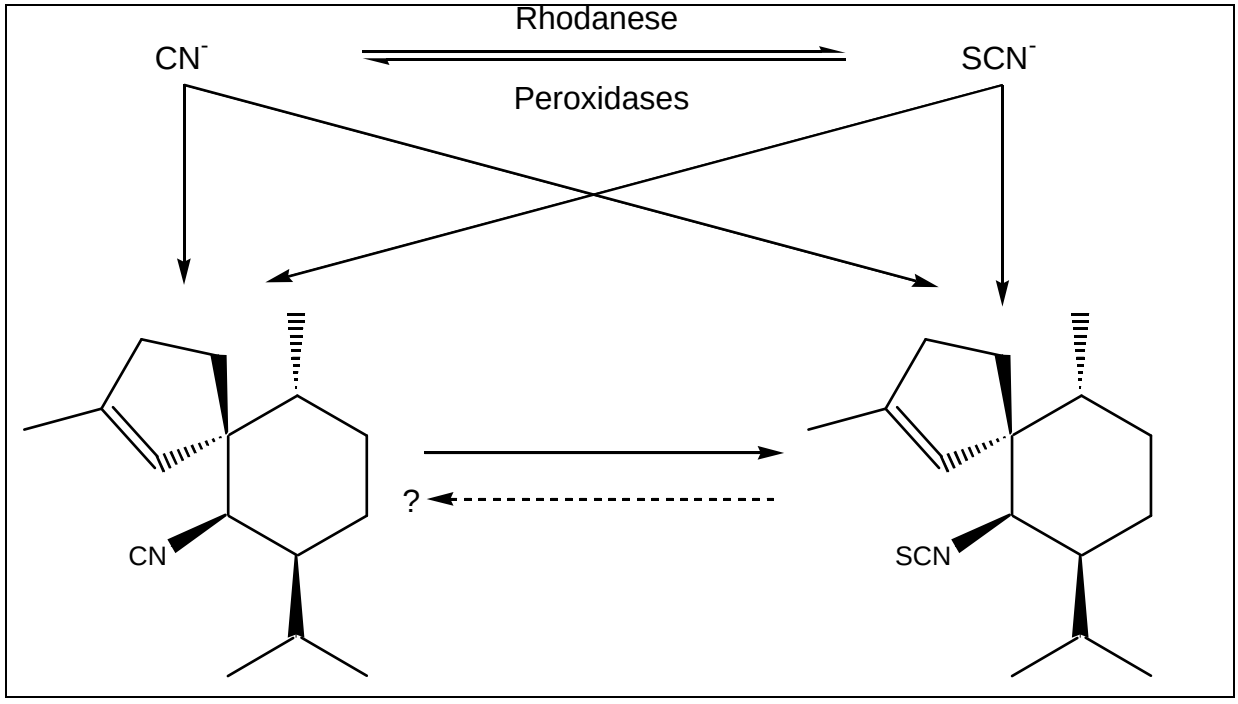


Figure 9. Proposed biosynthetic pathways in the sponge *A*. *cavernosa* [reproduced from Dumdei *et al*., 1997].

2 Materials and Methods

2.1 Research materials and location of collection

In the present study, the main research objects involve phyllidiid nudibranchs and their sponge-preys. Phyllidiid species have been reported to be sponge specialist predators. Feeding observations and sample collections were done at coral reefs of the Thousand Island National Park in Indonesia (Figure 10).

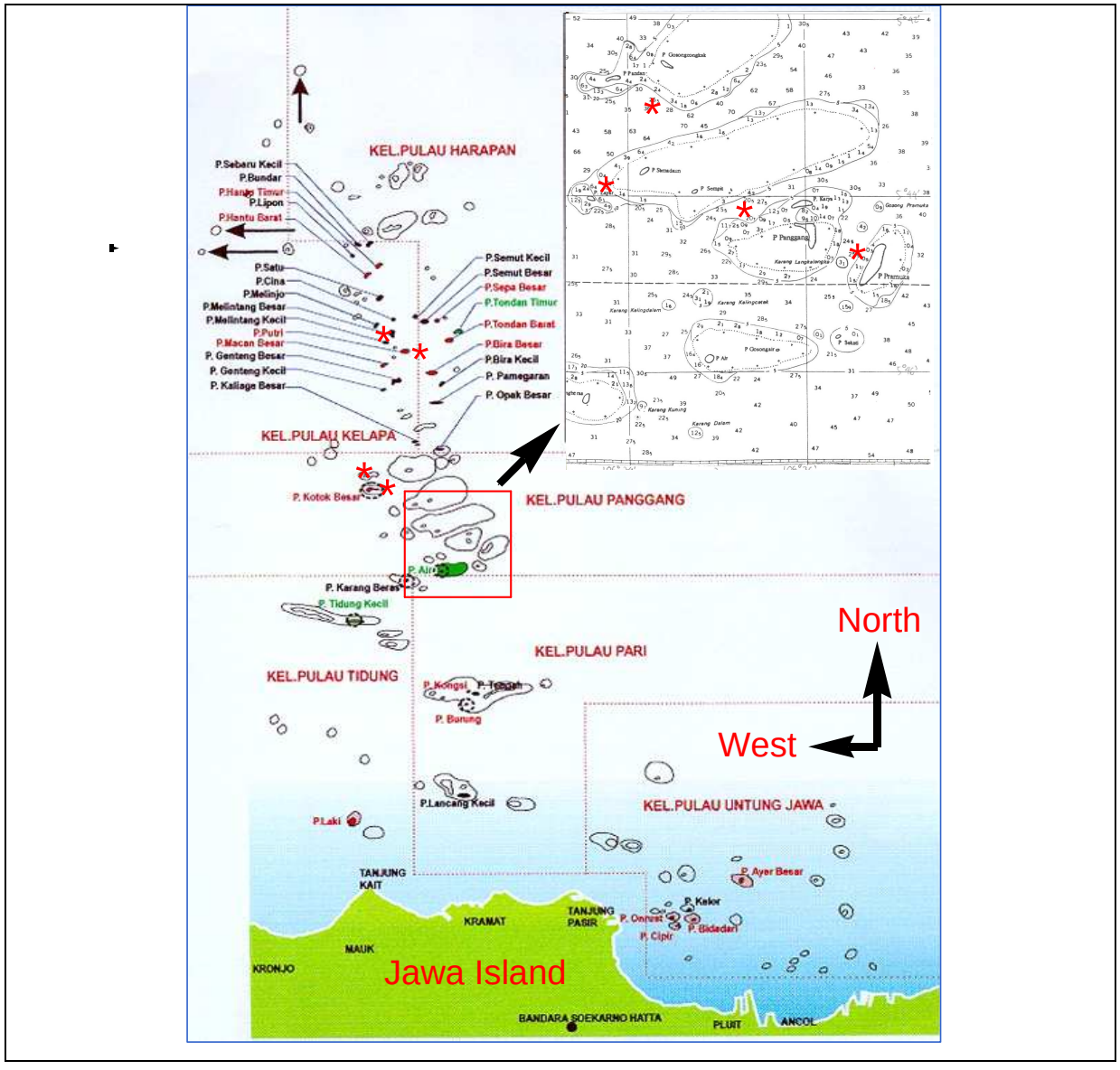


Figure 10. The map of the Thousand Islands, Indonesia. Those marked with a star show the collection location.

The Thousand Island National Park is located at 5˚24'-5˚45'S and 106˚25'-106˚40'E, in the vicinity of the Indonesian capital city, Jakarta. Some of the islands are inhabited; some are used for tourism as well as for conservation area (Ministry of Forestry and Estate Crops, 2000).

2.2 Collection of research objects

Samplings were performed twice. All samplings were done by mean of SCUBA. The first collection was done in June-July 2002 at the coral reefs of Pramuka Island. In the present study, Pramuka Island became the most importand island because most of the collected samples were used for chemical analysis, ecological and pharmacological experiments. All collected samples were immediately stored at -20^{o}C until they were shipped in ice boxes to the Institute of Life Science and Natural Product, BPPT, in Jakarta, for freeze drying. The second collection was done in April 2004 at the coral reefs of Pramuka Island, Melinjo Island, Putri Island, Kotok Besar and Kotok Kecil Island, Semak Daun Island, Karang Congkak Island, Opak Kecil Island, Karang Lebar reefs, and Karang Balik Layar reefs. Sponge-preys of the different phyllidiid species were collected after an extensive feeding observation. All samples were freeze-dried and brought to the Institute for Pharmaceutical Biology and Biotechnology, University of Düsseldorf in Germany for chemical study and identification. A voucher specimen of each of the phyllidiid species was stored in the Department of Biology, Faculty of Mathematics and Science, University of Indonesia, Indonesia; whereas a voucher specimen of each sponge species was deposited in the Zoological Museum Amsterdam, Netherland. Some vouchers which were proposed as new species were deposited in the Department of Biology, Faculty of Mathematics and Science, University of Indonesia, Indonesia.

2.3 Feeding observation

Feeding of phyllidiid species has been very well described by Brunckhorst in 1993. Phyllidiid species evert their pharyngeal bulb while feeding upon a sponge (Brunckhorst, 1993; personal observation). In the present study, every single phyllidiid species which was found on a sponge was abruptly taken away from a sponge and then the evertion of pharyngeal bulb was recognized. The one that was

observed to evert its pharyngeal bulb was then recognized as feeding on that particular sponge.

For *ex situ* feeding observation, two sponge-preys which were observed *in situ* predated by *P*. *varicosa* and *P*. *pustulosa* along with some phyllidiid species (2 ind. of *P*. *ocellata*, 2 ind. of *P*. *zeylanica*, 2 ind. of *P*. *varicosa*, 2 ind. of *P*. *shireenae*, 2 ind. of *P*. *pustulosa*) were collected alive and then were placed into a continuous water flow fiberglass aquarium (Figure 11). The sponge of *Acanthella cavernosa* was also kept alive in the aquarium. Feeding observations were done after three days acclimatization. The behaviour of the collected phyllidiid species was also recorded.

Figure 11. Fibre aquarium used for feeding and behaviour of phyllidiid species: A. The inlet of fresh sea water; B. The outlet; C. The sponge *Acanthella cavernosa*; D. The sponge *Axinyssa*.

2.4 Identification of samples

2.4.1 Phyllidiid species

Nudibranch species belonging to the family Phyllidiidae were collected and used for this study. Phyllidiid species collected in the present study were identified in nature through their external morphology and the colouration pattern of their mantle. Those which did not have the typical morphology, as described in Brunckhorst's monograph (1993), were excluded from the collection for further chemical analysis. Ten phyllidiid species from four genera have been collected from the coral reef of Thousand Islands, Indonesia. These include *Phyllidia elegans*, *P. ocellata*, *P. varicosa*, *Phyllidiella lizae*, *P. pustulosa*, *P. rudmani*, *P. zeylanica*, *Phyllidiopsis krempfi*, *P. shireenae*, and *Fryeria menindie*.

2.4.1.1 *Phyllidia elegans*

This species was described to have a black background at the notum while the notal tubercles are creamish pale green, which may have only one or a few yellow-capped tubercles. The tubercles tend to be isolated but their base may merge. Black rays run among densely smaller tubercles at the mantle edge. The rhinophores are yellow. The foot sole has a median longitudinal black stripe (Figure 12).

2.4.1.2 *Phyllidia ocellata*

The notum background is gold in color. The tubercles are usually isolated and may be colored gold or grey. A black ring area encircles some tubercles or is extended and is joined together covering almost the entire notum except the mantle edge area. The ventral surface is grey and has no distinctive marking on its foot sole (Figure 12).

2.4.1.3 *Phyllidia varicosa*

The notum of the species have many conical or angular, sometimes compound tubercles which form three or six longitudinal ridges. The ridges may be broken or continuous. A central ridge is always present. It has a blue grey color at the ridges

and base of the tubercles, but capped in yellow. Toward the margins of the notum there are usually numerous, short, transverse ridges interspersed by black rays. The foot and gills are grey in color. It possesses yellow rhinophore and a black longitudinal foot stripe at the foot sole (Figure 12).

2.4.1.4 *Phyllidiella lizae*

This species is an elongated ovate species which possesses a pale pink background notum, pale pink tubercles and irregular, narrow black lines on the dorsum. The notal tubercles are also pale pink in color, simple, rounded, and usually isolated. Narrow black lines of variable length occur irregularly on the dorsum; usually they cross over each other forming an “X” on the median area (Figure 12).

2.4.1.5 *Phylidiella pustulosa*

The body shape is elongating ovate with pale grey, sometimes pink or green, tubercles on a black notum. The three group tubercles that are clustured or amalgamated in the median area of the notum characterize the species. The mantle margin is edged in pale grey, sometimes pink or grey, which is also very typical for the species. The rhinophores are colored black (Figure 12).

2.4.1.6 *Phyllidiella rudmani*

This species has a pale pink or grey notum background. It has two longitudinal black stripes which are neither joined anteriorly nor posteriorly. The tubercles are arranged in longitudinal rows but never form ridges and are pale pink or grey in color. The apical and basal half of the rhinophores is black and pale pink, respectively (Figure 12).

2.4.1.7 *Phyllidiella zeylanica*

The background of the notum is black with pale pink or grey tubercles. The tubercles are joined and form ridges which may be continuous or interrupted. There are two

longitudinal black bands which are joined anteriorly and posteriorly. The rhinophores are colored black (Figure 12).

2.4.1.8 *Phyllidiopsis krempfi*

The species is charaterized by a predominantly pale grey or pink coloration, and broad based multicompound tubercles with paler apices. There are two primary longitudinal black lines which extend around and join in front of the rhinophores. A few black lines extend from the primary black lines to the mantle edge. Some other short black lines of variable size occur irregularly around the mantle edge. The rhinophores are pink on the anterior face and around the base, but black on the apex and posterior face (Figure 12).

2.4.1.9 *Phyllidiopsis shireenae*

The dorsum of the species has generally a very pale shade of pink or white and has a number of small, rounded tubercles. There are two primary longitudinal black lines which extend around and joint in front of the rhinophores. Two black lines run across the dorsal crest and are linked to the primary black lines. The rhinophores are pale pink in colour (Figure 12).

2.4.1.10 *Fryeria menindie*

This species is characterized by their large-isolated tubercles capped in gold on a black notum background. The base of the tubercles is blue-grey. Wide, crescentic, blue-grey areas with a few small gold capped tubercles occurr arround the mantle margin. Between each crescentic blue-grey area, there is a black ray which extends to the edge of the mantle. The rhinophores are gold in color (Figure 12).

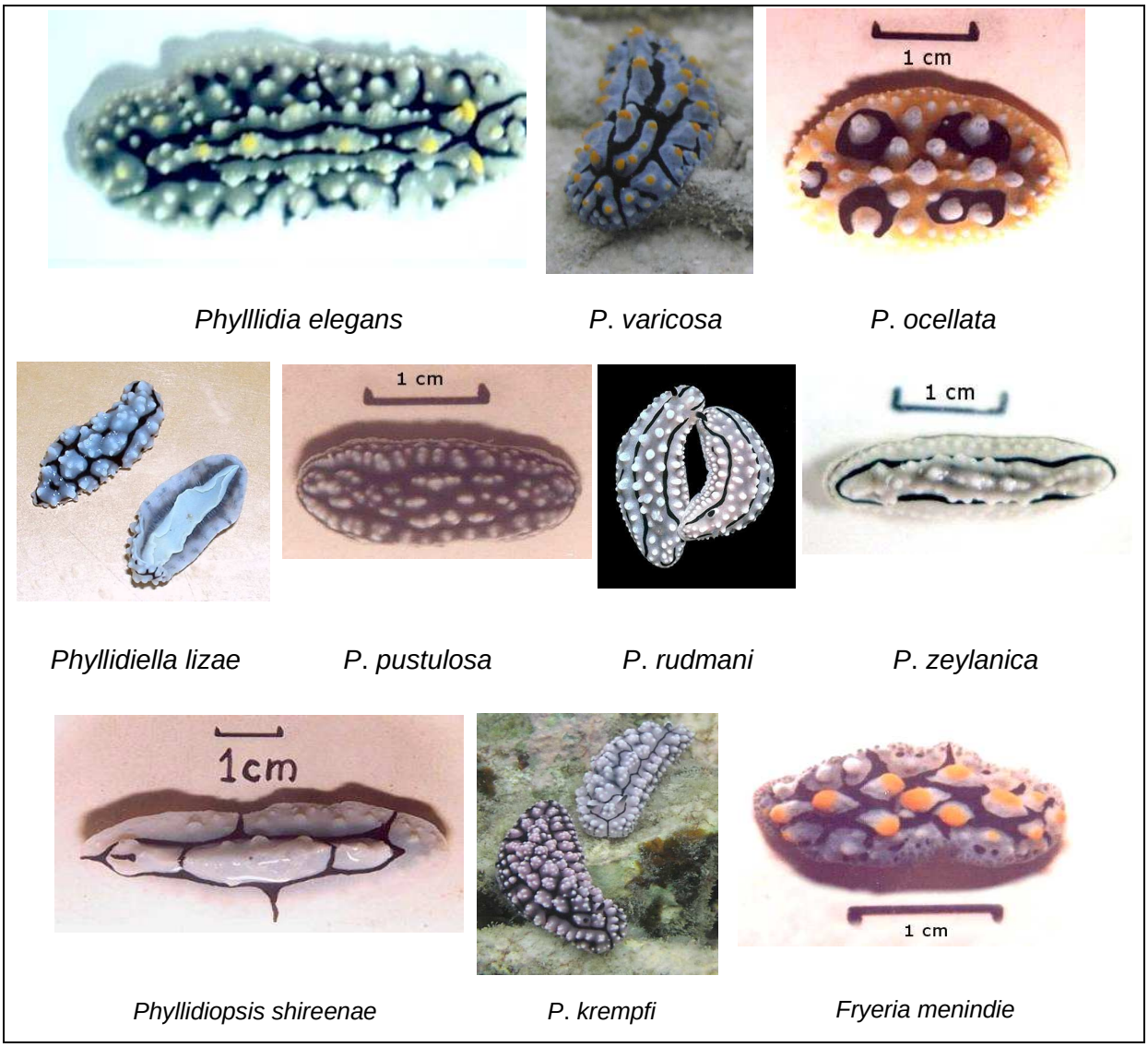


Figure 12. Phyllidiid species collected in Thousand Islands, Indonesia.

2.4.2 Sponge species

Sponge samples were collected through a predation-guided sponge sampling method. All samples were freeze-dried and brought to the Institute for Pharmaceutical Biology and Biotechnology, University of Düsseldorf in Germany for chemical study and identification. Identification of sponges was done by the author under the supervision of Dr. R.W.M van Soest of the Zoological Museum Amsterdam, Netherland.

For successful sponge identification it is necessary to assemble as much information regarding the specimen both in its living or freshly collected state. However, it is impossible to be certain of the identity without making a microscopic preparation showing the spicules and the skeletal structure (Bergquist, 1978; Boury-Esnault & Rützler, 1997).

Spicule preparation

A fragment (~0.5 cm^3) of the siliceous sponge was boiled in an eppendorf with about 1 mL of fuming nitric acid (HNO_3) until the cellular material is dissolved and the liquid solution is clear (~5-7 hours). The dissolved cellular sponge material was placed in a centrifuge at 3000 rpm for 3-5 minutes. The nitric acid was then decanted and the samples suspended in destilled water. Spicule pellet was dislodged by shaking the eppendorf and then was again placed in a centrifuge at 3000 rpm for 3-5 minutes. The destilled water was then pipetted off and replaced by ethanol. Spicule pellet was dislodged by shaking the eppendorf and then was again placed in a centrifuge at 3000 rpm for 3-5 minutes. This process was done several times to make sure that all water was totally replaced by ethanol. At the end of the process, the disloged spicule pellet was then mounted to special glass slide for Scanning Electron Microscope (SEM).

Preparation of skeleton structure

This is necessary to study the skeletal structure of a sponge. A thin hand sections from a sponge voucher, perpendicular and pararel to the surface, was made with a fresh, thin, razor blade. The sections were placed on a glass slide then were

mounted in Canada balsam [Merck] and covered with a glass slip. The preparative slides were allowed to dry on a heating plate. For a voucher which was preserved in alcohol, the sections were allowed to dry before mounting process.

2.4.2.1 *Axinyssa aplysinoides*

The sponge has a compressed-massive and compressed-lobate growth form (Figure 13). Surface is glabrous (smooth) but slightly hispid from the projection of the ends of some of the oxeot spicules. Externally, the color of the live animal is dark purple while in the internal surface is whitish purple but turns to very pale yellow after being preserved in alcohol. The specimen has no ectosome skeleton. The choanosomal skeleton is also arranged at random. At the periphery, the spicules are protuding and at right angles to the surface. The spicules have long oxeas, usually sharply pointed at both ends, straight but more often slightly curved. A fully grown spicule is 1.09 mm by 0.1175 (median size 0.94 mm) and 1.15 mm by 0.1175 (median size 1.01 mm). The two specimens belonging to *A*. *aplysinoides* were coded as PZ02 & PK04-I.

2.4.2.2 Group A (*Axinyssa 6* n. sp.)

This group consists of two specimens coded as FM04 and PP04-XIV. These two sponge vouchers were proposed as new species *Axinyssa 6* n. sp. because of their distinct growth form and their full grown size spicules were the shortest (0.78 and 0.80 mm) among the entire division. They were taxonomically excluded from *A*. *aplysinoides* due to their bundled microconules spiculation.

FMO4 was described as a holotype. It has sessile, encrusting-flattened growth form. Live specimen is yellowish brown (Figure 13); and its internal surface is pale yellow. It has a firm and breakable consistency. The surface is microconulose with some big openings which probably are the oscula. The sponge sample is approximately 15 mm in height and the length is about 60 mm. The specimen has no ectosomal skeleton. Their choanosomal skeleton has no definite fibres. Numerous spicules occur irregularly scattered so that the whole skeleton at random. At the periphery, the spicules are arranged in bundles at right angles to and protruding slightly beyond the surface causing microconulations. The sponge sample was

collected from the reefs of Karang Congkak. *In situ*, the specimen was observed to be predated by *Fryeria menindie*.

2.4.2.3 Group B (*Axinyssa 4* n. sp.)

This group consists of seven specimens which were coded as PP04-III, VIII, XVI; PV04-II, IX, X; and PK02. They all exhibited bundled projecting spicules forming a microconulus and the longest spicule size range is 0.87 – 0.95 mm. This group was described to be new species belonging to *Axinyssa 6* n. sp. Sponge voucher PP04-VIII was identified as a holotype specimen of this group (Figure 13).

The specimen is sessile, lobate, and the color of dried material is brown while fresh material is dark purplish brown color. The surface is microconulose with many small openings which are probably the ostia; Some big openings, probably the oscula are also present. Two big irregular openings that make a groove near the periphery of the specimen were also present. The inner side of the specimen has also numerous big openings of which some end to the groove on the surface. A thin pale brown organic layer covers the surface of the specimen sometimes covering some of the big openings. The presence of big openings at the surface and in the inner side of the specimen made its consistency cavernose. The height of the specimen is about 20 mm and its length is about 35 mm. Full grown spicule size is 0.92 mm by 0.1175 at a median size of 0.88 mm. There is no ectosomal skeleton present. The choanosomal skeleton has no definite fibres. Numerous spicules occur irregularly scattered so that the whole skeleton in arranged randomly. At the periphery, the spicules are arranged in bundles at right angles to the surface and protruding slightly beyond the surface causing microconulations. The sponge sample was collected from Kotok Besar Island. *In situ*, the specimen was observed to be predated by *P. pustulosa*.

2.4.2.4 Group C (*Axinyssa 5* n. sp.)

This is the largest group which consisted of 34 sponge specimens. This group has similar size range of the longest spicule as *A. aplysinoides* (1.02 - 1.23 mm by 0.175) but has bundled projecting spicules forming a microconulus. PV04-XIII was assigned as the holotype specimen of *Axinyssa 5* n. sp. (Figure 13).

The sponge sample is sessile and lobate. The dried material is very pale brown while the fresh material is dark purplish brown in color. The surface microconulose has many small openings which are probably the ostia and some big oscula are also present. Many shallow depressions were present making the surface filled with gullies. The inner side of the specimen has some small openings and big openings but in general these openings were much less compared to those present at the surface of the specimen. A thin pale brown organic layer covers the surface of the specimen sometimes hiding some big opening. Height of specimen present is about 20 mm and the length is about 2.3 mm. Full grown spicule size is 1.02 mm by 0.1175 with median size of 0.90 mm. There is no ectosomal skeleton present. The choanosomal skeleton has no definite fibres. Numerous spicules occur irregularly scattered so that the whole skeleton appears at random. At the periphery the spicules are arranged in bundles at right angles to the surface and protruding slightly beyond the surface causing microconulations. The sponge sample was collected from Semak Daun Island. Phyllidid *P*. *varicosa* was observed to be feeding *in situ* on the sponge sample.

2.4.2.5 Group D (*Axinyssa 1* n. sp.)

This group consists of six specimens coded as PK04-II, IV; PZ04-I, VIII, XI; and PP04-XI. All specimens have the same spiculation as *A*. *aplysinoides*. They were splitted from *A*. *aplysinoides* and were described as new species because of their distinct growth form and their full grown spicules size range were much longer (1.32 - 1.47 mm by 0.175). PK04-II was assigned as the holotype specimen of *Axinyssa 1* n. sp. (Figure 13).

The sponge sample possesses a branching growth form. Live specimen is pale yellowish brown while dried specimen is pale yellow in color. Consistency is firm. Surface is hispid with only few ostia. Internally, the specimen has 1-3 big openings that run tangentially to the surface following the branching track. Height of a single specimen is about 5 mm and it has a length of about 33 mm. There is no ectosomal skeleton present. Fibres are also absent. Fully grown spicule size is 1.29 mm by 0.1175 (median size 1.15 mm). Numerous spicules occur irregularly scattered so that the whole skeleton is at random. At the periphery, the spicules are arranged loosely at right angles to the surface and protruding slightly beyond the surface causing a

hispid condition at the surface of the sponge. The sponge specimen was collected from Melinjo Island. Phyllidiid *P*. *krempfi* and *P*. *pustulosa* were observed feeding on this sponge sample.

2.4.2.6 Group E (*Axinyssa 2* n. sp.)

This group has only one representative specimen which was coded PK04-III. It has a flattened growth form with small fistule-like projection at each corner end (Figure 13). Living specimen is pale yellowish brown while dried specimen is pale yellow in color. Consistency is firm. The surface is hispid with some small openings, probably the ostia. The inner part of the specimen has some big openings but no small openings are present. Height of a single specimen is about 10 mm and its length is about 32 mm.

There is no ectosomal skeleton present. Fibres were also absent. Its full grown spicule size is about 1.40 mm by 0.1175 with a median size of 1.24 mm). Numerous spicules occur irregularly scattered so that the whole skeleton is at randomly arranged. At the periphery, the spicules are arranged loosely at right angles to the surface and protruding slightly beyond the surface creating a hispid condition. The sponge was collected from Semak Daun Island. Phyllidiid *P*. *krempfi* was observed to be feeding *in situ* on the sponge sample.

2.4.2.7 Group F (*Axinyssa 3* n. sp.)

This group has also only one representative specimen coded as PZ04-III. The sponge sample is sessile and lobate. The dried material is very pale brown and the fresh specimen is purplish brown in color. The sponge has a hard but brittle consitency. The surface is microconulose with some small ostia and one big osculum. The inner side of the specimen has a lot of small openings and some big openings of which three of them end to the big opening to the external surface. A thin pale brown organic layer covers the surface of the specimen closing the osculum. Height of single specimen is about 15 mm and its length is about 25 mm (Figure 13).

There is no ectosomal skeleton present. The choanosomal skeleton has no definite fibres. The full grown spicule size is about 1.53 mm by 0.1175 with a median size of 1.28 mm). Numerous spicules occur irregularly scattered so that the whole

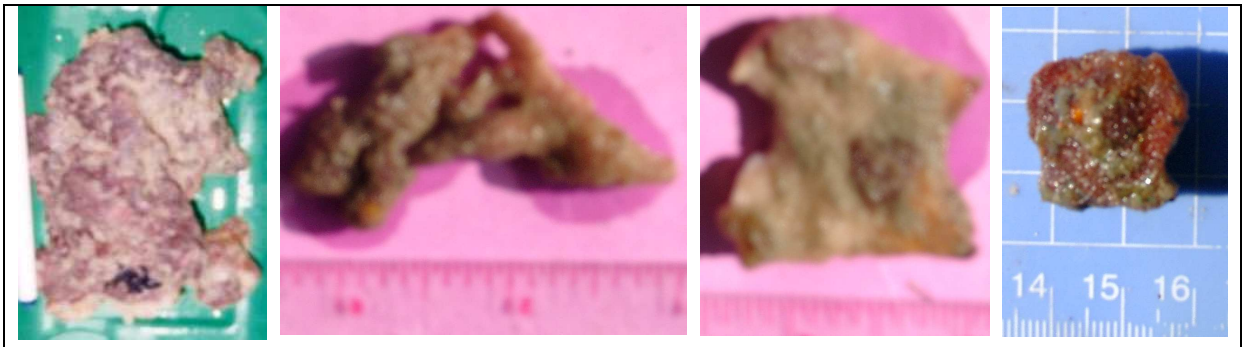
skeleton becomes randomly arranged. At the periphery, the spicules are arranged in bundles at right angles to the surface and protruding slightly beyond the surface causing microconulations. The sponge specimen wass collected from the reefs of Karang Congkak. The specimen was predated by *P*. *zeylanica* as observed *in situ*.

2.4.2.8 *Acanthella cavernosa*

The sponge is erect and cavernose in habit (Figure 13). The surface is hispid due to protruding long stylote spicule of choanosomal skeleton. Fresh specimen is bright orange but very pale yellow in dried condition. Its ectosome has no specialized skeleton. The choanosomal skeleton is made up of condensed tracks or axes of sinulose strongyles or strongyloxeas from which large stylote spicules toward the periphery and beyond the surface caused a hispid condition.

2.4.2.9 *Dragmacidon* sp.

The sponge sample is flattened or compressed-lobate (Figure 13). Its surface is more or less smooth with short conules or tubercles. Its ectosome has no specialised skeleton. Its choanosomal skeleton plumoreticulation has ascending plumose tracts. The sponge has no axial or extra-axial regions. Its megascleres are mainly made up of stylote with some oxeot spicules or just occasionally occur. Microscleres are not present.



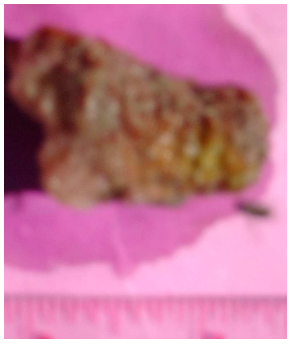
A. aplysinooides

Axinyssa 1 n. sp.

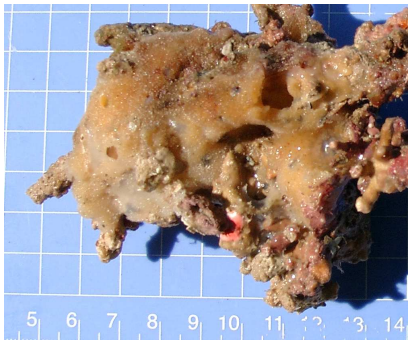
Axinyssa 2 n. sp.

Axinyssa 3 n. sp.

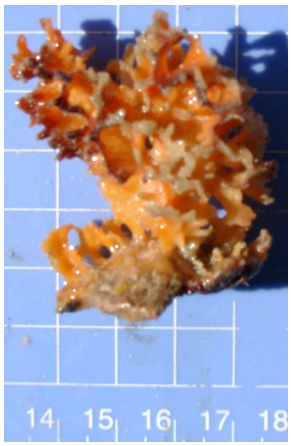
Axinyssa 4 n. sp.



Axinyssa 5 n. sp.



Axinyssa 6 n. sp.



Acanthella cavernosa

Dragmacidon sp.

Figure 13. Photograph of sponge-preys of phyllidiid species in the Thousand Islands National Park.

2.5 Isolation of isocyanide compounds

2.5.1 Chromatographic methods

2.5.1.1 Thin layer chromatography

TLC was performed on precoated TLC plates with Si gel 60 F_{254} (layer thickness 0.2 mm, E. Merck. Darmstadt, Germany) with Hexane: EtOAc (90:10; or/and 95:5). TLC on reversed phase (RP)-C18 F_{254} (layer thickness 0.25 mm, E. Merck, Darmstadt, Germany) was used for controlling fractions getting from RP-C18 normal column transferred by and using the solvent system Acetonitrile:MeOH (50:50).

The compounds were detected by spraying the TLC plates with anisaldehyde reagent follwed by heating at 110 ^{o}C. Anisaldehyde/H_2SO_4 Spray Reagent (DAB 10) consists of Anisaldehyde (5 parts), Glacial Acetic acid (100 parts), Methanol (85 parts), Conc. H_2SO4 (5 parts). The first 3 parts were mixed, to which 5 parts of concentrated H_2SO_4 were added slowly. The reagent was stored in an amber-colored bottle and kept refrigerated until use.

TLC was used to monitor the identity of each of the fractions and the qualitative purity of the isolated compound. It was also utilized to optimize the solvent system that would be applied for column chromatography.

2.5.1.2 Vacuum liquid chromatography

Vacuum liquid chromatography (VLC) is a useful method for initial isolation procedure for large amounts of sample. In this study VLC was used to wash all crude extracts from fatty acid by using 100% n-hexane and to separate target compounds from steroid by adding 5% of EtOAc in hexane. The apparatus consists of a VLC glass column with an inner diameter of 3 to 8 cm, which depends on the amount of crude extract. Silica gel 60 was packed into the column at a height of 5 cm under applied vacuum. The sample used was incorporated with a small amount of silica gel using a volatile carrier solvent and then dried and keep under reduced pressure by using a dessicator overnight. The loaded silica was then packed onto the top of the prior-packed VLC column. Elution was activated with vacuum and the column was allowed to run dry after each fraction was collected.

2.5.1.3 Normal column chromatography

VLC Fractions containing target compounds were repeatedly separated through normal column chromatography using silica gel 60 as a stationary phase and 100% n-hexane and/or a combination of hexane:EtOAc (95:5) as a solvent system.

2.5.1.4 Flash chromatography

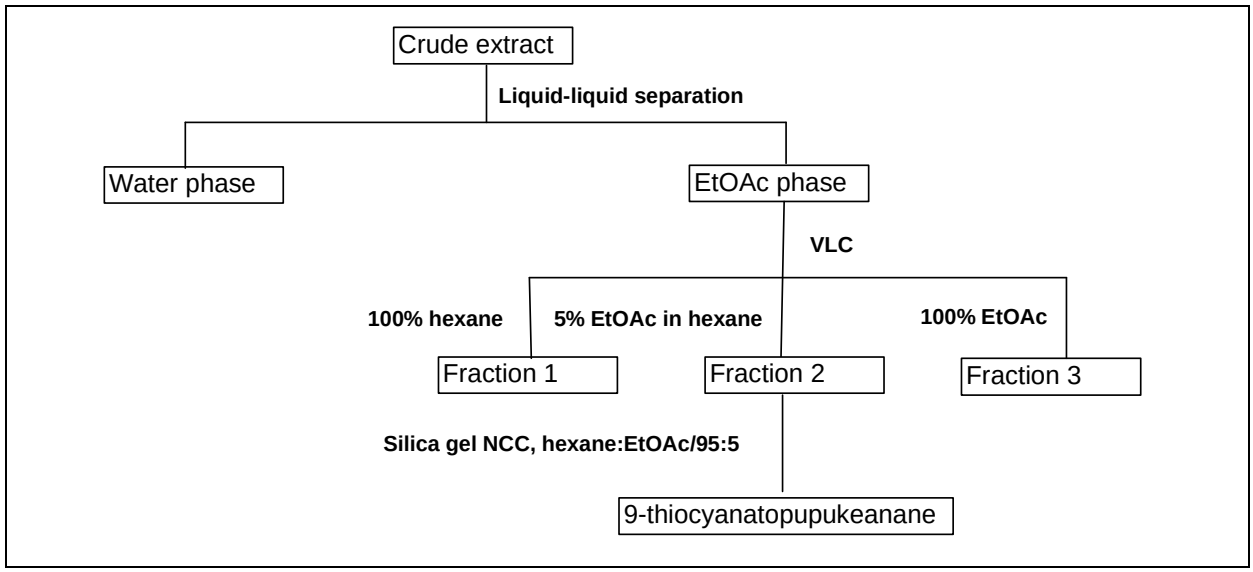
Flash chromatography (Biotage)® was used for further purification of the target compoundsrapid isolation toward impurity fractions from a normal column chromatography. Utilizing this method was also very useful when dealing with very little amount of the crude extract. Sample was dissolved in a small volume of eluent and loaded into a pre-packed silica gel column GF_{254} (160 mm lenght; Ø 12 mm) by using a special syringe. The flow of mobile phase (100% hexane or hexane:EtOAc/95:5) was activated by an air pump and the eluent were collected into different fractions.

2.5.1.5 Gas chromatography

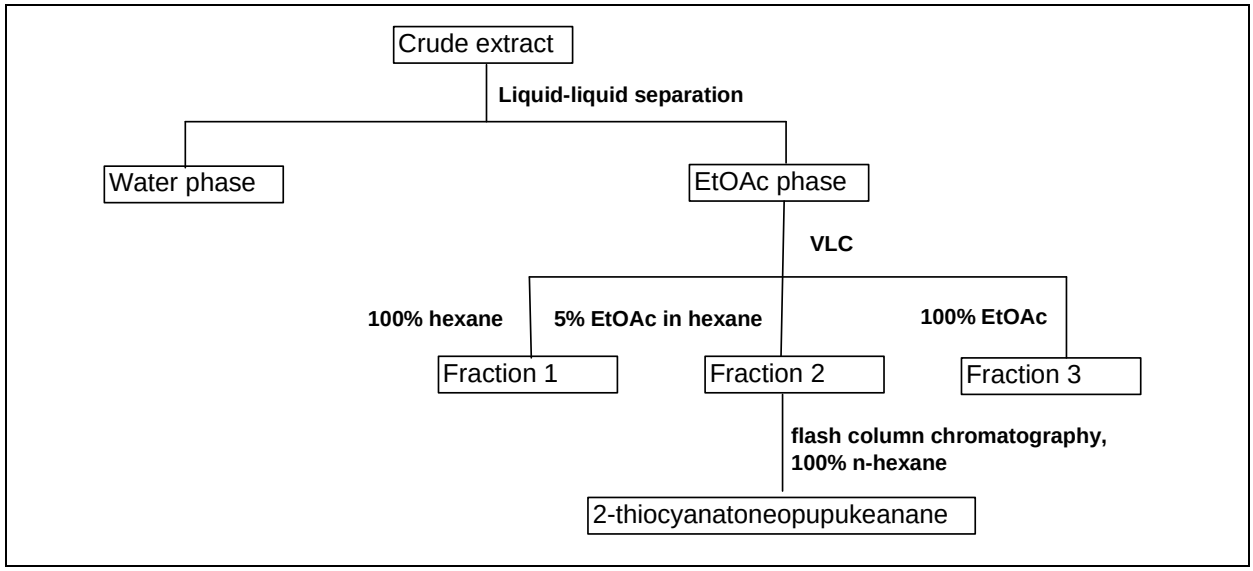
Gas chromatography was used to detect and monitor the presence of interesting peaks from extracts and fractions and to evaluate the purity of the isolated compounds as well as to quantify the concentration of the different secondary metabolites in the total extracts. Samples were injected in the Agilent 6850 GC System using a special program with a gradient of 150 ^{o}C at 0 min to 300 ^{o}C at 75 min.

2.5.2 Procedure for the isolation of the secondary metabolites

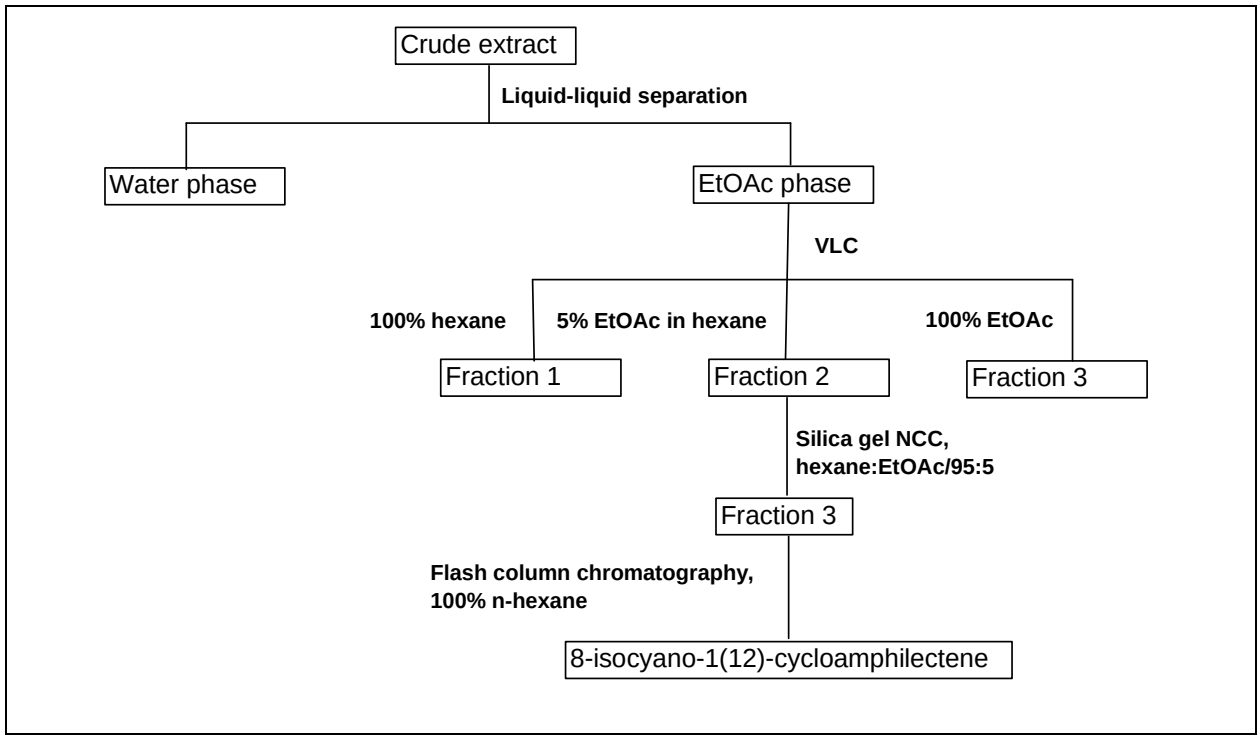
2.5.2.1 Isolation of the mixture 9α- and 9β-thiocyanatopupukeanane



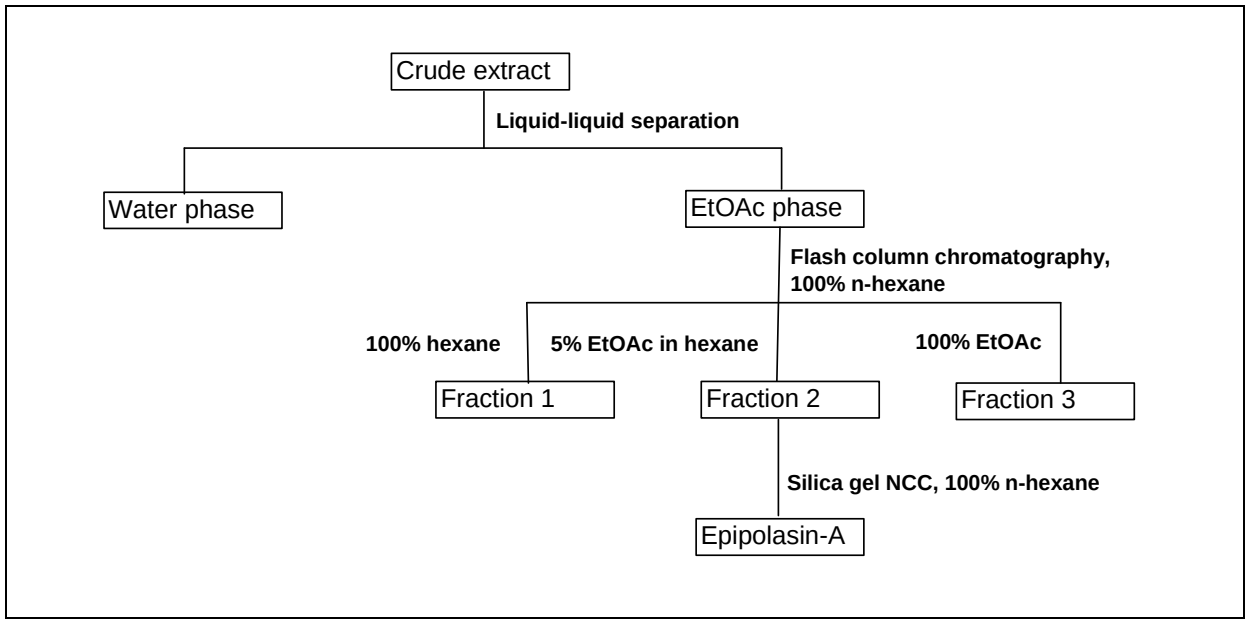
2.5.2.2 Isolation of 2-thiocyanatoneopupukeanane



2.5.2.3 Isolation of 8-isocyano-1(12)-cycloamphilectene



2.5.2.4 Isolation of Epipolasin-A



2.5.3 Structure elucidation of the isolated secondary metabolites

2.5.3.1 Mass spectrometry (MS)

EIMS (electron impact mass spectroscopy) analysis involves vaporizing a compound in an evacuated chamber and then bombarding it with electrons having 25-80 eV (2.4-7.6 MJ/mol) of energy. The high energy electron streams not only ionize an organic molecule (requiring about 7-10 eV) but also cause extensive fragmentation (the strongest single bonds in organic molecules have strengths of about 4 eV). The advantage is that fragmentation is extensive, giving rise to a pattern of fragment ions which can help to characterize the compound. The disadvantage is the frequent absence of a molecular ion. Mass spectra (EI) measurements were done on a Finnigan MAT 8430 mass spectrometer by Dr. Peter Tommes of HHU Düsseldorf.

High Resolution MS. High resolution is achieved by passing the ion beam through an electrostatic analyser before it enters the magnetic sector. In such a double-focusing mass spectrometer, ion masses can be measured with an accuracy of about 1 ppm. With measurement of this accuracy, the atomic composition of the molecular ions can be determined. HRMS(EI) measurement was performed by Dr. Albrecht Berg of Hans Knoll Institut für Naturstofforschung, Jena.

2.5.3.2 Nuclear magnetic resonance spectroscopy (NMR)

NMR measurements were done by Dr. Victor Wray at the Gesellschaft für Biotechnologiesche Forschung (GBF) in Braunschweig and by Dr. Peters at the Institut für Anorganische Chemie of the Heinrich-Heine Universität Düsseldorf. ^{1}H and ^{13}C NMR spectra were recorded at $300^{o}K$ pm Bruker DPX 300, ARX 400 or AVANCE DMX 600 NMR spectrometers. All 1D and 2D spectra were obtained using the standard Bruker software. This sample is dissolved in a deuterated solvent ($CDCl_3$), the choice of which is dependent on the solubility of the sample. Solvent signals at 3.30 ppm and 49.0 ppm (CD_3OD) and at 2.49 ppm and 39.5 ppm (DMSO-d_6) were considered as internal standard (reference signal). The observed chemical shift (δ) values were given in ppm and the coupling constants (*J*) in Hz.

2.5.3.3 Optical activity

Optical rotation was determined on a Perkin-Elmer-241 MC Polarimeter by measuring the angle of rotation at the wavelength of 546 and 579 nm of a mercury vapor lamp at room temperature (25^{o}C) in a 0.5 mL cuvette with 0.1 dm length. The specific optical rotation was calculated using the expression:

$$[\alpha]_{D}^{20} = \frac{[\alpha]_{579} \text{ x } 3.199}{4.199 - \frac{[\alpha]_{579}}{[\alpha]_{546}}}$$

where $[\alpha]_{D}^{20}$ = the specific rotation at the wavelength of the sodium D-line, 589 nm, at a temperature of 20^{o}C.

$[\alpha]_{579}$ and $[\alpha]_{546}$ = the optical rotation at wavelength 579 and 546 nm respectively, calculated using the formula:

$$[\alpha]_{\lambda} = \frac{100 \text{ x } \alpha}{l \text{ x } c}$$

where α = the measured angle of rotation in degrees,

l = the length in dm of the polarimeter tube,

c = the concentration of the substance expressed in g/100 mL of the solution

2.6 Organ-specific distribution of isolated compounds

In order to know the distribution of the isocyanide compounds in the body of phyllidiids, four representative of freshly collected phyllidiid species (6 individuals of *P*. *elegans*, 19 individuals of *P*. *pustulosa*, 9 individuals of *P*. *krempfi*, and 3 individuals of *P*. *shireenae*) were dissected into three parts: mantle, foot, and digestive glands (Figure 14). After lyophylization, samples were repeatedly extracted with acetone and then with MeOH. The resulting total extracts (combination of acetone and MeOH extract) were then subjected to solvent-solvent partitioning

between EtOAc and H_20. The resulting EtOAc extracts then were used for GC analysis.

Each isolated compound was injected to the Agilent 6850 GC system in a series of different concentration. The amount of individual cyanide terpenes in the body part of each phyllidiid species was then quantified through a calibration curve for each of the respective isolated natural product of interest.

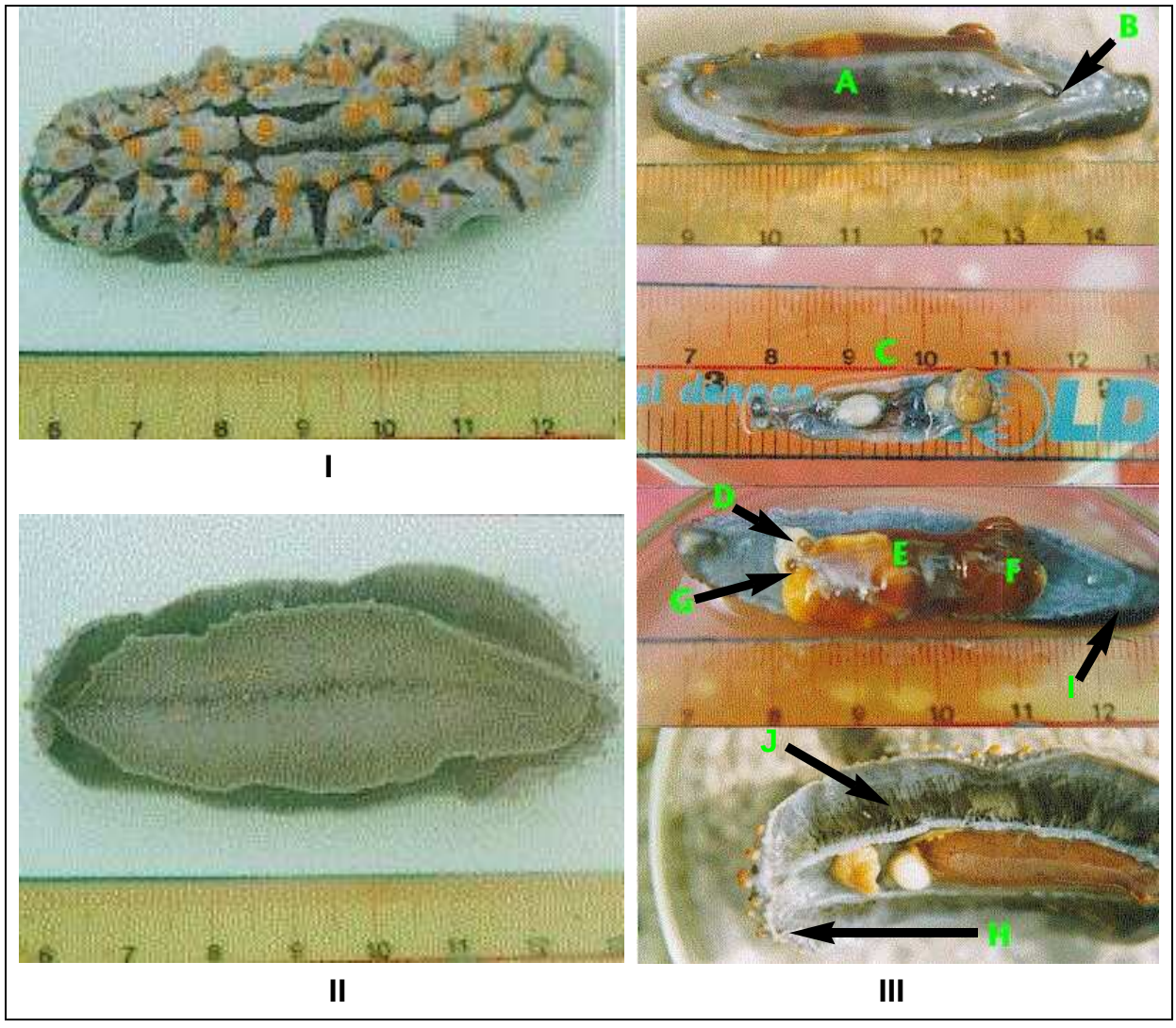


Figure 14. Intact specimen of *P. varicosa*: I. Dorsal (mantle) view; II. Ventral (foot) view. Dissected specimen: a. visceral sheet of digestive tract; b. anus; c. digestive organ; d. hemi-testis; e. ovo-testis; f. digestive gland, g. nidamental gland; h. mantle; i. foot; j. gills.

2.7 Feeding deterrent experiments

2.7.1 Qualitative feeding deterrent experiments of phyllidiid species intact

It has been known that phyllidiid species are rarely attacked by any potential predators (Brunckhorst, 1993). In order to get a preliminary assessment of this statement, a qualitative feeding deterrent experiment of phyllidiid species intact was performed *in situ*. Marine coral reef fishes were assumed to be potential predators because most of them were observed to "attack" everything that pass through a water column of a coral reef. As much as 37 individual of phylidiiid species were collected alive by SCUBA (see Table 3). To initiate the experiment, a four cm-piece of fresh octopus's arm was passed through the water column (2-3 m in depth) where common coral reef fishes were usually found. After the initiation, collected phyllidiid species were then passed the water column. At the end of the experiment, again a piece of fresh octopus's arm was passed through the water column as a control. The reaction of fishes and the fate of the controls (pieces of octopus's arm) and treatments (phyllidiid species) were recorded. Experiments were done in four different locations along the barrier reefs of Pramuka Islands.

Table 3. The list of phyllidiid species that were used as treatments in the qualitative feeding deterrent experiments.

1st experiment	2nd experiment	3th experiment	4th experiment
P. varicosa (2 ind.)	*P. varicosa* (2 ind.)	*P. varicosa* (2 ind.)	*P. varicosa* (2 ind.)
P. pustulosa (3 ind.)	*P. pustulosa* (2 ind.)	*P. pustulosa* (2 ind.)	*P. pustulosa* (3 ind.)
P. zeylanica (2 ind.)	*P. zeylanica* (1 ind.)	*P. zeylanica* (1 ind.)	*P. zeylanica* (1 ind.)
P. ocellata (1 ind.)	*P. ocellata* (1 ind.)	*P. rudmani* (2 ind.)	*P. rudmani* (1 ind.)
P. krempfi (2 ind.)	*P. rudmani* (1 ind.)	*P. krempfi* (1 ind.)	*P. krempfi* (2 ind.)
	P. krempfi (3 ind.)		

2.7.2 Quantitative feeding deterrent experiments of isolated compounds

Common reef fishes were caught by gill nets and were kept in a continous fresh sea water fiber aquarium. Each individual was kept in a single plastic cage to ensure that all feeding activities could be controlled. For five days, all fishes were adapted to feed on commercial fish pellet which consisted of three colors: green, brown, and red, each pellet weighs 0.02 g (dw). Fish pellets were given to each fish three times (at 7 am, 11 am, and 5 pm) and the amount of pellets of each color consumed by each fish was noted. Afterwards, 20 µg of the pure compounds were incorporated into the pellets and fed to the fishes. An EtOAc and blank control were also used.

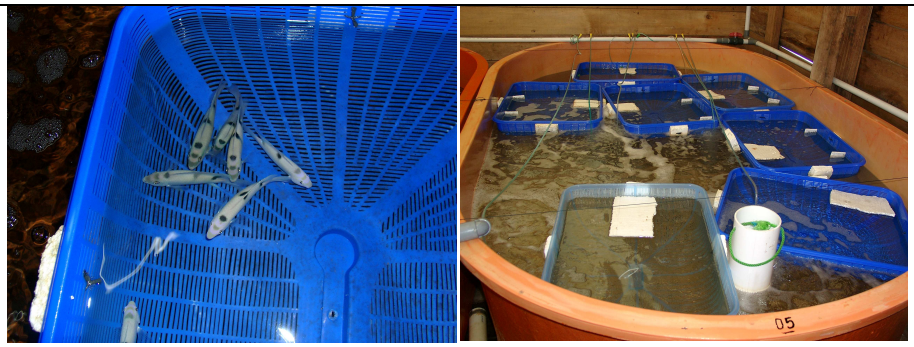


Figure 15. Fishes and fibre aquarium that used in the feeding experiments

2.8 Bioassay

The methods for the detection of biological activity of natural product mixtures can best be divided into two groups for screening purposes: general screening bioassays and specific screening bioassays. The search for specific pharmacologic activites often overlooks other useful activities which are not detected or are ignored, in the screening process. Furthermore specific test methods are often cumbersome and expensive. Hence, for bioassay-guided isolation of biologically active secondary metabolites, inexpensive ‘bench-top’ bioassays for rapid screening of extracts and fractions have to be employed. Since most active plant principles are toxic at elevated doses, a possible approach to developing an effective general bioassay is to simply screen for substances that are toxic to a zoologic system. Once compounds have been isolated, a battery of specific and more sophisticated bioassays could then be employed.

2.8.1 Brine-shrimp assay

This technique is an *in vivo* lethality test employing crustaceans, called brine shrimps (*Artemia salina* Leach). It has been previously utilized in various bioassay systems in the analysis of pesticide residues, mycotoxins, stream pollutants, anesthetics, dinoflagellate toxins, morphine-like compounds, toxicity of oil dispersants, cocarcinogenicity of phorbol esters and toxicants in marine environments [Meyer *et al*., 1982]. This test takes into account the basic premise that pharmacology is simply toxicology at a lower dose, and that toxic substances might indeed elicit its interesting pharmacologic effects at a lower non-toxic dose [McLaughlin, 1991]. The procedure determines LD_{50} values in μg/ml of active compounds in the brine medium.

Sample preparation. The test samples were dissolved in an organic solvent and the appropriate amount is transferred to a 10-ml sample vial. One mg of each two different ratios of the epimeric mixture of compounds **1** and **2** (30:70 and 50:50 respectively) was used. A series of concentrations of the ratio 30:70 (10-1000 μg) was also used to determine LD_{50} values.The samples were then dried under nitrogen and the dried samples were reconstituted with 20 μL DMSO. Control vials containing DMSO were also prepared.

Hatching the eggs. Brine shrimp eggs (Dohse, Aquristik GmbH, Bonn, Germany) were hatched in a small tank filled with artificial sea water which was prepared with a commercial salt mixture (Sera Sea-Salt, Aquaristik GmbH, Bonn, Germany) and distilled water. After 48 hours, 20 nauplii were collected by pipette (counted macroscopically in the stem of the pipette against a lighted background) and transferred into each test sample vial. Artificial sea water was then added to make 5 mL. The vials were maintained under illumination. Survivors were counted, with the aid of a magnifying glass after 24 hours and the percent deaths at each dose and control were determined.

2.8.2 Antibacterial activity

Microorganisms. Three bacterial species: *Bacillus subtilis*, *Staphylococcus aureus* (gram positive bacteria), and *Escherichia coli* (gram negative bacterium) and a yeast *Saccharomyces cerevisiae* as well as three fungal species: *Candida albicans*, *Cladosporium herbarum*, and *C*. *cucumerinum* were applied as test microbes in the present study. The two *Cladosporium* species are plant pathogens.

Culture preparation. The agar diffusion assay was performed according to the Bauer-Kirby-Test (DIN 58940, Bauer et al. 1966). Prior to testing, a few colonies (3 to 10) of the organism to be tested were subcultured in 4 ml of tryptose-soy broth (Sigma, FRG) and incubated for 2 to 5 h to produce a bacterial suspension of moderate cloudiness. The suspension was diluted with sterile saline solution to a density visually equivalent to that of a $BaSO_4$ standards, prepared by adding 0.5 ml of 1% $BaCL_2$ to 99.5 ml of 1% H_2SO_4 (0.36 N). The prepared bacterial broth is inoculated onto Müller-Hinton-Agar plates (Difco, USA) and dispersed by means of sterile beads.

Agar diffusion assay. A mixture ratio of 30:70 for epimer **1** versus **2** was dissolved in dichloromethane and loaded onto sterile filter-paper discs of 5 mm diameter (Oxoid Ltd.) at a dose of 10 and 20 μg. The impregnated discs were placed on agar plates previously seeded with the selected test organisms. Solvent blanks were run against each test organisms. The plates were incubated at 37^oC for 24 h, and then antimicrobial activity was recorded as the clear zone of inhibition surrounding the disc, of which the diameter was measured in mm. A test sample is considered active when the zone of inhibition is greater than 7 mm.

2.9 Chemicals used

2.9.1 General laboratory chemicals

Anisaldehyde (4-methoxybenzaldehyde)	Merck
L-(+)-Ascorbic acid	Merck
Glacial acetic acid	Merck
Formaldehyde	Merck
Hydrochloric acid	Merck
Sodium hydroxide	Merck
Concentrated sulfuric acid	Merck
Trifluroacetic acid (TFA)	Merck
Canada Balsam	Merck

2.9.2 Solvents

Acetone
Acetonitrile
Chloroform
Dichloromethane
Dimethylsulfoxide
Ethanol
Ethyl Acetate
Hexane
Methanol

Solvents were purchased from the Institute of Chemistry, University of Duesseldorf. They were distilled prior to use and spectral grade solvents were used for spectroscopic measurements.

2.9.3 Chromatography:

Precoated TLC plates (AluO, Silica Gel 60 F254, layer thickness 0.2 mm	Merck
Pre-coatred TLC plates (Glass), RP-18, F254 S, layer thickness 0.25 mm	Merck
Silica Gel 60, 0.04-0.063 mm mesh size	Merck

2.10 Equipments used

Balances	Mettler AT 200;Mettler AT 250; Mettler PE 1600; Sartorius RC210P
Centrifuge	TGA-Ultracentrifuge, Rotor 65.13; Konton
Fraction collector	ISCO Cygnet
Freeze Dryer	LYOVAC GT2 Pump TRIVAC D10E
Hot plates	Camag
Syringe	Hamilton 1701 RSN
Mill	Moulinex 354
Magnetic Stirrer	Variomag Multipoint HP
Mixer	Braun
PH-Electrode	Inolab; Behrotest pH 10-Set
Rotary Evaporator	Buchi Rotavap RE111; Buchi Rotavap R-200
Drying Ovens	Heraeus T 5050
Sonicator	Bandelin Sonorex RK 102
UV Lamp	Camag (254 and 366 m,)
Vacuum Exsicator	Savant SpeedVac SPD111V Savant Refrigerator Vapor Trap RVT400 Pump Savant VLP80
Microscope	
Slide and cover glass	
Razor blade	
Scanning Electromagnetik Microscope (SEM)	

3 Results

3.1 Identification of research objects

3.1.1 Phyllidiid species

Family Phyllidiidae is characterized by the replacement of the dorsomedian-circumanal gill circlet which is common in other dorid nudibranchs, with a series of ventrolateral gill leaflets. All collected species in the present study were readily identified through the external morphology and color pattern of the mantle accordingly described in the Brunckhorst's monography (1993). All pictures of the species collected in this study were sent to the sea slugs forum (www.seaslugsforum.net) to get a feed back from other nudibranchs scientists, naturalists, and Brunckhorst himself. Descriptions of all phyllidiid species were given in the section 2.4.1.

3.1.2 Sponge-preys of phyllidiid species

In the present study, all sponges which were predated by phyllidiid species belong to the order Halichondrida as taxonomically described by van Soest & Hooper (2002). Ceractinomorpha Demonspongia possesses styles, oxeas, strongyles, or intermediate spicules of widely diverging size, and are not functionally localized. Microscleres (not always present) are microoxeas and/or trichodragmas. Skeletons are peripherally tangential or undifferentiated and main skeleton is composed of a criss-cross of spicules, or compressed into a distinct axis, or with plumose, either plumo-reticulate or dendritic mineral skeleton. The fibre system could either be poorly developed or absent.

By using the taxonomical key to families under the order Halichondrida as given below, most sponge specimens have characteristic skeletal structures that are taxonomically classified under the family Halichondriidae of the genus *Axinyssa*. Of the total 61 sponge samples, 51 were identified to belong under the genus *Axinyssa*. Four sponge samples were characterized to belong to the family Dictyonellidae and six sponge vouchers were identified to belong to the family Axinellidae.

KEY TO FAMILIES in the ORDER of HALICHONDRIDA

(1) Ectosomal specialization present, either in the form of a tangential crust or single spicule layer, a palisade of smaller and/or larger megascleres or erect or scattered microscleres; surface smooth, but may be wrinkled or thrown up into folds and depressions ..……2

No ectosomal specialization, surface velvety, or hispid due to single projecting spicules, or fleshy-organic, without tangential spicules …….. 3

(2) At the surface there is a tangential layer or a palisade of spiny smaller oxeas or strongyles …… **Desmoxyidae**

No spiny megascleres or microscleres; surface skeleton consists either of tangential single spicules, or palisades c.q. bouquets of smaller spicules mingled with larger choanosomal spicules …….. **Halichondriidae**

(3) Surface velvety or hispid, due to projecting single spicule tracts; sponges firmly resilient ……....4

Surface fleshy-conulose, with organic skin thrown up into conules by choanosomal fibres or spicules; comes off in flakes when attempted to obtain a surface peel …….. **Dictyonellidae**

(4) Thinly encrusting, not exceeding 1 cm thickness, strongly hispid, due to erect monactinal megascleres, with heads embedded in a basal layer of interlacing megascleres…….…… **Bubaridae**

Elaborate growth forms; surface microhispid, often velvety 5

(5) Choanosomal skeleton reticulate, often with axial and extra-axial differentiation …… **Axinellidae**

Choanosomal skeleton confused …….…. **Halichondriidae** (genus *Axinyssa*)

[source: van Soest & Hooper, 2002]

Sponges of the genus *Axinyssa* collected in this study have two types of spiculation. The first type has a randomly arranged skeleton with a slightly hispid surface due to the projection of the ends of some loose oxeot spicules (Figure 16a), while the second type has a randomly arranged skeleton with a microconulose surface due to the projection of the ends of some bundled oxeot spicules (Figure 16b). These two types were then further classified into seven species according to the size of full grown spicules (see Table 4 and the key to species of *Axinyssa* spp.)

1. Surface is slightly hispid due to the projection of the ends of some loose oxeot spicules …… **2**
   Surface is microconules due to the projection of the ends of some bundled oxeot spicules …….... **3**
2. Branching compressed-lobate or simple lobate growth form; life specimen is orange to brown purplish orange; fully grown spicule sizes are 1.29-1.47 mm by 0.1175 (median size 1.15-1.23 mm) ……. ***Axinyssa 1*** **n. sp.**
   Compressed-massive and compressed-lobate growth form; life specimens are dark brown purple but are very pale yellow after being preserved in alcohol; fully grown spicule is 1.09 mm by 0.1175 (median size 0.94 mm) and 1.15 mm by 0.1175 (median size 1.01 mm) ……. ***Axinyssa aplysinoides***
   Flattened growth form with small fistule-like at each corner end; life specimen is pale yellowish brown; fully grown spicule size is 1.40 mm by 0.1175 (median size 1.24 mm) …….. ***Axinyssa 2*** **n. sp.**
3. Lobate life form; purplish brown in live condition; the longest spicule is 1.53 mm by 0.1175 (median size 1.28 mm) …….. ***Axinyssa 3*** **n. sp.**
   Life form varies, fully grown spicule size is less than 1.30 mm by 0.1175 … **4**
4. Lobate or semi errect life form; life specimens are dark purplish brown or pale purple; fully grown spicule sizes are longer than 1.00 mm but less than 1.30 mm. Longest oxeot spicules are 1.02-1.25 mm by 0.1175 .. ***Axinyssa 4*** **n. sp.**

   Lobate growth form; life specimens are dark purplish brown; fully grown spicules sizes are longer than 0.80 but less than 1.00 mm; longest oxeot spicules are 0.87-0.95 mm by 0.1175 ……..... ***Axinyssa 5*** **n. sp.**

   Compressed lobate or encrusting-flattened growth form; life specimens are dark purplish brown or yellowish pale brown; fully grown spicules size are 0.80 mm or even less …….. ***Axinyssa 6*** **n. sp.**

Table 4. The taxonomical division of *Axinyssa* specimens according to the size of their fully grown spicules.

		Longest size of fully grown spicule (mm)								
		0.70<x≤0.80	0.80<x≤0.90	0.90<x≤1.00	1.00<x≤1.10	1.10<x≤1.20	1.20<x≤1.30	1.30<x≤1.40	1.40<x≤1.50	1.50<x≤1.60
Median Size of fully Grown Spicule (mm)	0.60<x≤0.70	**(POR 17054)**; FM04								
	0.70<x≤0.80	PP04-XIV **(A)**	PP04-III; PV04-X	PP04-XVI; PV04-II						
	0.80<x≤0.90		**(B)**	PP04-VIII; PV04-IX	**(POR 17287)**; PV04-I, VIII, XII, XIII					
	0.90<x≤1.00			PK02	[*PK04-I*]; PP04-I, II, VII PV04-III, XVI; PZ04-VI	PS02; PV04-XI, XIV; PP04-X; PZ04-VII, X	PZ04-IV **(C)**	PK04-IV		
	1.00<x≤1.10				PV04-VI, XVII PZ04-V PV00	[*PZ02*]; PV04-XV; PP04-V, IX PZ04-II, IX	PP02; PV04-IV, V; PP04-IV, VI, XII	**(D)**		
	1.10<x≤1.20						PK04-II	PZ04-VIII, XI	PZ04-I	
	1.20<x≤1.30							PK04-III **(E)**	PP04XI	PZ04-III **(F)**

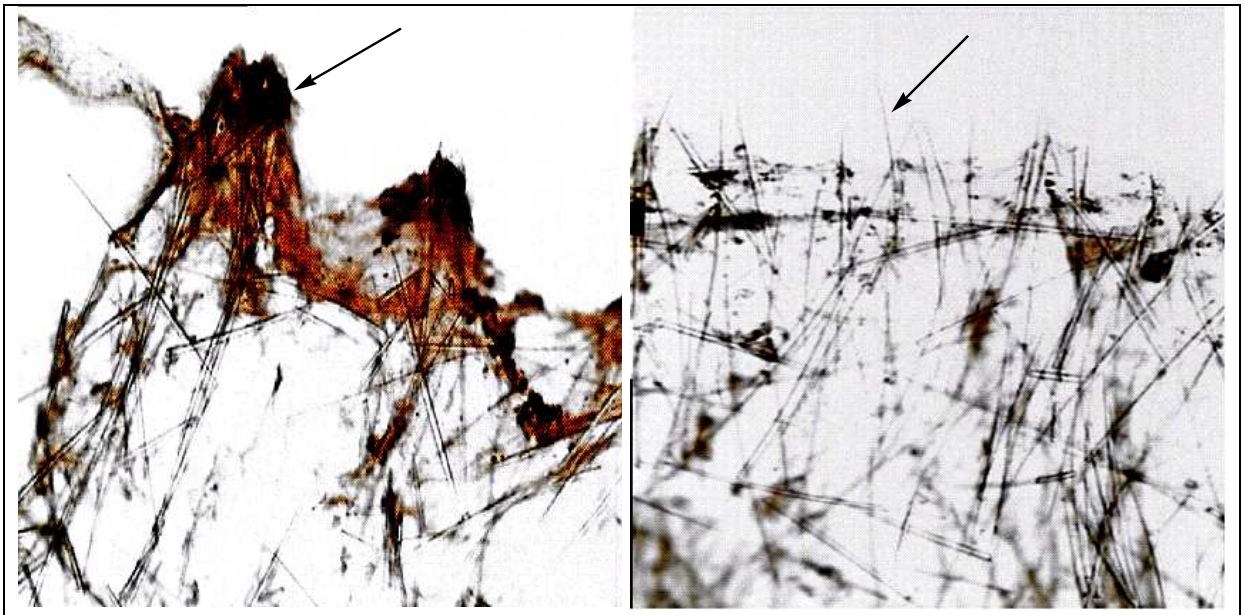


Figure 16. Types of spiculation in the sponge *Axinyssa* spp. collected in the Thousand Islands National Park, Indonesia: a. microconulose spiculation; b. hispid spiculation. Arrow shows the spicule projection above the surface.

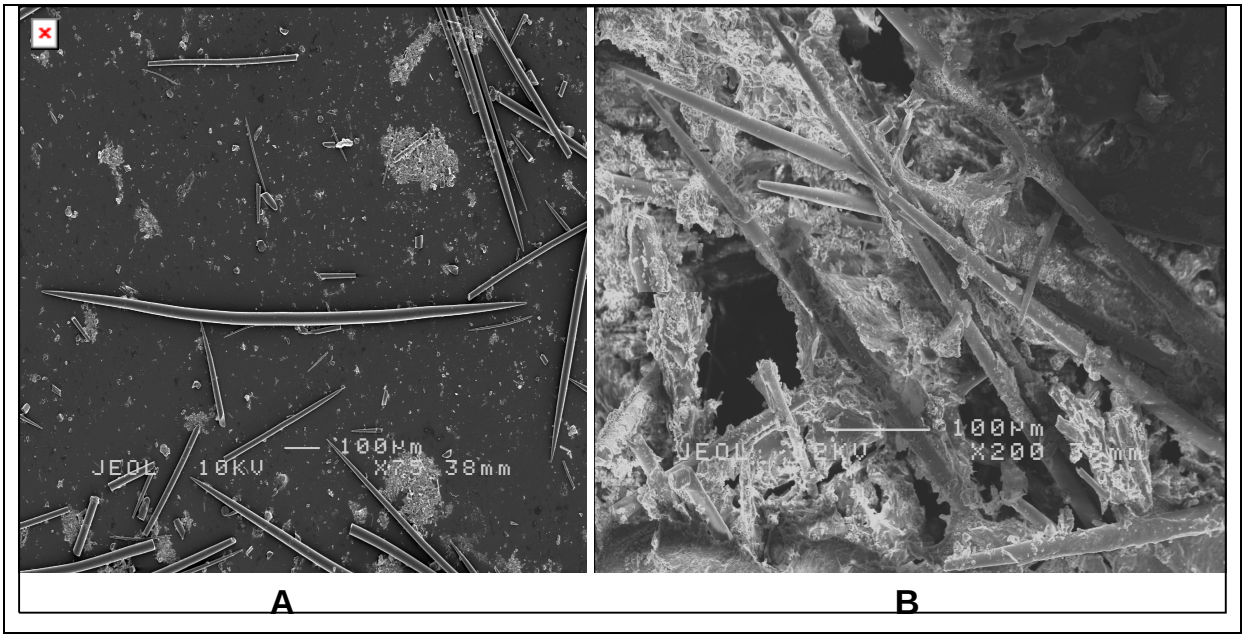


Figure 17. Scanning Electron Microscope of spicule of *Axinyssa 3* n. sp. A. A slightly curved, smooth, gradually sharp-pointed oxeot spicule; B. The appearance of a collagenous ground substance in between the scattered spicules.

The sponge *Dragmacidon* sp. collected in this study has a plumose reticulation type of spiculation. All sponge specimens belonging to these species have megascleres that are mainly made up of stylote with some oxeot spicules that allow them to be identified under the genus *Dragmacidon*. The sponge specimens of *Acanthella cavernosa* that were collected were readily classified from their color and habitus of the live specimens. Microscopic analysis of their spiculation then further unambiguously confirmed their taxonomy as *A*. *cavernosa*. The descriptions of all sponge species collected in the present study were given under section 2.4.2.

3.2 Feeding and behaviour observations in the aquarium

Feeding of phyllidiid species has been very well described by Brunckhorst in 1993. Phyllidiid species evert their pharyngeal bulb while feeding upon a sponge (Brunckhorst, 1993). In this study, the evertions of phyllidiid's pharyngeal bulb were also observed in 62 predation records in the field. A documentation of the evertion of phyllidiids's pharyngeal bulb is shown for *P*. *varicosa* and *P*. *zeylanica* in Figure 18 and Figure 19. The phyllidiids were observed feeding on two different sponge pieces of *Axinyssa 5* n. sp. Traces of feeding on the sponge-preys of *P*. *varicosa* was also

well documented (Figure 18). These two documentations are reported for the first time.

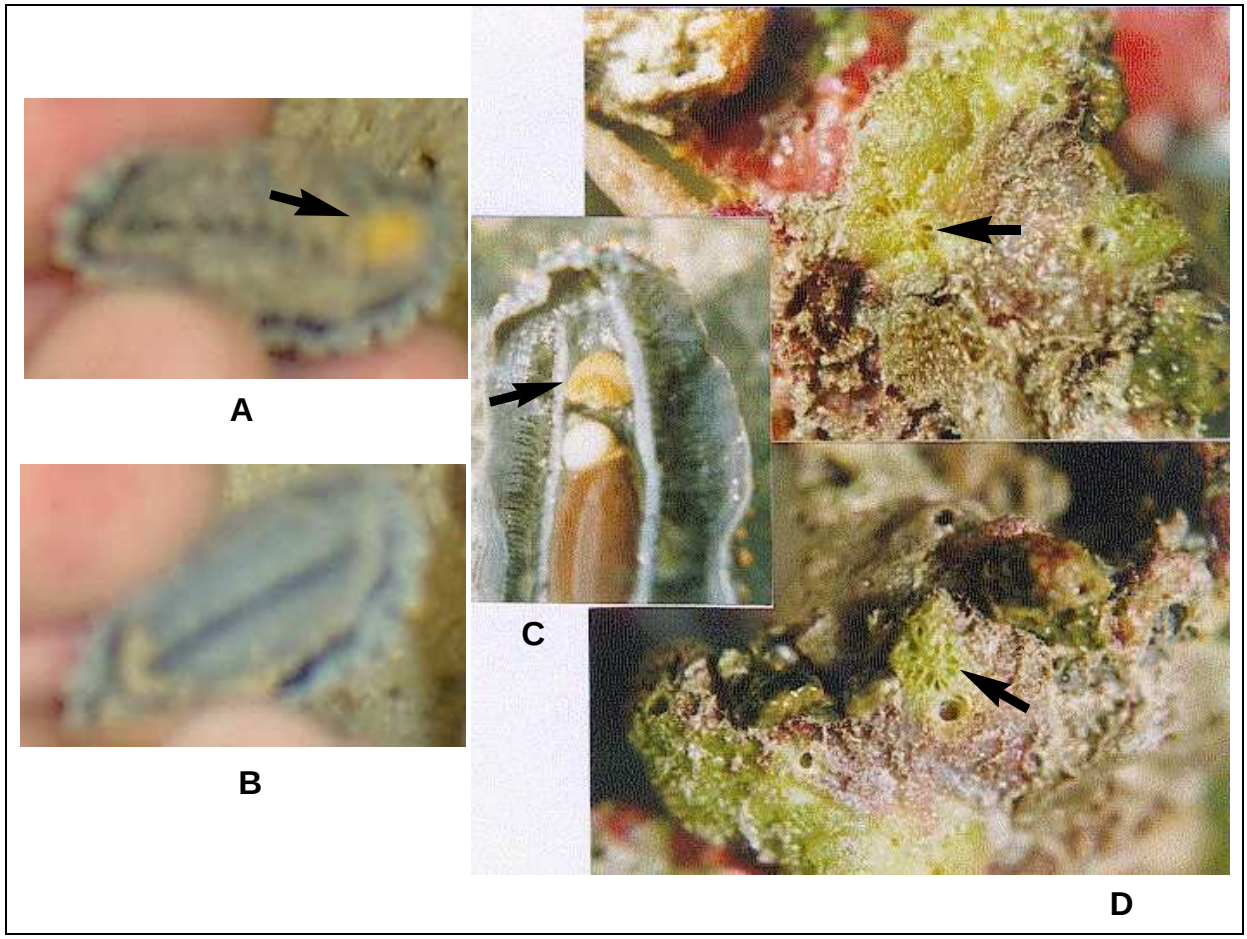


Figure 18. The documentation of the evertion of *P*. *varicosa*'s pharyngeal bulb and feeding traces found on its prey: A. The arrow shows the evertion of *P*. *varicosa*'s pharyngeal bulb. Photograph was taken right after *P*. *varicosa* was abruptly separated from its sponge-prey; B. After a few minutes, the pharyngeal bulb was totally withdrawn into the mouth; C. Pharyngeal bulb was exposed in dissected specimen; D. Feeding traces found on its sponge-prey as shown by the arrow.

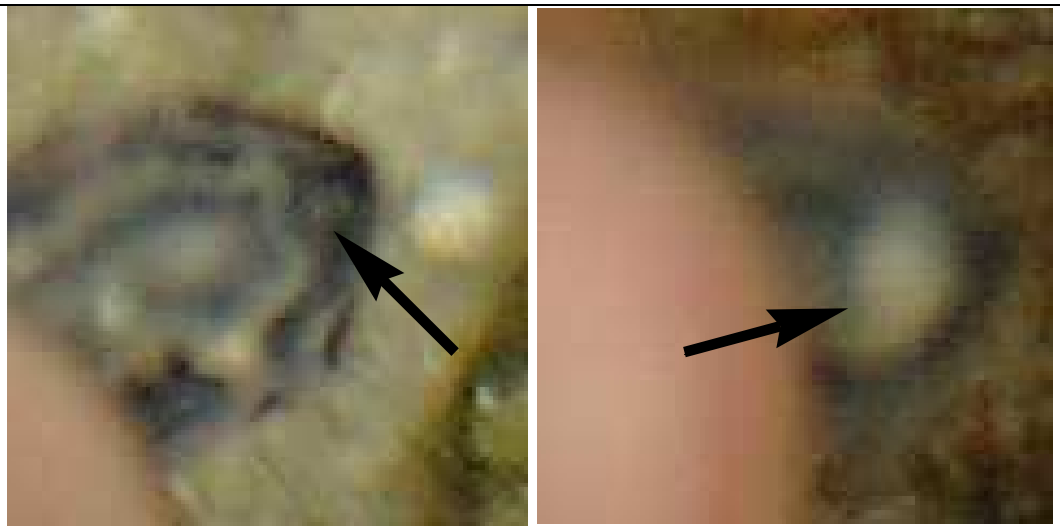


Figure 19. Photographs documenting the evertion of *P*. *zeylanica*'s pharyngeal bulb.: A. After a few minutes, the pharyngeal bulb was totally withdrawn into the mouth (see arrow); B. The evertion of *P*. *zeylanica*'s pharyngeal bulb (see the arrow). Photograph was taken right after *P*. *zeylanica* was abruptly taken out from its sponge

As shown in this study, phyllidiid species tend to be specialist predators on sponges of the order Halichondrida. Identification of the collected sponge-preys showed that six phyllidiid species (*Phyllidiella pustulosa*, *P*. *zeylanica*, *Phyllidia varicosa*, *Phyllidiopsis krempfi*, *P*. *shireenae*, and *Fryeria menindie*) tend to be specialist predators on sponges of the genus *Axinyssa* (fam. Halichondriidae) (see Figure 20); three phyllidiid species (*Phyllidia ocellata*, n=1; *Phyllidiella lizae*, n=2; *P*. *rudmani*, n=1) tend to be specialist predators on the sponge *Acanthella cavernosa* (fam. Dictyonellidae); while one species (*Phyllidia elegans*, n=6) tends to be consistently feeding on the sponge *Dragmacidon* sp. (fam. Axinellidae).

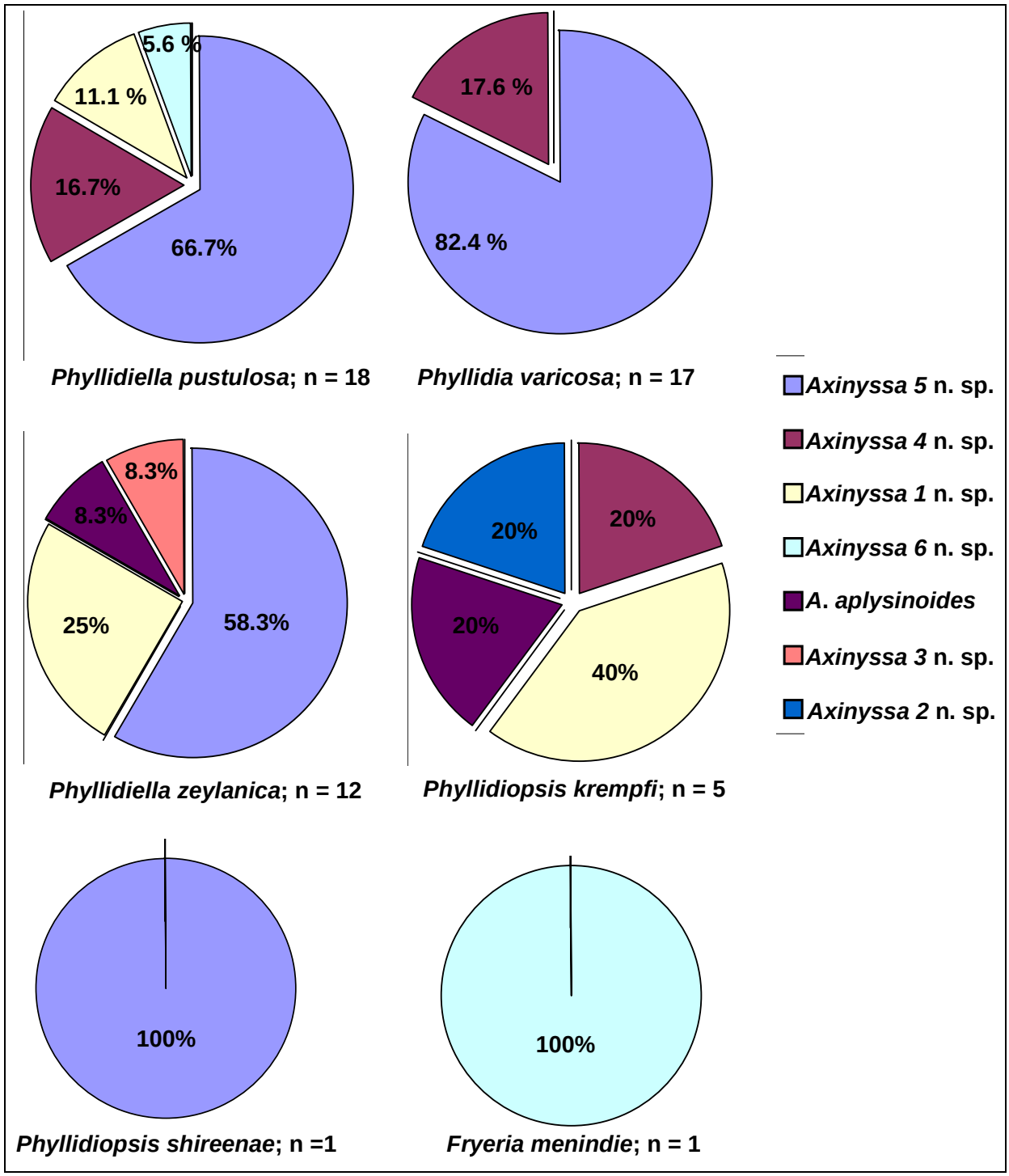


Figure 20. Feeding preference of phyllidiid species against the sponge *Axinyssa* spp. Note: data were obtained from *in situ* observation of 52 predation records as well as *ex situ* observation of 2 predation records.

Most phyllidiid species kept in a fiber aquarium were observed just crawling and resting either on the wall of the aquarium, on dead corals, or on the sponge *Axinyssa 5* n.sp. The nudibranchs were rarely observed actively crawling on the sand surface unless when they transfer from one substrate to another. No feeding activities were recorded during the 24-hour observation period.

P. *varicosa* was observed predating on the sponge *Axinyssa 5* n.sp. which was observed *in situ* to be the sponge-prey of *P*. *pustulosa*. On the other hand *P*. *zeylanica* predated on the sponge *Axinyssa 5* n.sp. which was observed *in situ* to be predated by *P*. *varicosa*. Both predations were recorded on the 11th day of observation. However, no feeding activities were observed occurring on the sponge *Acanthella cavernosa*. It was observed that phyllidiids have no ability to conceal themselves under dead corals or to seek refuge in the presence of coral fishes (three wrass fishes, one spine foot, one trigger fish, and one leather-jacket fish) actively swimming in their environment.

Some abnormal activities were also recorded, which included mating between the different species i.e. between *Phyllidiella pustulosa* and *P*. *zeylanica* as well as between *Phyllidia ocellata* and *P*. *elegans* (Figure 21). These were observed when the organisms lift up their mantle edge to expose their gills (Figure 22), and when they lie upside down on the water surface (Figure 22). *P*. *pustulosa*, *P*. *ocellata*, and *P*. *shireenae* were also observed to lay egg masses on the aquarium wall (Figure 21). However it was not proven if they were fertile or sterile egg masses. Predation of those phyllidiid egg masses by fishes in the aquarium may suggest that it does not contain any isocyanide compounds or the concentration of these compounds was too low. No records of predation by fishes on the phyllidiids were observed. Even a large hermit crab (*Pagurus* sp.) that lived in the aquarium gave no records of predation.

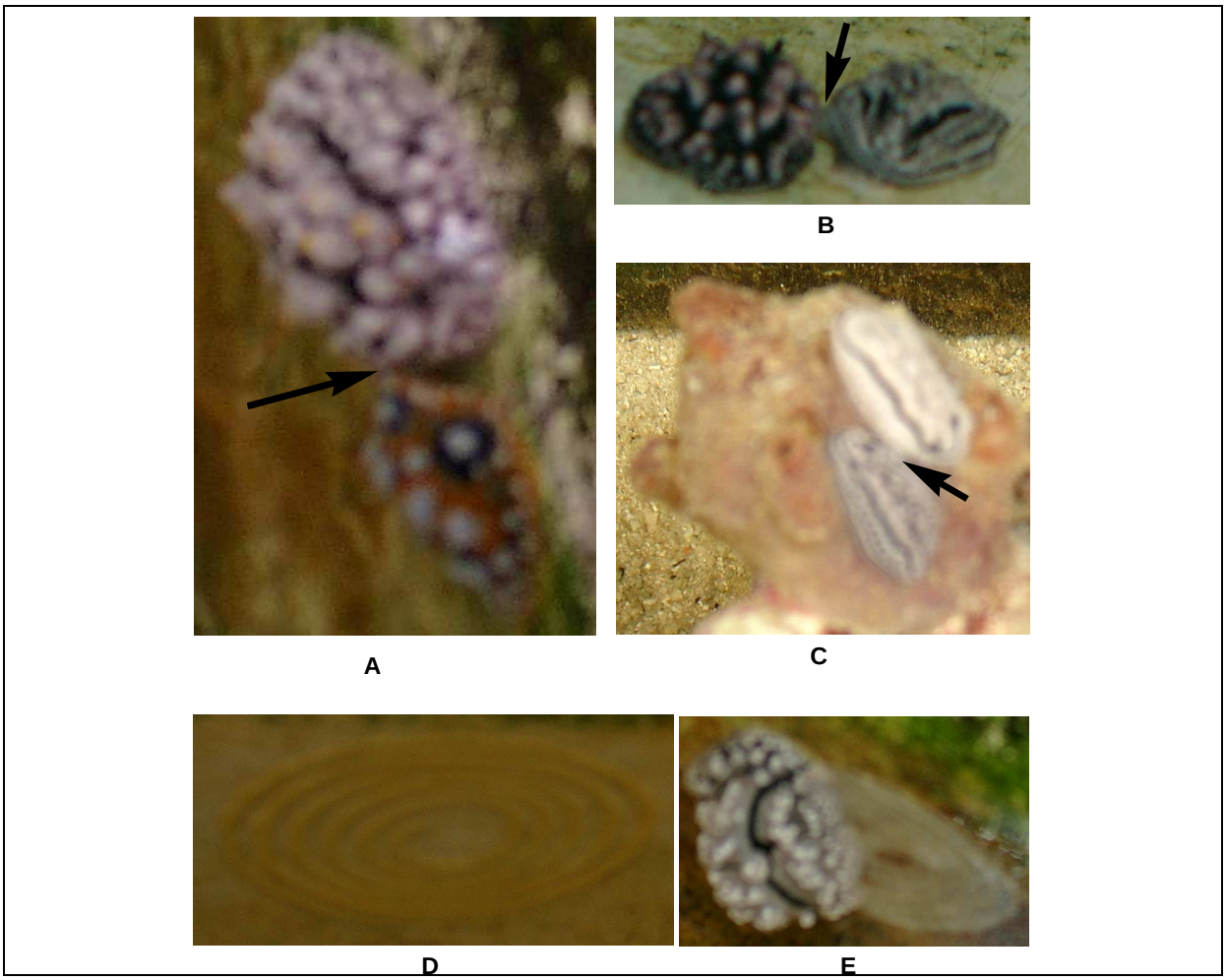


Figure 21. (A) Mating between *P*. *elegans* and *P*. *ocellata*; (B) Mating between *P*. *pustulosa* and *P*. *zeylanica*; (C) Mating between two *P*. *zeylanica* specimens; (D) spiral-like egg mass of *P*. *ocellata*; (E) *P*. *krempfi* laying its egg mass. Note: the arrows show the interchange of semi-penis of both individuals.

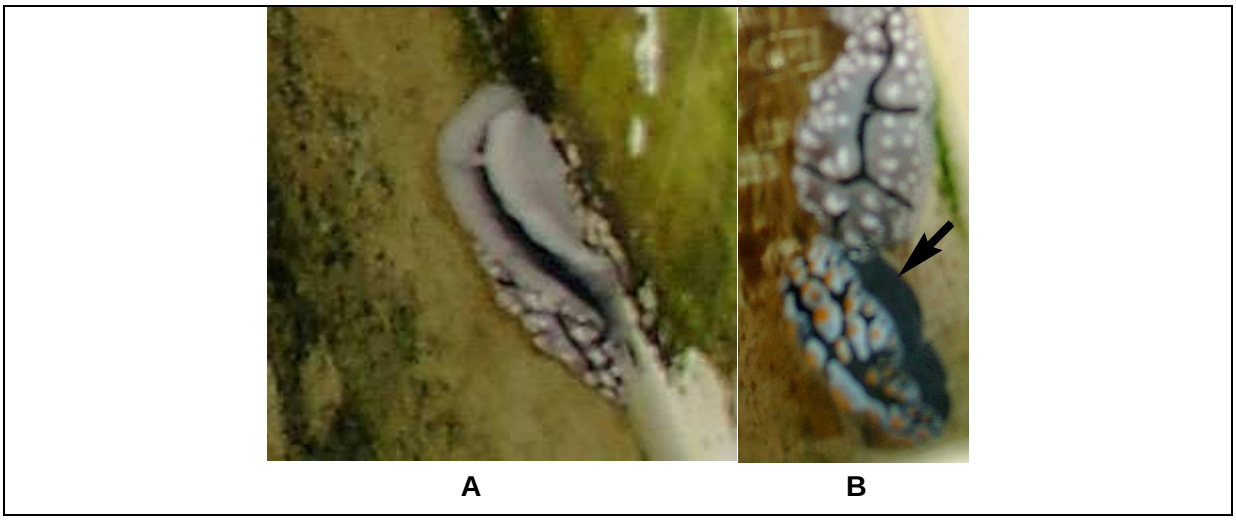
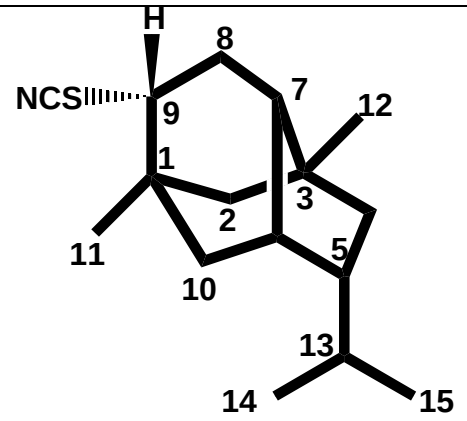


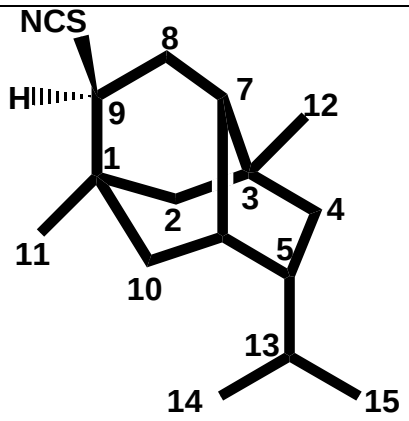
Figure 22. Some unusual behaviour of phyllidiids in an aquarium. (A) *Phyllidiopsis krempfi* initiated to lie upside down on the water surface; (B) *Phyllidia varicosa* lifted up its mantle edge of to expose its gills (see arrow)

3.3 Isolated isocyanide compounds

3.3.1 Isolated compound 9α- & 9β-thiocyanatopupukeanane (1, 2; new compounds)



Compound **1**



Compound **2**

Characteristic : colorless oil
Molecular Formula : $C_{16}H_{25}SN$
Molecular weight : 263

The compounds were isolated as an epimeric mixture. The presence of the two isomers was detected by GC coupled to a FID detector at Rt 16.8 (**2**) and 17.4 (**1**) (Figure 23). Both compounds gave a molecular weight of 263, and their MS spectra also indicated identical fragmentation patterns. The base peak was observed at *m/z* 205, demonstrating the loss of a –SCN function. HREIMS data established the molecular formula $C_{16}H_{25}SN$. The co-occurrence of the two epimers, **1** and **2,** was clearly evident from their ^{1}H and ^{13}C NMR spectra showing separated resonances for H-9, C-9, C10, and C-2 for each of the epimers. Proton and carbon assigments for each isomer were inferred by 2D $^{1}H,^{1}H$-COSY, HMBC, and HMQC (Table 5 for compound **1** & Table 6 for compound **2**). Full assignments of the ^{1}H-NMR data were accomplished through a *J*-resolved experiment. The ROESY data confirmed the presence of two epimers at C-9.

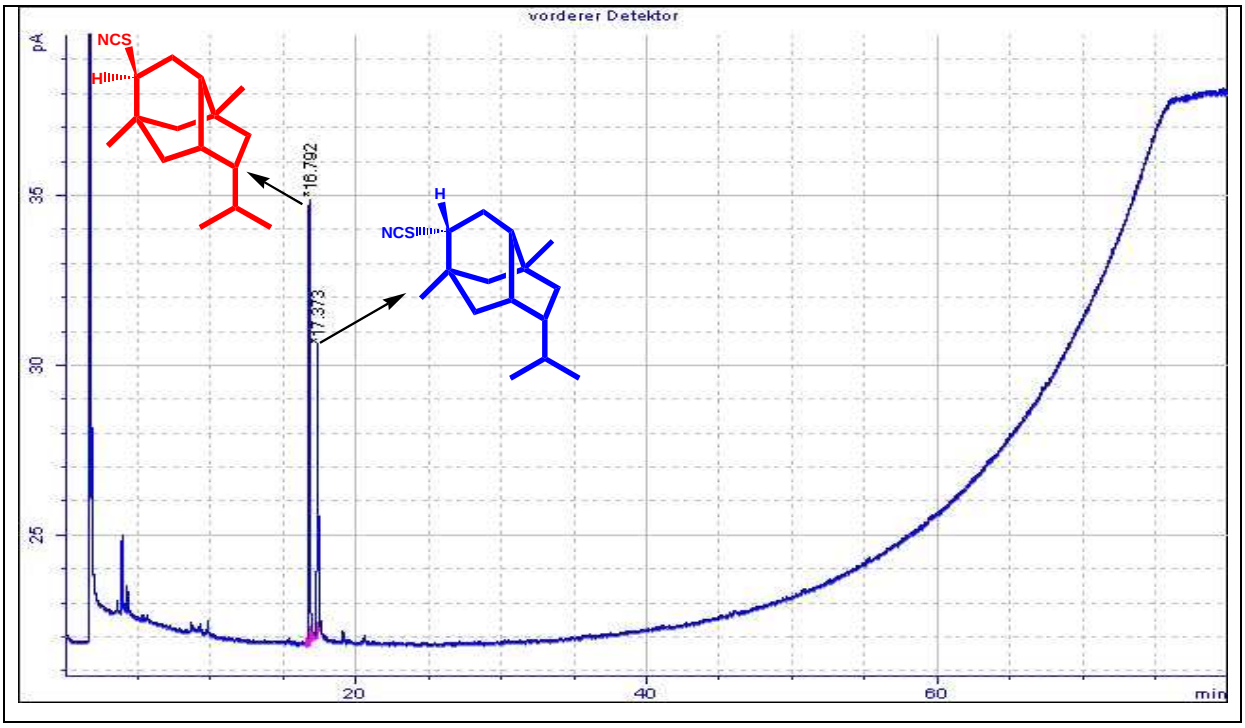


Figure 23. GC chromatogram of the epimers **1** and **2**.

The basic structures of **1** and **2**, as well as their ^{1}H and ^{13}C NMR data, were comparable to those of 9-isocyanopupukeanane and 9-isothiocyanato-pupukeanane, which were previously isolated from phyllidiid *Phyllidiella rosans* (also known as *Phyllidia bourgini*) and another sponge species of the genus *Axinyssa* and its predator *P. pustulosa*, respectively (Fusetani *et al*., 1990; Simpson *et al*., 1997). The presence of a thiocyanate function at C-9 as opposed to an isothiocyanate group was confirmed by inspection of the ^{13}C NMR spectrum and comparison with previously reported data (Burreson *et al*., 1975a; Hagadone *et al*., 1979; Fusetani *et al*., 1990; Simpson *et al*., 1997). The ^{13}C chemical shifts for epimers **1** and **2** were comparable with those of 9-isothiocyanatopupukeanane and 9-isocyano-pupukeanane epimers. The major difference in chemical shift was observed for C-16 of the respective congeners. For 9-isothiocyanatopupukeanane, the chemical shift at ca. δ 125 referred to the presence of an isothiocyanate group (Simpson *et al*., 1997), while the chemical shift at ca. δ 155 as in 9-isocyanopupukeanane epimers indicated the occurrence of an isocyanide moeity (Burreson *et al*., 1975a; Fusetani *et al*., 1990; Simpson *et al*., 1997). For epimers **1** and **2**, the carbon shift at δ 113.5 characterized the presence of a thiocyanate function as described for 2- and 4-thiocyanatoneopupukeanane (Pham *et al*., 1991; Simpson *et al*., 1997). Epimers **1** and **2** can be distinguished from each other by the difference in their ^{13}C chemical

shifts for C-2 (δ 46.9 for **1**, δ 55.0 for **2**) and C-10 (δ 35.5 for **1**, δ 27.3 for **2**). This pattern of differences was also observed in the co-occurrence of 9-isocyanopupukenane epimers in the phyllidiid *P*. *rosans* (as *P*. *bourgini*) and *P*. *pustulosa* (Fusetani *et al*., 1990; Fusetani *et al*., 1991). The signals for C-2 and C-10 of **2** are compatible with those in 9-isocyanopupukeanane and 9-isocyanatopupukeanane, indicating the same configuration of their functional groups. The presence of the two epimers was observed from the 2D COSY spectrum as presented by a four-bond "w" coupling between H-9 (δ_H 3.40) and H-2 (δ_H 1.00) in **1**, while epimer **2** showed a similar coupling between H-9 (δ_H 3.30) and H-10 (δ_H 1.16). Coupling constants were evaluated from 2D *J*-resolved ^{1}H spectrum: 2.3 Hz ($^4J_{9\text{-}2B}$) for epimer **1** while epimer **2** gave a coupling constant of 2.5 Hz ($^4J_{9\text{-}10B}$). Additional evidence of the presence of the two epimers was also provided by the significant differences in the coupling constants for CH_2-8 to H-9 in the respective epimers. For epimer **1**, H-9 gave coupling constants of 10.6 Hz ($^3J_{8A\text{-}9}$) and 6.6 Hz ($^3J_{8B\text{-}9}$), while **2** gave coupling constants of 10.8 Hz ($^3J_{8A\text{-}9}$) and 4.2 Hz ($^3J_{8B\text{-}9}$).

The relative stereochemistry in both epimers was confirmed from a 2D ROESY spectrum. The correlation of δ_H 3.40 (H-9) with δ_H 1.38 (CH_2-10B) attested to the α-configuration of the –SCN group in **1**, which indicated that H-9 was *cis* to H-10B. Correlation of δ_H 3.30 (H-9) with δ_H 1.25 (CH_2-2B) confirmed the β-configuration of the –SCN group in **2**, where H-9 was *cis* to H-2B. Similar responses were also observed in the NOESY spectra of the 9-isocyanopupukenane epimers (Fusetani *et al*., 1990; Fusetani *et al*., 1991). Therefore, epimers **1** and **2** were elucidated unambiguously as 9α-thiocyanatopupukeanane and 9β-thiocyanatopupukeanane, respectively.

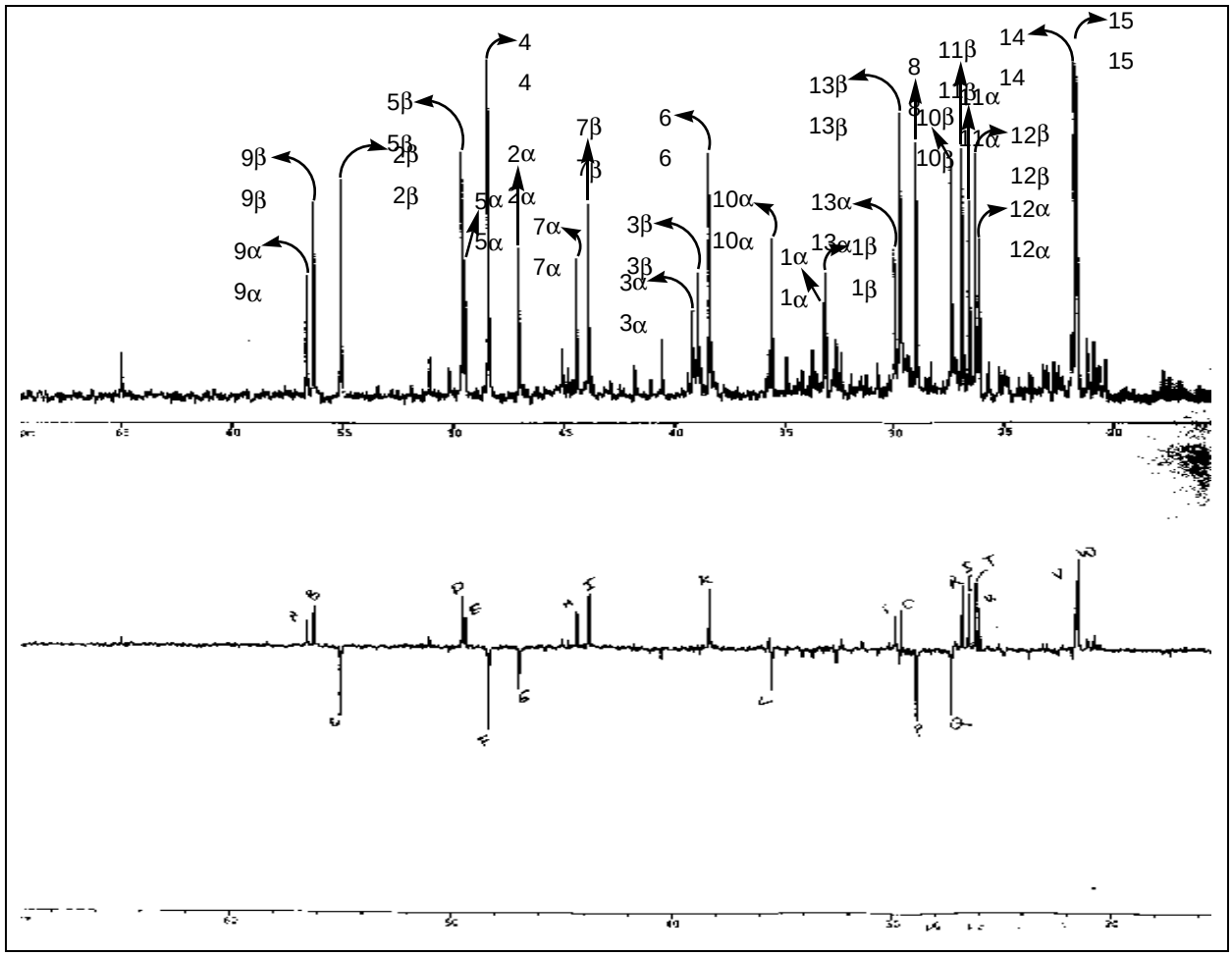


Figure 24. ^{13}C NMR and DEPT spectra of the isolated compounds **1** and **2**.

Table 5. NMR data for compound **1**.

	δ_C (mult.)	δ_H (mult., *J*)	HMBC (δ_H to δ_C)
1	33.1 (s)		
2	46.9 (t)	1.58 (ddd, 14.1, 3.5, 1.8)	C-1, C-3, C-5, C-6, C-9
		1.00 (ddd, 14.0, 2.3, 1.3)	
3	39.1 (s)		
4	48.3 (t)	1.83 (ddd, 13.3, 10.3, 2.8)	C-2, C-3, C-5
		1.14 (dd, 13.5, 8.0)	
5	49.3 (d)	1.40 (dddd, 12.5, 10.4, 8.2, 4.8)	
6	38.3 (d)	2.11 (br dddd, 6.3, 5.5, 4.5, 3.5)	C-4
7	44.3 (d)	1.33 (ddd, 6.2, 3.8, 2.5)	
8	28.9 (t)	2.35 (ddd, 15.5, 10.5, 3.8)	C-1, C-6, C-7, C-9
		1.72 (ddd, 15.5, 6.8, 2.5)	
9	56.5 (d)	3.40 (ddd, 10.6, 6.6, 2.3)	C-1, C-8, C-11, C-16
10	35.5 (t)	1.46 (ddd, 13.5, 5.5, 3.5)	C-11, C-1, C-3, C-7, C-4
		1.38 (dd, 13.0, 3.5)	
11	26.5 (q)	0.93 (s)	C-1, C-2, C-10
12	26.0 (q)	1.01 (s)	C-2, C-3, C-4, C-7
13	29.7 (d)	1.52 (ddq, 12.5, 6.4, 6.4)	
14	21.6 (q)	0.84 (d, 6.4)	C-5, C-15
15	21.5 (q)	0.84 (d, 6.4)	C-5, C-13, C-14
16	113.5 (s)		

Table 6. NMR data for compound **2**.

	δ_C (mult.)	δ_H (mult., *J*)	HMBC (δ_H to δ_C)
1	33.0 (s)		
2	55.0 (t)	1.37 (dd, 3.8, 1.8)	C-1, C-3, C-4, C-7, C-11
		1.25 (ddd, 13.5, 3.5, 1.5)	
3	38.9 (s)		
4	48.3 (t)	1.85 (ddd, 13.3, 10.3, 2.8)	C-2, C-3, C-5
		1.16 (dd, 13.5, 8.0)	
5	49.6 (d)	1.44 (dddd, 12.5, 10.5, 7.8, 4.9)	
6	38.3 (d)	2.09 (bm)	C-4
7	43.7 (d)	1.35 (ddd, 6.0, 3.0, 2.5)	
8	28.9 (t)	2.40 (ddd, 15.7, 10.8, 3.0)	C-1, C-6, C-7, C-9
		1.89 (ddd, 15.7, 4.4, 3.0)	
9	56.2 (d)	3.30 (ddd, 10.8, 4.2, 2.5)	C-1, C-2, C-8, C-11, C-16
10	27.3 (t)	1.65 (ddd, 14.8, 10.3, 3.5)	C-11, C-1, C-3, C-7, C-4
		1.17 (dd, 14.5, 2.5)	
11	26.8 (q)	0.92 (s)	C-1, C-2, C-10, C-9
12	26.2 (q)	0.98 (s)	C-2, C-3, C-4, C-7
13	29.6 (d)	1.56 (ddq, 12.5, 6.4, 6.4)	C-5, C-14, C-15
14	21.6 (q)	0.85 (d, 6.4)	C-5, C-13, C-15
15	21.5 (q)	0.83 (d, 6.4)	C-5, C-14
16	113.5 (s)		

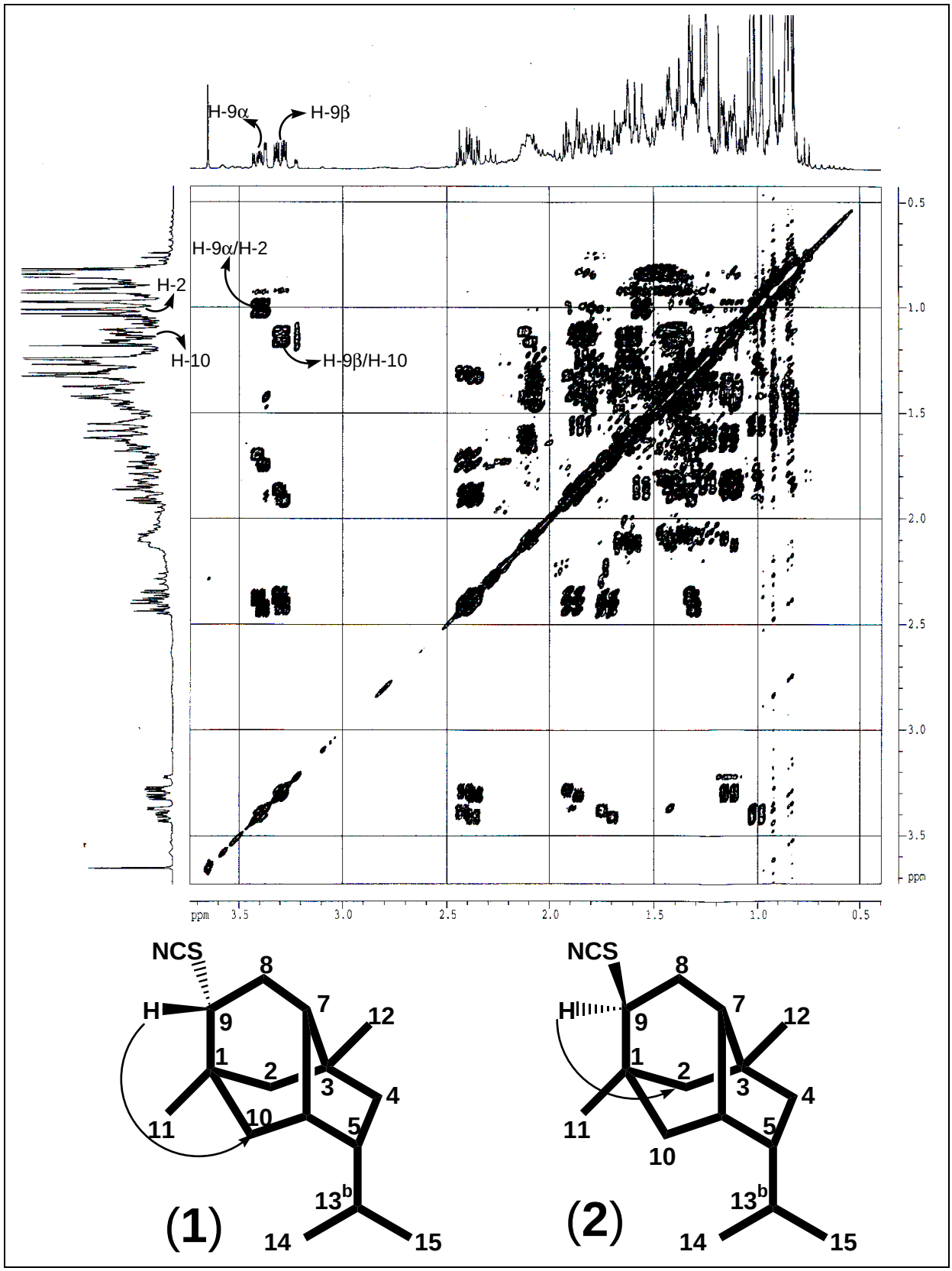


Figure 25. COSY spectrum of compounds **1** and **2**.

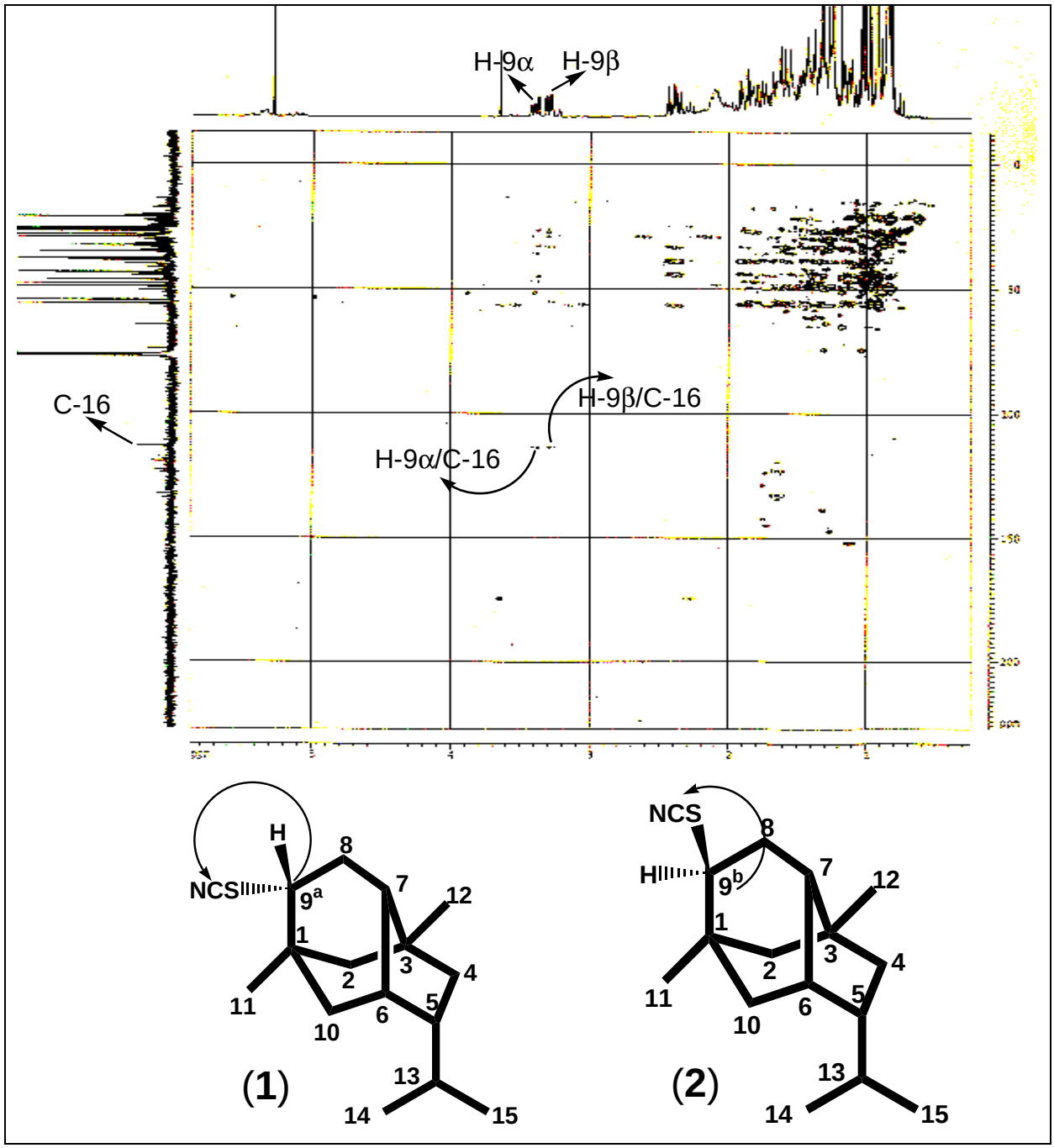


Figure 26. Full HMBC NMR spectrum and direct evidence of the correlation between H-9α with C-16 for compound **1** and H-9β with C-16 for compound **2**.

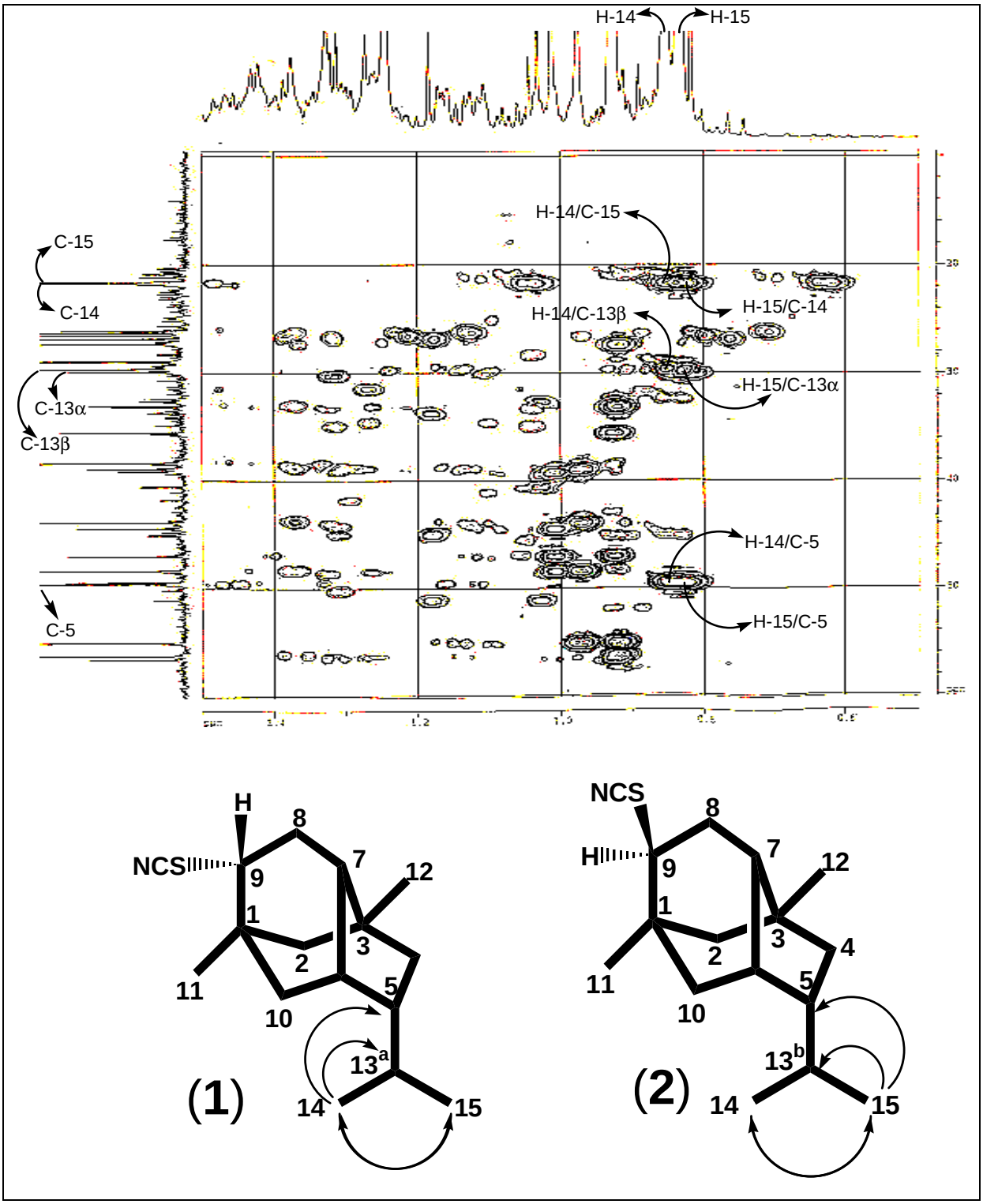


Figure 27. Expansion of HMBC NMR spectrum to show direct evidence of the existence of isopropyl unit in compounds **1** and **2**.

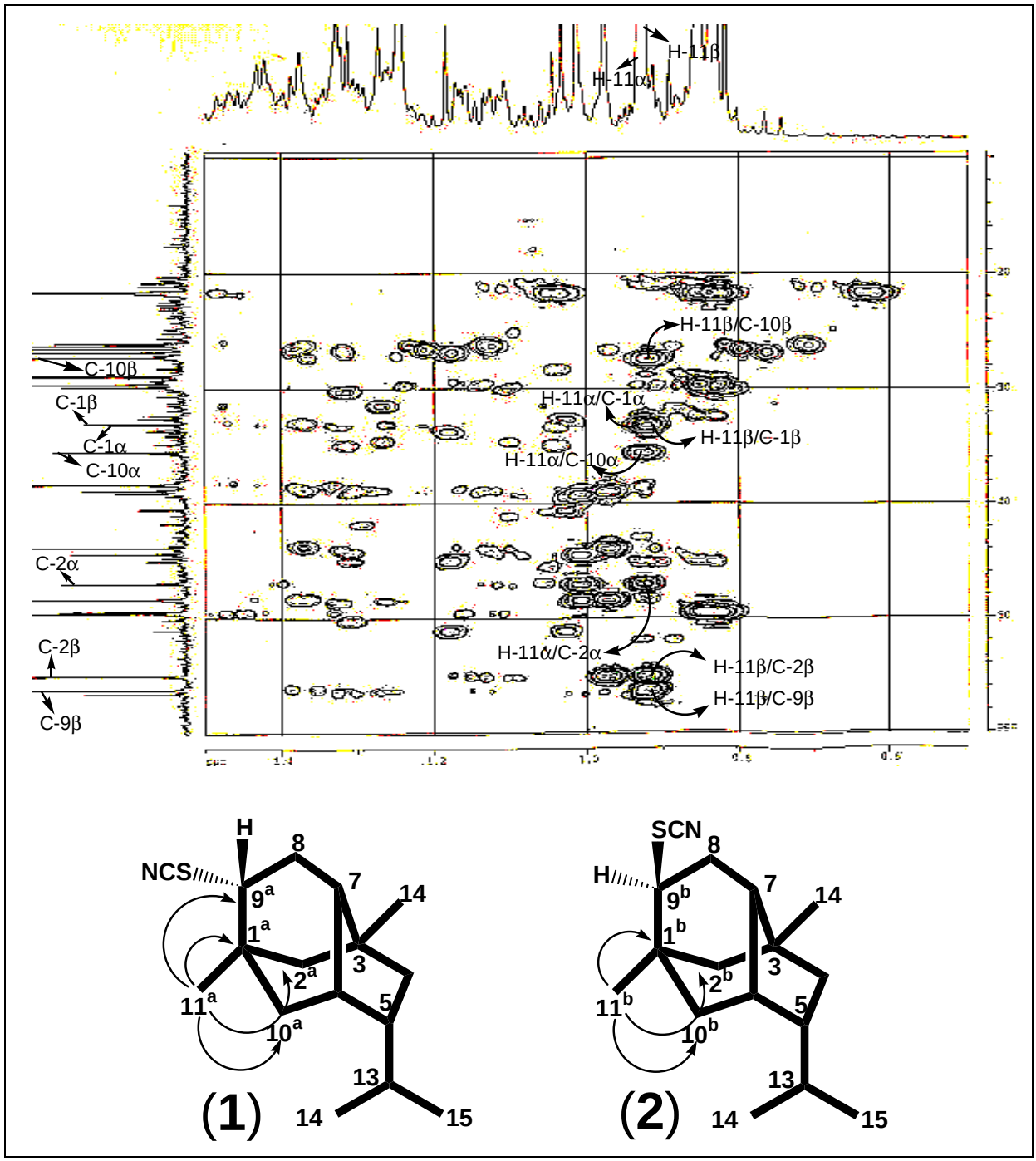


Figure 28. Expansion of HMBC NMR spectrum to confirm direct evidence of methyl position (C*H*$_3$-11) in compounds **1** and **2**.

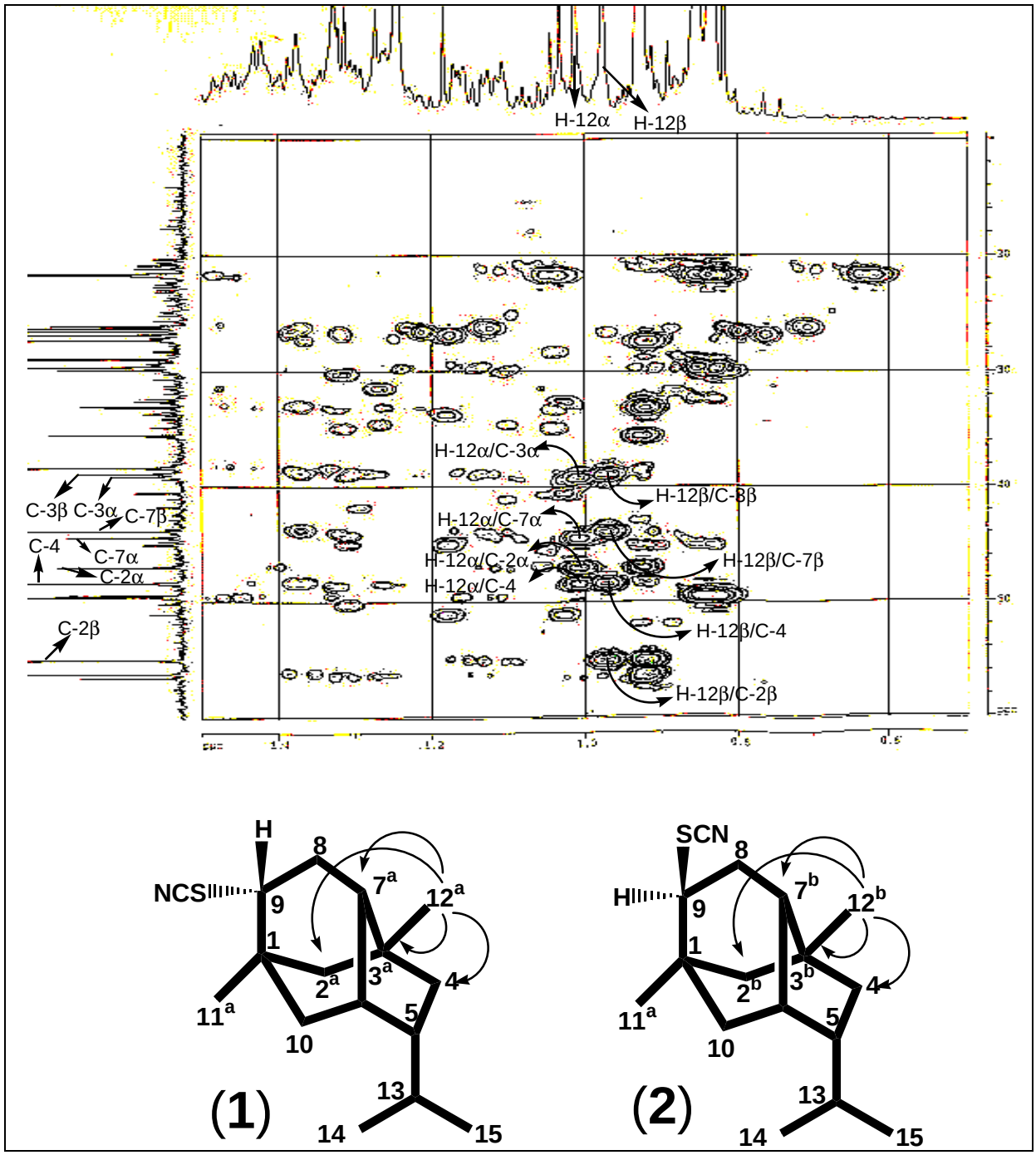


Figure 29. Expansion of HMBC NMR spectrum to confirm direct evidence of methyl position (C*H*$_3$-12) in compounds **1** and **2**.

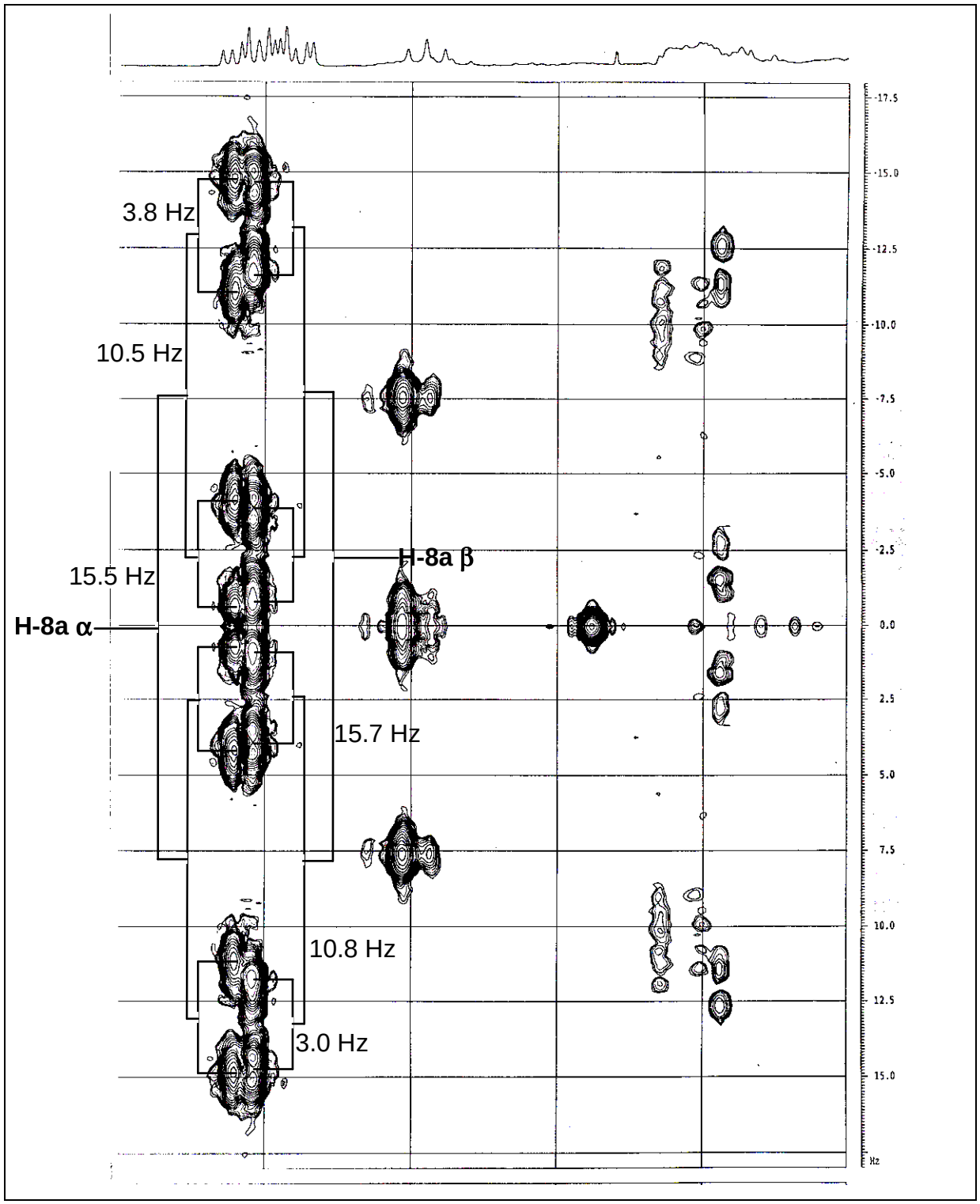
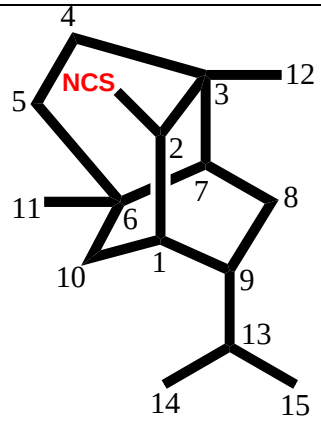


Figure 30. Expansion of *J*-resolved NMR spectrum to show the multiplicity and coupling constant of proton H-8a in compound **1** and proton H-8a in compound **2**.

3.3.2 Isolated compound 2-thiocyanatoneopupukeanane (3; known compound)



Compound **3**

Characteristic : colorless oil
Molecular Formula : $C_{16}H_{25}SN$
Molecular weight : 263

Sesquiterpene 2-thiocyanatoneopupukeanane was first isolated from the sponge *Phycopsis terpnis* (Fam. Axinellidae) by the group of Scheuer in 1991, followed by the group of Faulkner (1992) from the sponge *Axinyssa aplysinoides* (Fam. Halichondridae) (Pham *et al*., 1991; He *et al*., 1992). More recently, the group of Garson was also able to isolate 2-thiocyanatoneopupukeanane from Australian sponge *Axinyssa* sp. nov. (Fam. Halichondridae) (Simpson *et al*., 1997).

In the present study, 2-thiocyanatoneopupukeanane (**3**) was isolated from the sponge *Axinyssa aplysinoides* (Fam. Halichondridae). In this study, compound **3** was found in all sponge species predated by phyllidiid species in the coral reefs of Thousand Island, Indonesia. Compound **3** was isolated as a colorless oil. The presence of **3** was detected by GC coupled to a FID detector at R.T. 15.4 minute (Figure 33). The EIMS spectrum showed the molecular ion peak at *m/z* 263 (Figure 32). The important fragment at *m/z* 205 showed that the backbone was similar to epimers **1** and **2**. This fragment also indicated the loss of a -SCN functional group. The structure of **3** was established as 2-thiocyanatoneopupukeanane from 1D and 2D NMR spectra which were comparable to literature data (Pham *et al*., 1991; He *et al*., 1992; Simpson *et al*., 1997). The ^{13}C NMR and DEPT showed that compound **3** has 16 carbons which consist of four methyl signals, four methylene signals, five methine signals, and three quarternary carbons of which the signal at δ_C 113.9

confirmed again the presence of a thiocyanate group. The HMBC correlations between Me-11 (δ_H 1.02, s) with C-5, C-6, and C-10 established the position of attachment at C-6 whereas the correlations between Me-12 (δ_H 1.17, s) with C-2, C-3, and C-4 established the position of attachment at C-3. Furthermore, the cross peaks between Me-11 and Me-12 with C-7 established the connectivity between the two substructures (Figure 31 & Figure 36). The position of attachment of the isopropyl at C-9 was confirmed from the HMBC correlations of the two methyl doublets, Me-14 and Me-15 which also coupled to each other and with C-13 and C-9 (Figure 36). Inspection of COSY spectrum showed the two spin systems in compound **3**. The first spin system involved the methylene protons H_2-5 with H_2-4; whereas the second spin system started at the methine proton carrying –SCN functionality at H-2 with H-1, then to methylene proton H-10 which coupled with H-1, then to H-9, H-8b (δ_H 1.28, ddd, 15.1, 5.4, 2.8 Hz), and H-7 (δ_H 1.01, t, 3.16 Hz) as well as for coupling between the methine proton H-9 with H-13, then to H-14 (δ_H 0.86, d, 6.6 Hz) and H-15 (δ_H 0.93, d, 6.6 Hz) which consequently established the position of the attachment of the isopropyl function on compound **3** at C-9 as also confirmed in its HMBC spectrum (Figure 35). From these 1D and 2D NMR data, compound **3** was therefore elucidated unambiguously as 2-thiocyanatoneopupukeanane.

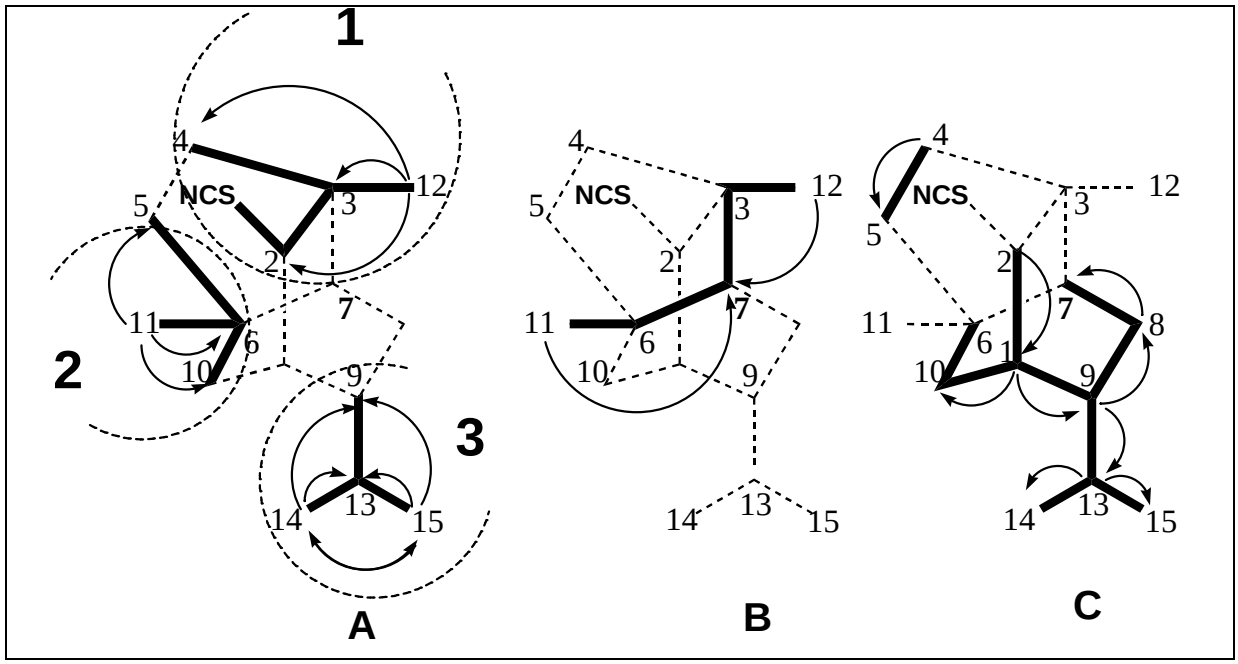


Figure 31. A. Three sub structures obtained from HMBC spectrum; B. The connectivity of substructure 1 and 2 obtained from HMBC spectrum; C. Two spin systems showed by COSY spectrum.

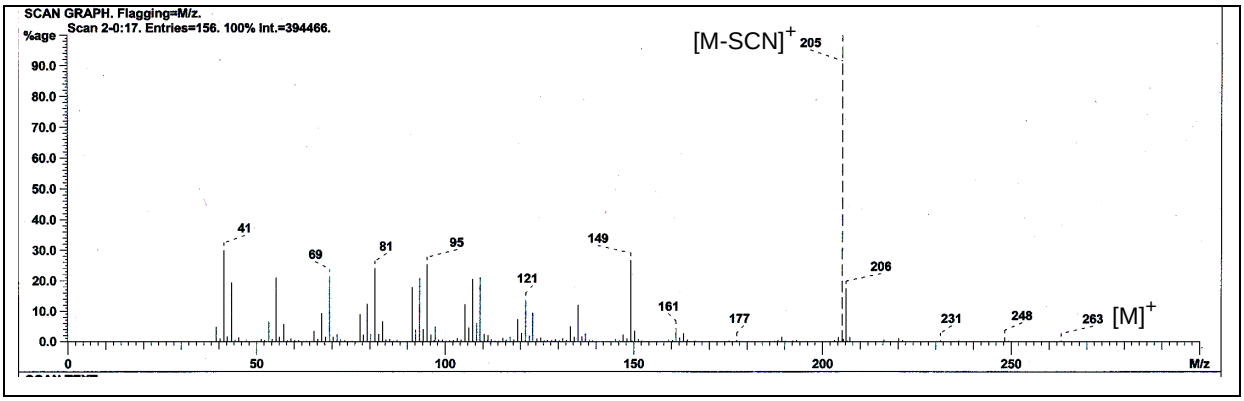


Figure 32. EIMS spectrum of compound **3**.

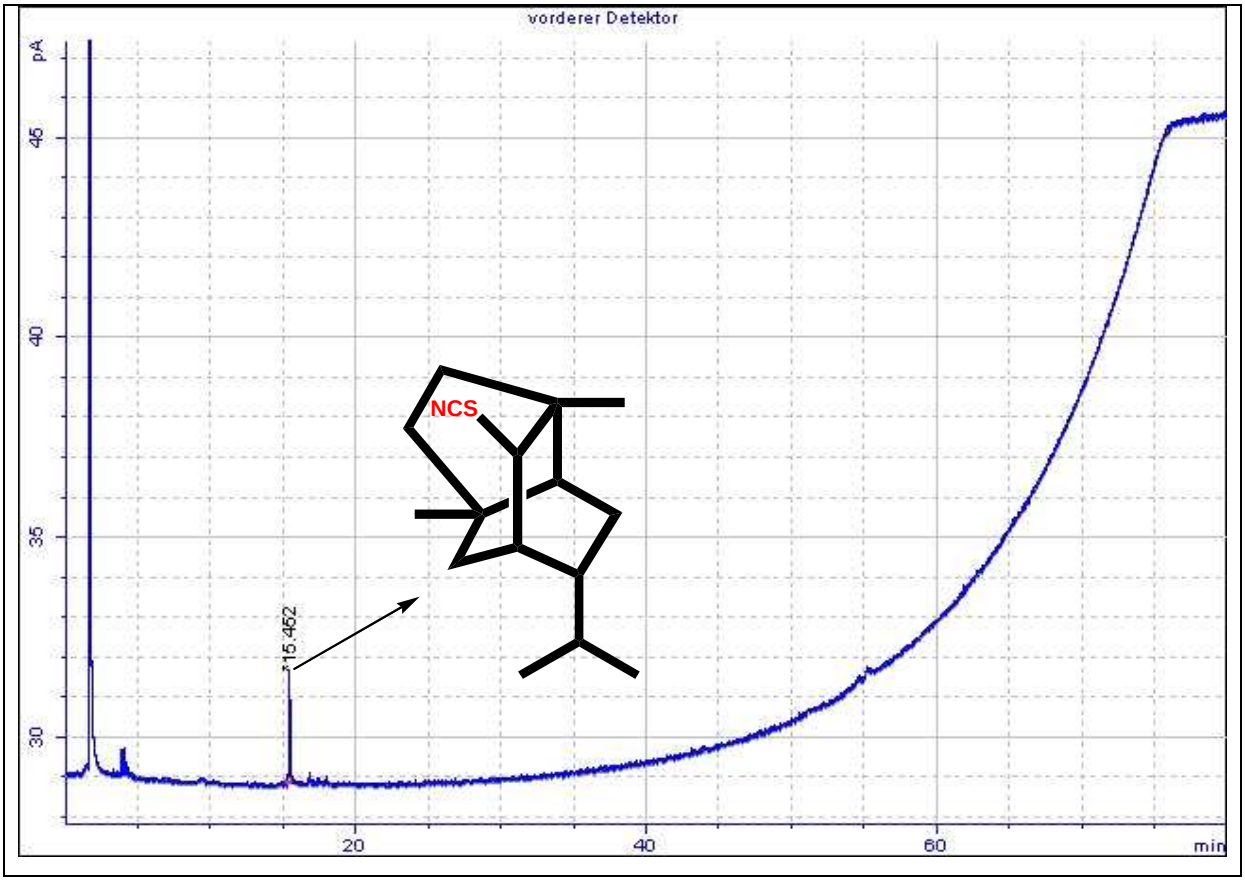


Figure 33. GC chromatogram of compound **3**.

Table 7. ^{13}C and ^{1}H NMR data of compound **3** with comparison to available literature data.

	^{13}C (δ_C, mult.)		^{1}H (δ_H, mult., *J* in Hz)	
	Literature (Pham *et al*., 1991)	compound **3**	Literature (Pham *et al*., 1991)	compound **3**
1	35.6	35.6, d	1.88, bs	1.91, bs
2	64.9	64.9, d	3.36, bs	3.38, bs
3	44.9	44.9, s	-	-
4	33.7	33.6, t	a. 1.79, m	a. 1.66, m
			b. 1.33, m	b. 1.37, m
5	40.5	40.5, t	a. 1.47, m	a. 1.47, m
			b. 1.33, m	b. 1.40, m
6	39.3	39.3, s	-	-
7	51.0	51.0, d	0.99, t, 3.15	1.01, t, 3.16
8	21.8	21.8, t	a. 1.79, ddd, 14.7; 10.9; 3.3	a. 1.81, ddd, 14.5; 11.0; 3.5
			b. 1.23, ddd, 14.8; 5.6; 2.8	b. 1.28, ddd, 15.1; 5.4; 2.8
9	45.0	44.9, d	0.98, m	1.01, m
10	32.6	32.6, t	1.40, m	1.44, m
11	26.2	26.2, q	1.02, s	1.04, s
12	26.5	26.5, q	1.17, s	1.19, s
13	32.4	32.4, d	1.41, m	1.44, m
14	20.8	20.8, q	0.83, d, 6.7	0.86, d, 6.6
15	21.2	21.2, q	0.91, d, 6.5	0.93, d, 6.6
16	114.2	113.9, s	-	-

Table 8. Important correlations in COSY & HMBC spectra of compound **3**.

	^{1}H	COSY (δ_H- δ_H)	HMBC (δ_H- δ_C)
1	1.91	H-10	
2	3.38	H-10, H-1	C-3, C-16
3	-		
4	a. 1.66	H-4b, H5a	
	b. 1.37		
5	a. 1.47		
	b. 1.40		
6	-		
7	1.01		
8	a. 1.81	H-7, H-9, H-8b	
	b. 1.28	H-9, H-7	
9	1.01	H-13	
10	1.44		
11	1.04		C-5, C-6, C-7, C-10
12	1.19		C-2, C-3, C-4, C-7
13	1.44	H-14, H-15	
14	0.86		C-9, C-13, C-15
15	0.93		C-9, C-13, C-14
16	-		

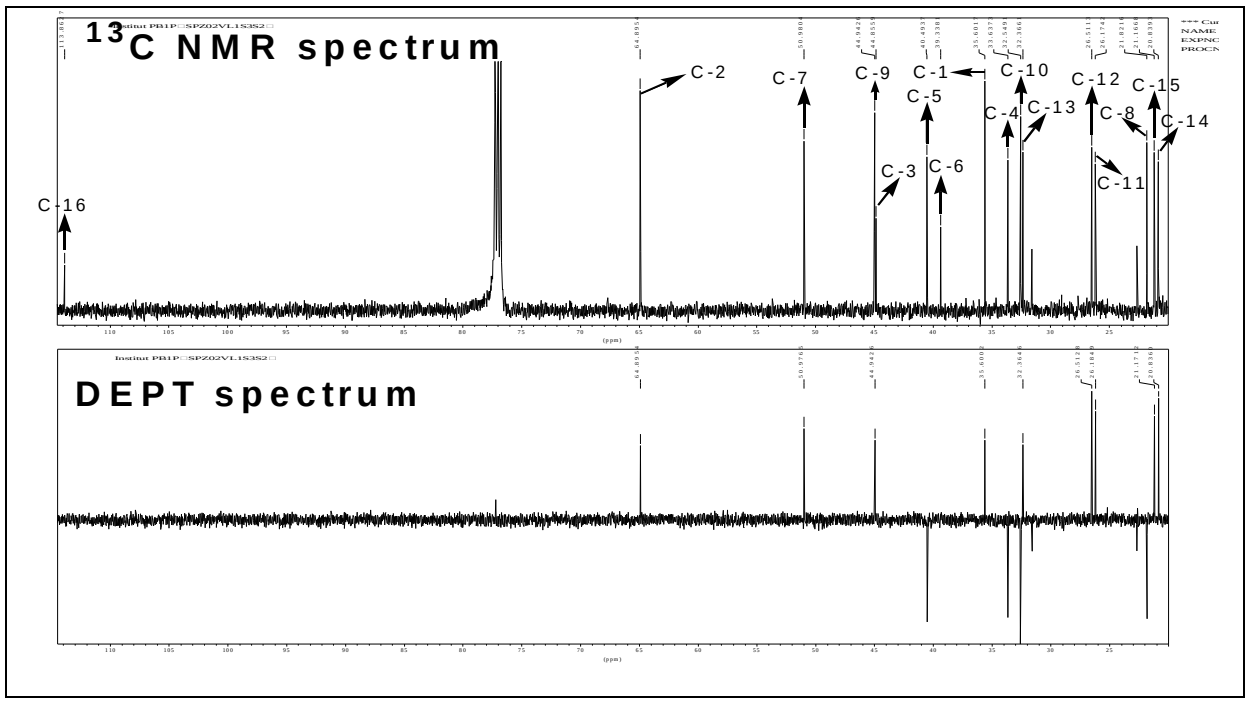


Figure 34. ^{13}C NMR and DEPT spectra of the isolated compound **3**.

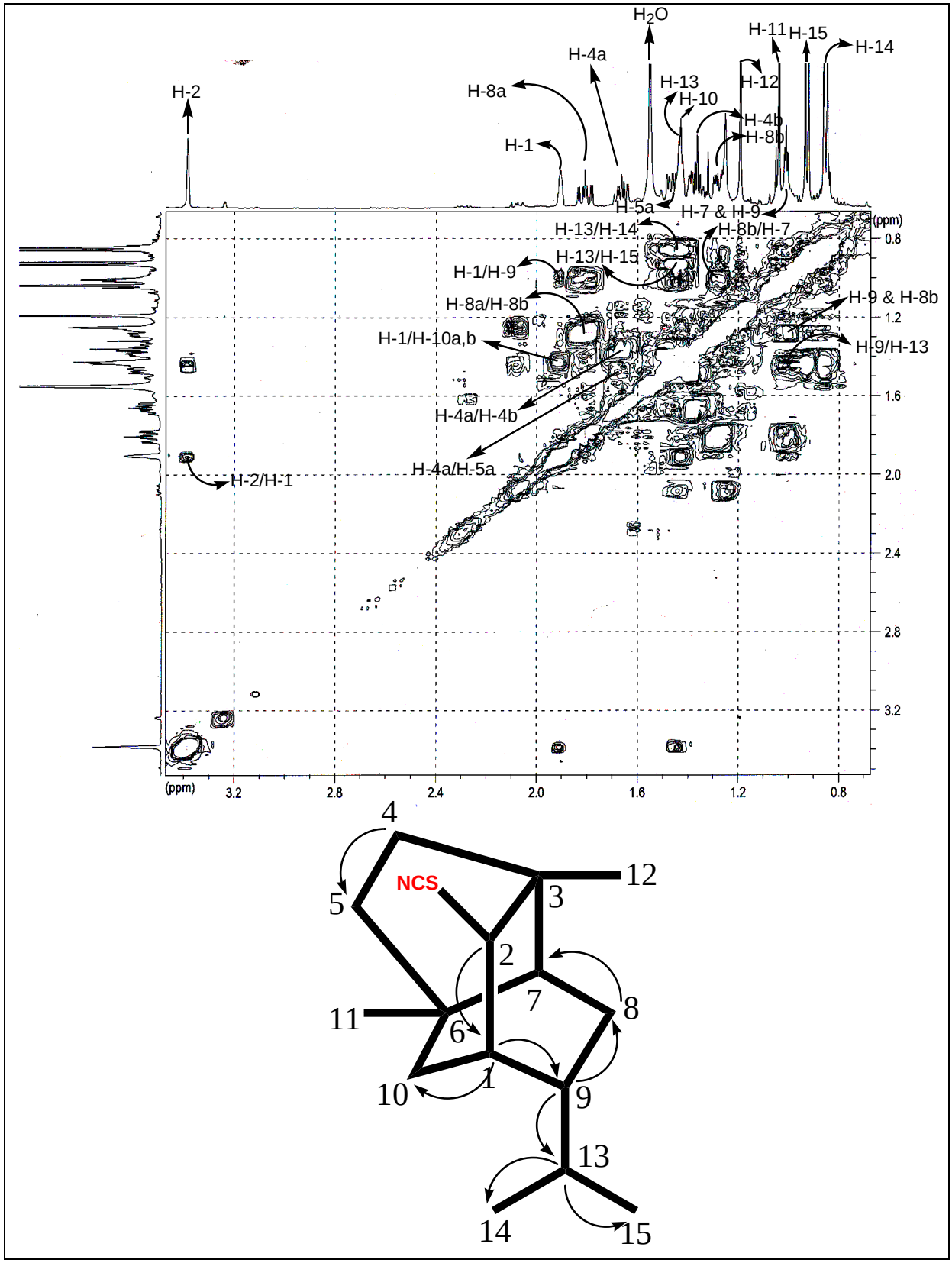


Figure 35. Full COSY spectrum of compound **3**. The two spin systems are shown.

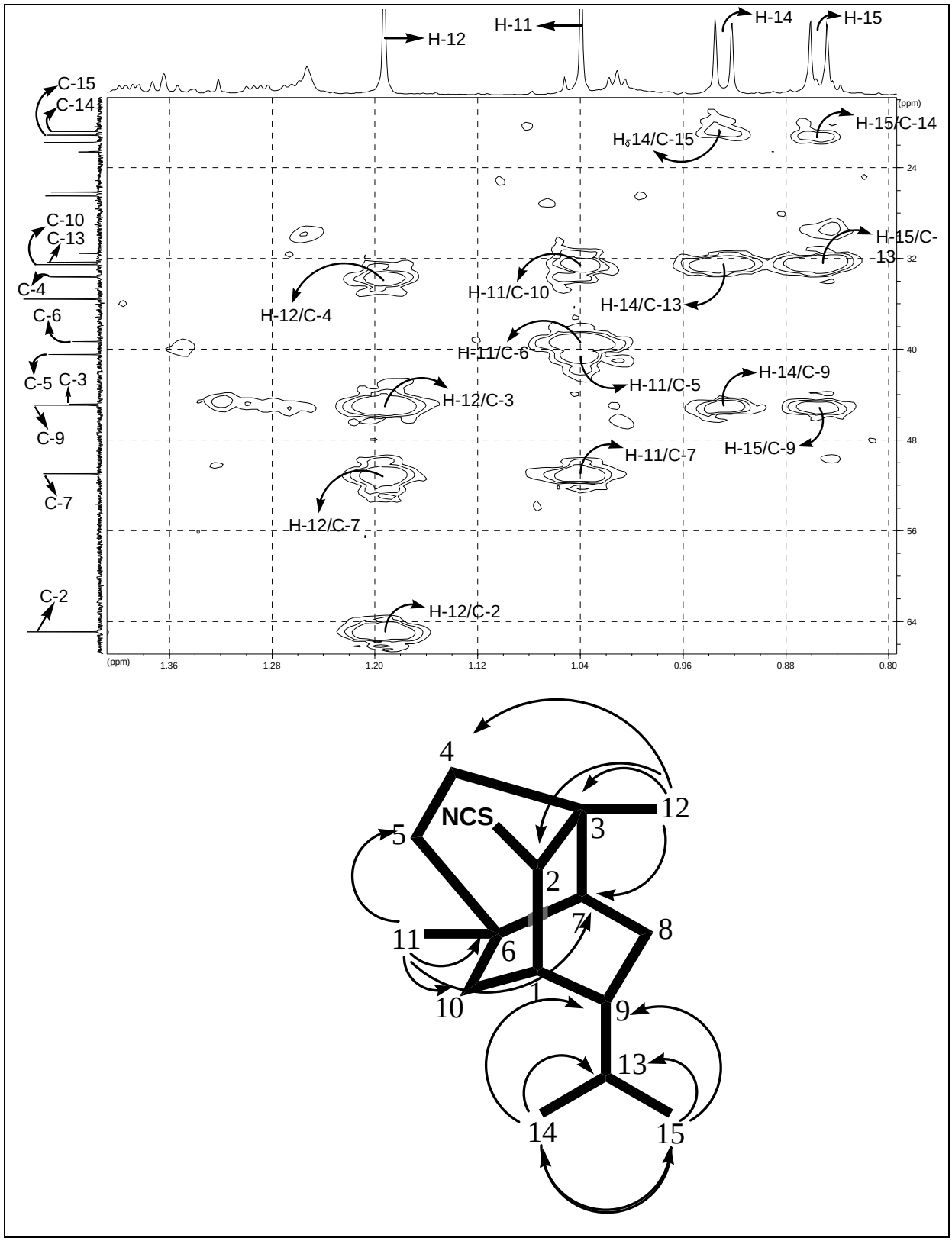


Figure 36. Expansion of HMBC NMR spectrum to confirm direct evidence of methyl positions (C*H*$_3$-11 and C*H*$_3$-12) and the existence of isoprophyl in the compound **3**.

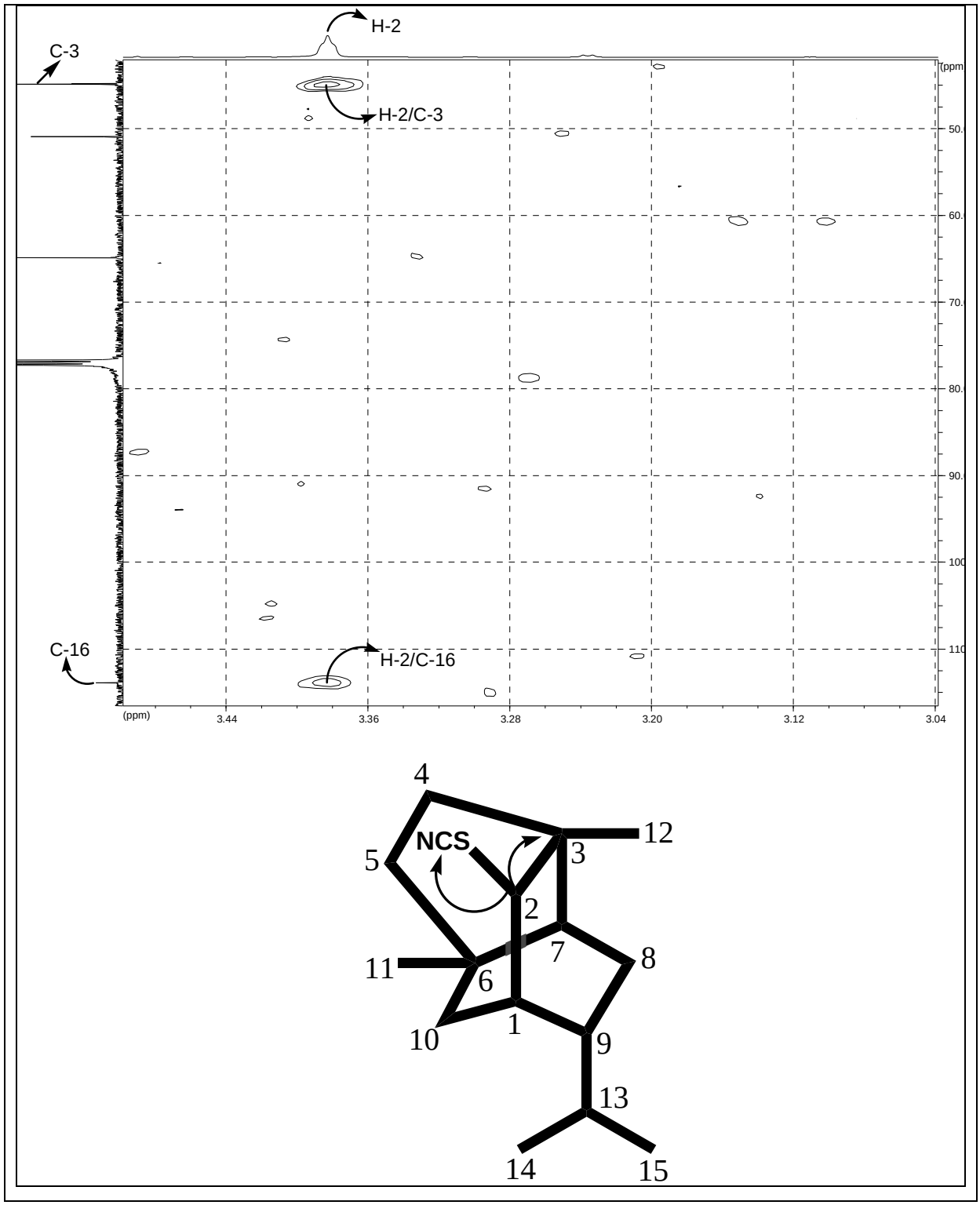
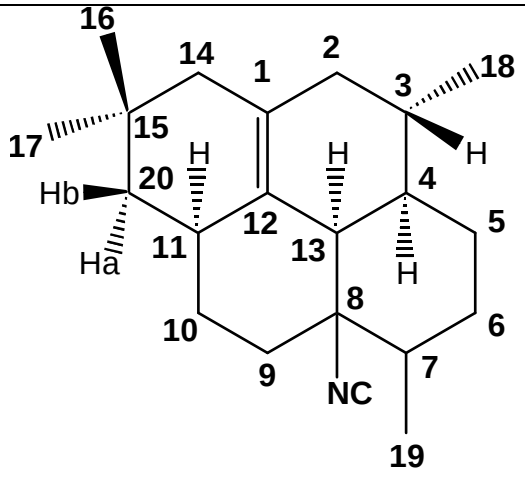


Figure 37. Direct evidence of the correlation between methine proton H-2 with quarternary carbon C-16 for compound **3** which confirms the existence and position of –SCN functional group.

3.3.3 Isolated compound 8-isocyano-1(12)-cycloamphilectene (4, known compound)



Compound **4**

Characteristic : colorless oil
Molecular Formula : $C_{21}H_{31}N$
Molecular weight : 297

8-Isocyano-1(12)-cycloamphilectene (**4**) was first isolated by Kazlauskas *et al*. in 1980. It was wrongly proposed as 8-isocyano-11(12)-cycloamphilectene on the basis of the interpretation of very limited spectral data [^{1}H NMR: ($CDCl_3$) 0.92 (apparent singlet 12 H); ^{13}C NMR: 154.8 (bs), 128.5 (s), 62.9 (t, *J* 4 Hz), 46.7 (d), 44.6, 43.6, 42.7, 40.0, 39.8, 35.8, 34.1, 32.2, 30.0, 29.8, 29.5, 28.7, 26.4, 26.1, 19.1 (q), 15.0 (q)]. The compound was presumably elucidated as a double bond isomer of 8-isocyano-10-cycloamphilectene whose structure was obtained by single crystal X-ray analysis, and by spectral comparison of the ^{13}C NMR data tetrasubstituted double bond, it was then concluded to be at Δ11(12) (see Figure 38). In 1987, the group of Faulkner reproposed the structure as 8-isocyano-1(12)-cycloamphilectene on the basis of X-ray analysis of a crystalline formamide which was prepared through hydrolysis catalized by acid of isolated 8-isocyano-1(12)-cycloamphilectene (Molinski *et al*., 1987).

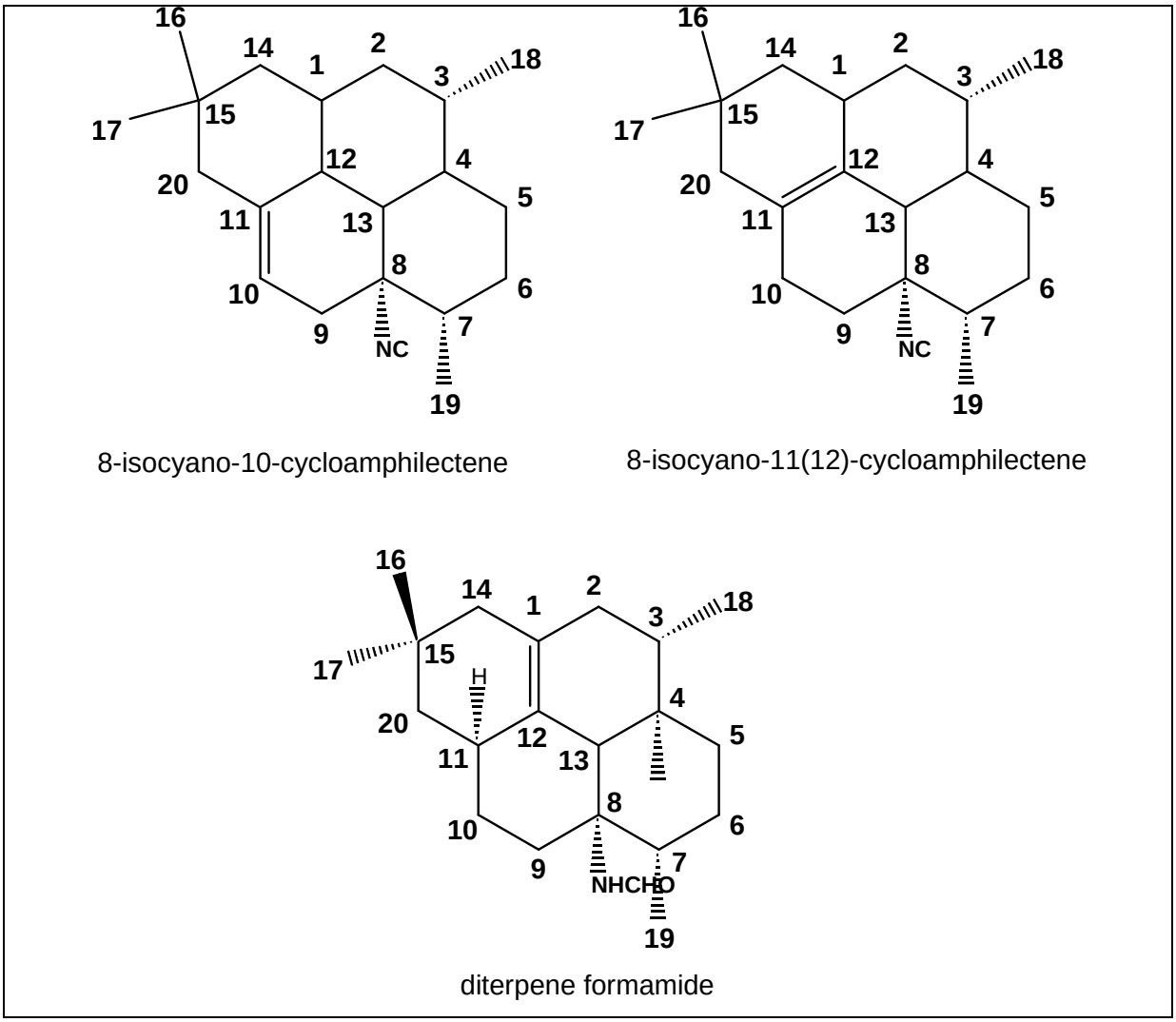


Figure 38. Related structures of compound **4**.

Compound **4** was isolated as a colorless oil from the sponge-prey of phyllidiid *P*. *elegans*, *Dragmacidon* sp. The presence of **4** was detected by GC coupled to a FID detector at R.T. 25.0 minute (Figure 39). The EIMS spectrum showed the molecular ion peak at *m/z* 297. The important fragment at *m/z* 271 indicated the loss of –NC group which revealed the presence of an isocyanide functionality. The chemical shift NMR data of ^{13}C of the isolated compound are very comparable to the reported data of diterpene formamide by Molinski *et al*. (1987) [1H NMR: ($CDCl_3$) 0.84 (s, 3H), 0.90, 0.91, 0.92 (2 d, s overlapping, 9 H), 2.28 (m, 3H), 5.47 (br. d, 1H, *J* = 12 Hz), 8.37 (d, 1H, *J* = 12 Hz); 13C NMR: 15.0 (q), 19.1 (q), 26.1 (t), 26.5 (q), 29.2 (t), 29.3 (s), 29.8 (t), 29.8 (q), 33.0 (d), 34.7 (d), 39.9 (t), 40.1 (d), 43.8 (t), 44.3 (d), 44.7 (t) 47.5 (d), 56.2 (s), 127.8 (s), 129.3 (s), 164.2 (d)]. The NMR full data of **4** is shown in Table 9.

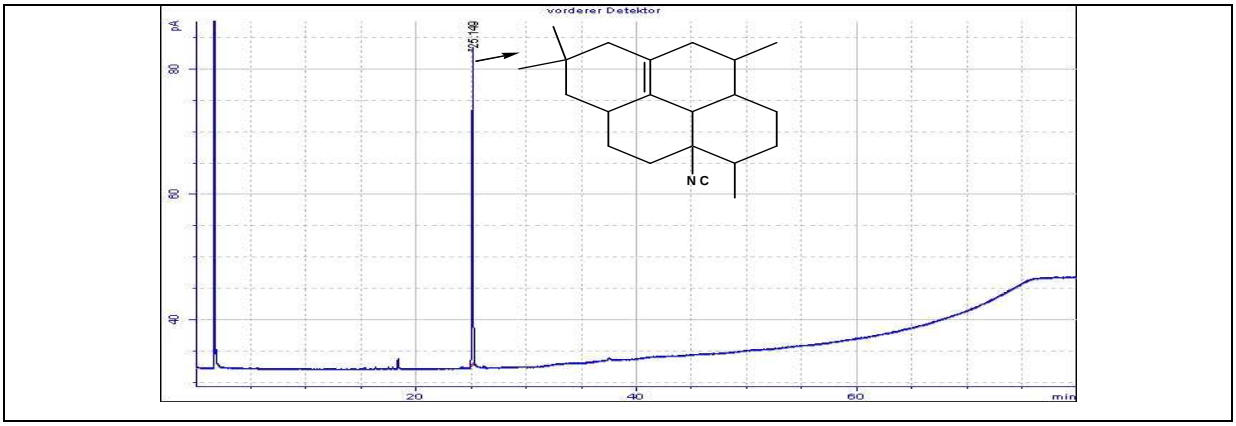


Figure 39. GC chromatogram of amphilectene isocyanide (**4**).

By inspection of the COSY spectrum of **4**, it is clearly seen that there are three spin systems in the compound. The first spin system involved the methylene protons on C-14 at δ 1.76 and 1.59 which coupled to each other while the second spin system is from H-11 (δ 2.54) which coupled to H_2-20 (δ 1.31 and 0.99) and H-11 (δ 2.54) which further coupled to H_2-10 (δ 1.61 and 1.26), then to protons H_2-9 (δ 2.18 and 1.73). The third spin system commenced at H_2-2 (δ 1.92 and 1.68) which coupled to H-3 (δ 1.47), then to H-4 (δ 1.27), H_2-5 (δ 1.95 and 0.86), H_2-6 (δ 1.55 and 1.38), H-7 (δ 1.37), H_3-19 (δ 1.01), and methane H-3 (δ 1.47) which coupled to H_3-18 (δ 0.91), and then H-4 (δ 1.27) which coupled to H-13 (δ 1.85).

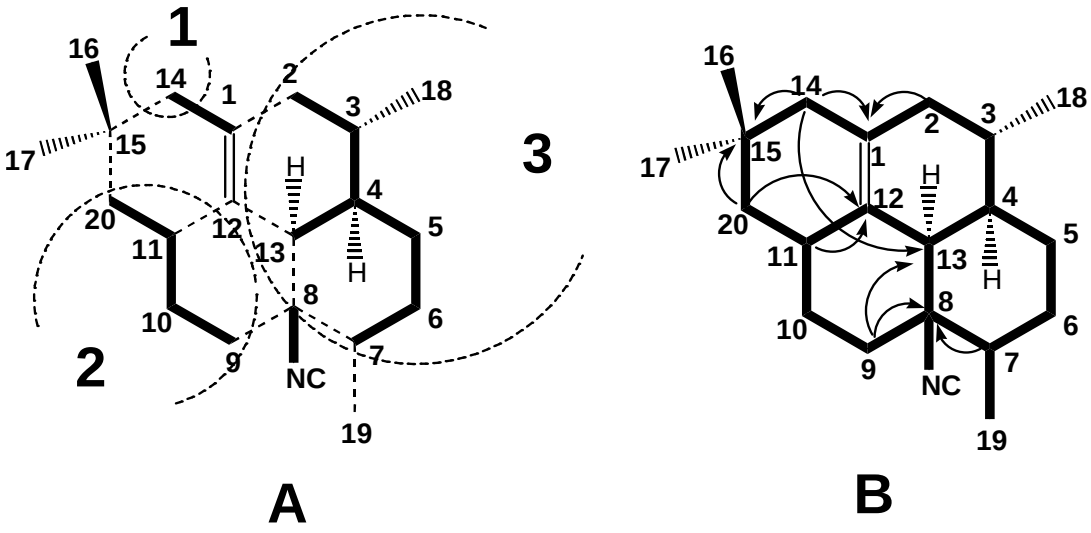


Figure 40. Spin systems in the compound **4**. (A) The three spin systems shown by COSY spectrum in compound **4**; (B) The important correlations shown by HMBC spectrum which connect spin systems in compound **4**.

The connection of each spin system in compound **4** can be cleary seen by the inspection of its HMBC spectrum. The first spin system which involved methylene H_2-14 is connected with the second one through the observed correlation between protons H_2-14 (δ 1.76 and 1.59) and H_2-20 (δ 1.31 and 0.99) to C-15 (δ 29.5, s) and is connected with the third spin system by the correlation from H_2-14 (δ 1.76 and 1.59) and H_2-2 (δ 1.92 and 1.68) to C-1 (δ 128.6, s) which also could confirmed the position of the double bond between C-1 and C-12 instead of C-11 and C-12 as wrongly proposed by Kazlauskas *et al*., (1980). The second spin system is connected with the third substructure through the correlation from H_2-9 (δ 2.18 and 1.73) and H-7 (δ 1.37) to the carbon C-8 (δ 63.0, s).

The HMBC correlation which connected the three spin systems to form the four conjugated cyclic amorphene backbone was the coupling between H_2-14 (δ 1.76 and 1.59) and H_2-9 (δ 2.18 and 1.73) to C-13 (δ 46.7, d) and the correlation between H_2-20 (δ 1.31 and 0.99) to C-11 (δ 29.8, d) and C-12 (δ 127.6, s) as well as the correlation between H_2-9 (δ 2.18 and 1.73) and H-7 (δ 1.37) to the carbon C-8 (δ 63.0, s) (see Figure 40, Figure 44, and Figure 45) The chemical shift of the quaternary carbon C-8 (δ 63.0, s) confirms the position of the isocyanide at C-8. The HMBC spectrum unambiguously showed the position of double bonds at Δ 1,12 in compound **4**. This was shown through the homoallylic coupling in the HMBC between H-14a (δ 1.76) to C-13 (δ 46.7). From the given 1D and 2D NMR data, compound **4** was therefore unambiguously elucidated as 8-isocyano-1(12)-cycloamphilectene.

Stereochemistry

The stereochemistry of compound **4** was first basically proposed through X-ray analysis of a crystalline formamide which was prepared through hydrolysis of isolated 8-isocyano-1(12)-cycloamphilectene using acidic condition (Molinski *et al*., 1987). In the present study, the relative configuration of compound **4** was assigned through a ROESY experiment. The ROESY correlation from CH_3-18 (δ 0.91) to H-4 (δ 1.27) and H-13 (δ 1.85) as well as correlation between H-11 (δ 2.54) and CH_3-17 (δ 0.94) confirmed that the stereochemistry is identical to that the formamide derivative reported in the literature (Molinski *et al*., 1987). The key correlation between H-20a (δ

1.31) and H-11 (δ 2.54) as well as correlation between H-20b (δ 0.99) and H-3 (δ 1.47) further confirmed that the protons H-4, H-11, H-13, H-20a, CH_3-18 and CH_3-17 have an α configuration whereas protons H-3 and H-20b have a β configuration. This relative configuration is compatible to that described in the literature of which the configuration was determined by X-ray analysis.

Compound **4** has an observed $[\alpha]_D$ value of + 9.5 (c. 0.35 in $CHCl_3$, 20℃) while the reported 8-isocyano-1(12)-cycloamphilectene has an optical rotation of + 39.6 (c. 0.6 in $CHCl_3$) (Kazlauskas *et al*., 1980).

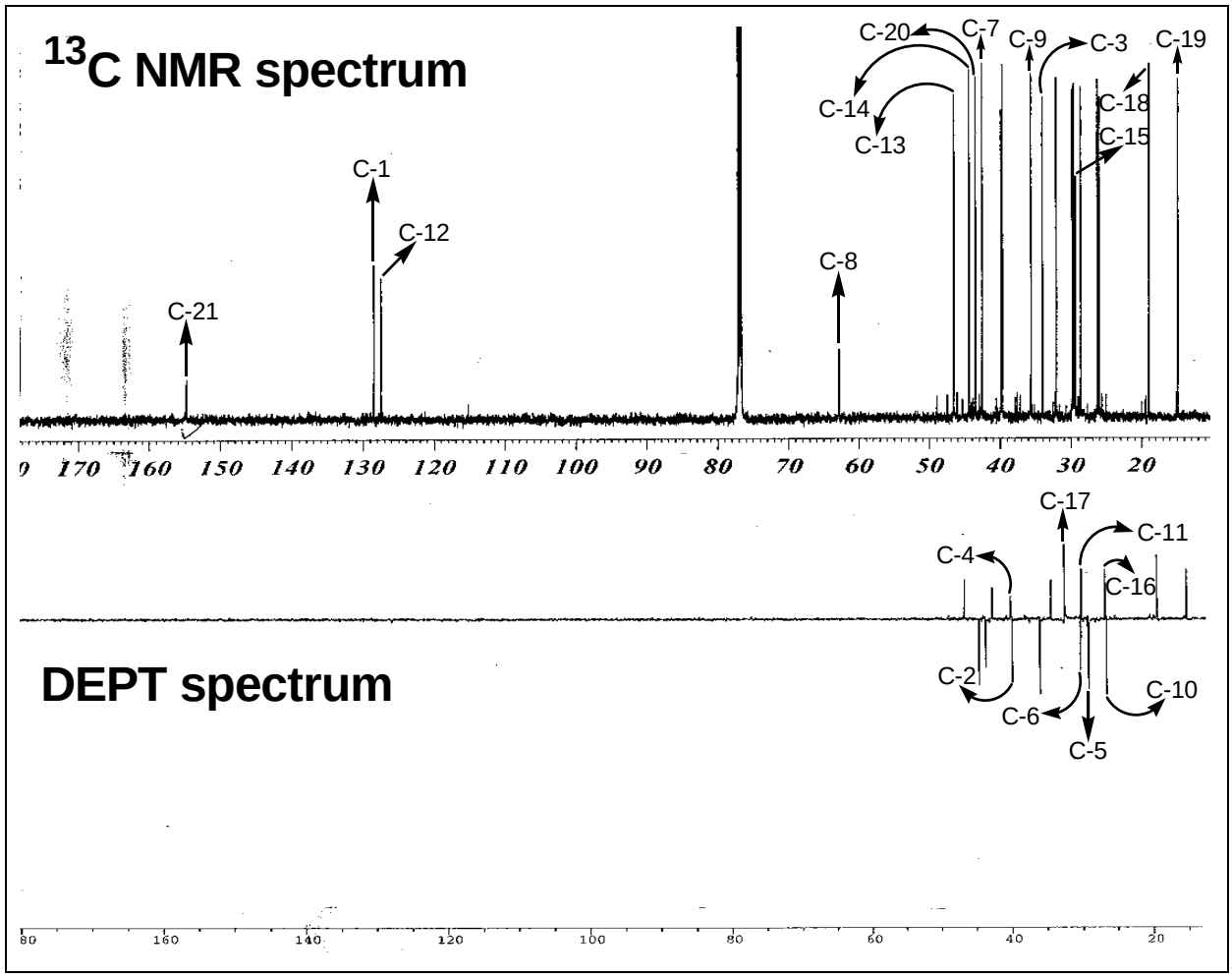


Figure 41. ^{13}C NMR and DEPT spectra of the isolated compound **4**.

Table 9. Full NMR data of compound **4**.

	^{13}C (δ_C, mult.)	^{1}H (δ_H, mult.)	COSY (δ_H- δ_H)	HMBC (δ_H- δ_C)	ROESY
1	128.6, s	-	-	-	-
2	39.9, t	a. 1.91, m	H-3	C-1, C-3, C-4, C-14	
		b. 1.68, m	H-3	C-18	
3	34.2, d	1.47, m	H-2, H-4, H-18	C-4, C-5, C-13, C-18	H-20b
4	40.0, d	1.27, m	H-13, H-3, H-5	C-2, C-3, C-13, C-18	H-18
5	28.8, t	a. 1.95, m	H-4, H-6	C-7, C-13	
		b. 0.86, m	H-4, H-6	C-4, C-6, C-7, C-13	
6	30.0, t	a. 1.55, m	H-5, H-7	C-4, C-5, C-7, C-19	
		b. 1.38, m	H-5	C-5, C-7, C-19	
7	42.7, d	1.37, m	H-6a	C-6, C-8	
8	63.0, s	-	-	-	-
9	35.8, t	a. 2.18, m	H-10a,b	C-7, C-8, C-10, C-13	
		b. 1.73, m	H-10a	C-8, C-10, C-13	
10	26.2, t	a. 1.61, m	H-9a,b	C-8, C-9, C-11, C-20	
		b. 1.26, m	H-9a	C-9, C-20	
11	29.8, d	2.54, m	H-20, H-10		H-17, H-20a
12	127.6, s	-	-	-	-
13	46.7, d	1.85, m	H-4,	C-5, C-9	H-18
14	44.6, t	a. 1.76, m	H-14b	C-1, C-12, C-13, C-15, C-16, C-17	
		b. 1.59, m	H-14a	C-1, C-12, C-16	
15	29.5, s	-	-	-	-
16	26.4, q	0.92, s		C-1, C-14, C15, C-17, C-20	
17	32.2, q	0.94, s		C-1, C-14, C15, C-16, C-20	
18	19.1, q	0.91, d	H-3	C-3, C-4	
19	15.0, q	1.01, d	H-7	C-7, C-8	
20	43.7, t	a. 1.31, m	H-11, H-20b	C-12, C-14, C-15, C-16, C-17	
		b. 0.99, m	H-11, H20a	C-12, C-14, C-17	
21	154.9, s	-	-	-	-

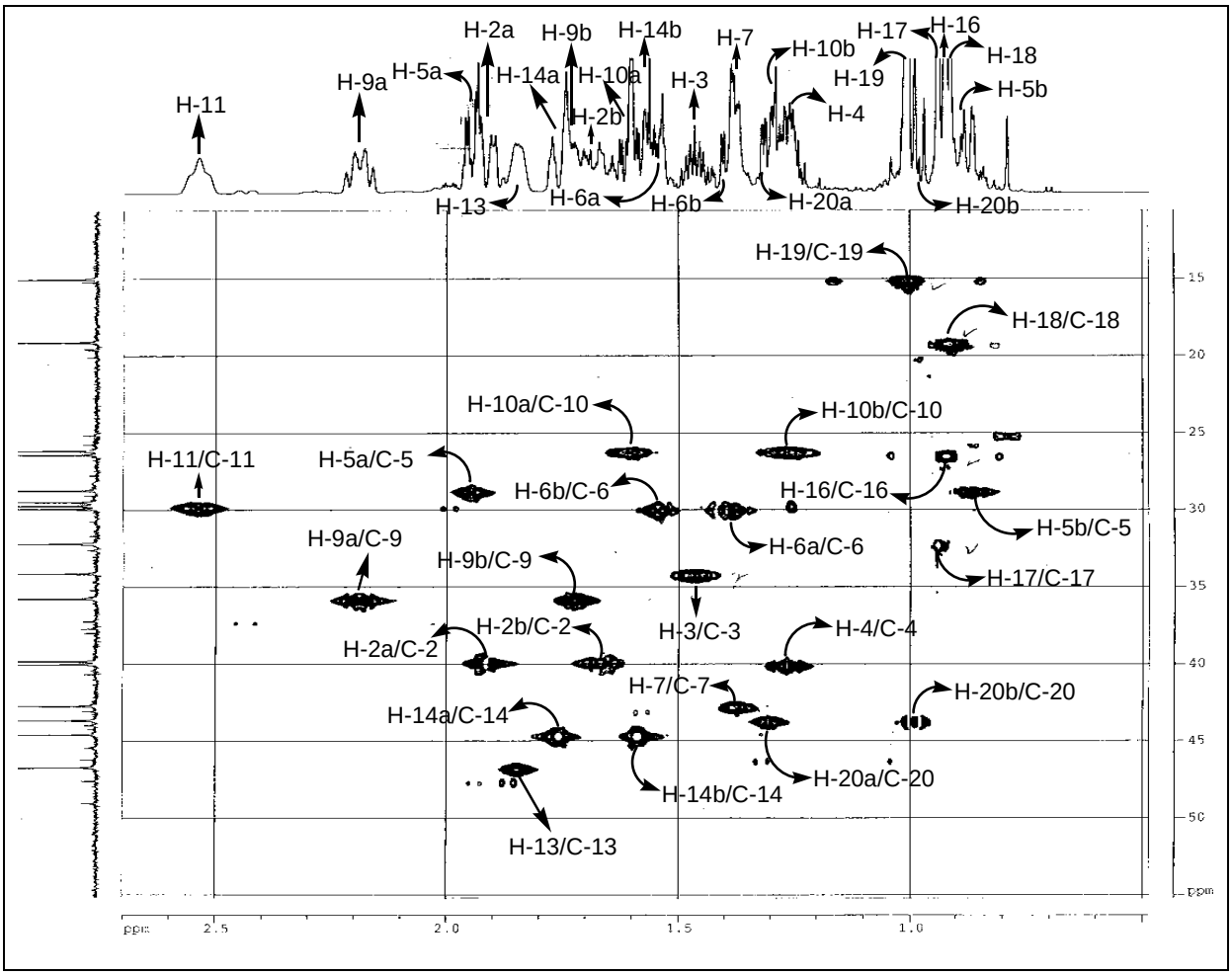


Figure 42. Spectrum of direct C-H correlation (HMQC) of the isolated compound **4**.

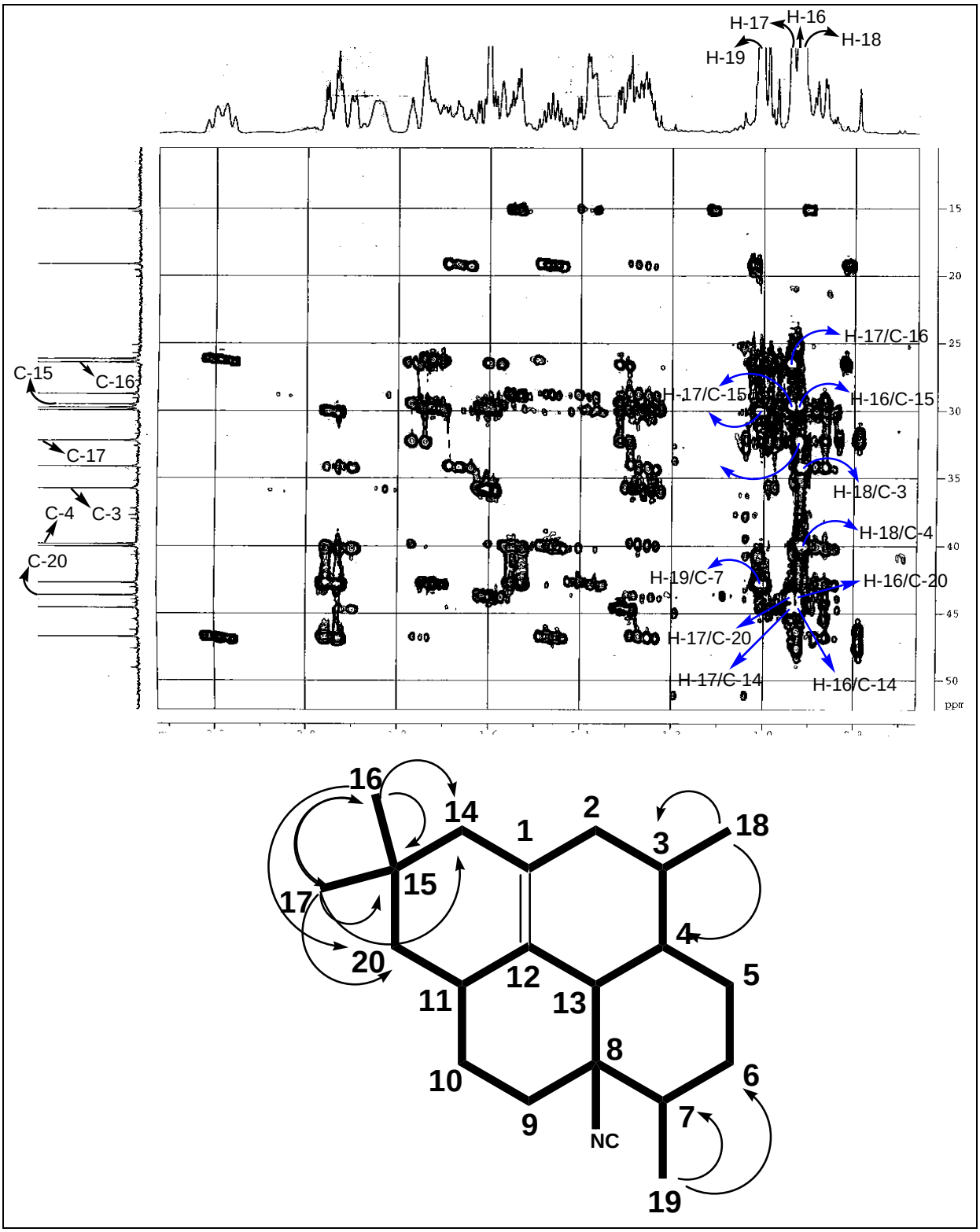


Figure 43. Expansion of HMBC NMR spectrum to show direct evidence of the position and existence of methyl groups in compound **4**.

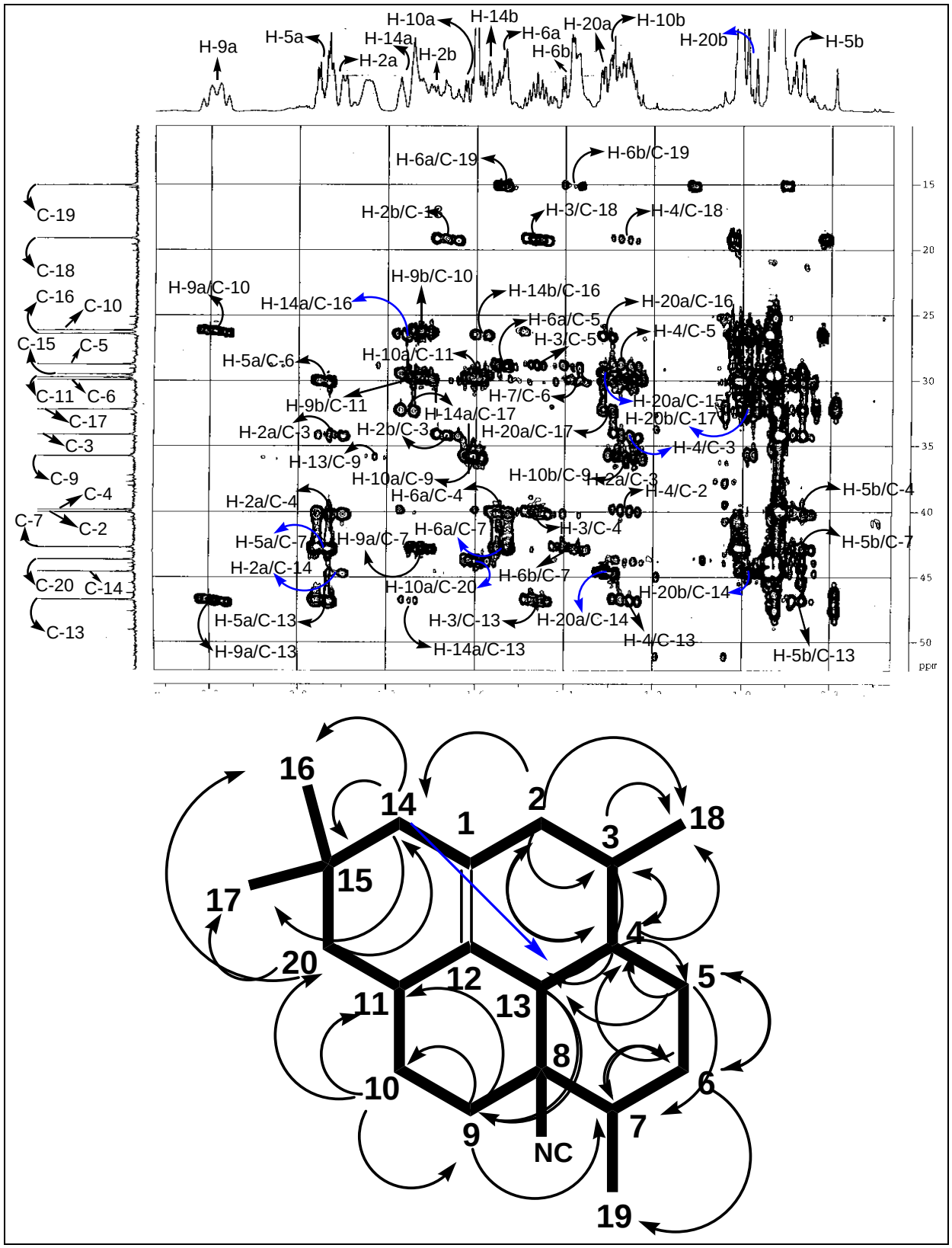


Figure 44. Expansion of HMBC NMR spectrum to show correlation of the methylene protons with the neighboring carbons in compound **4**.

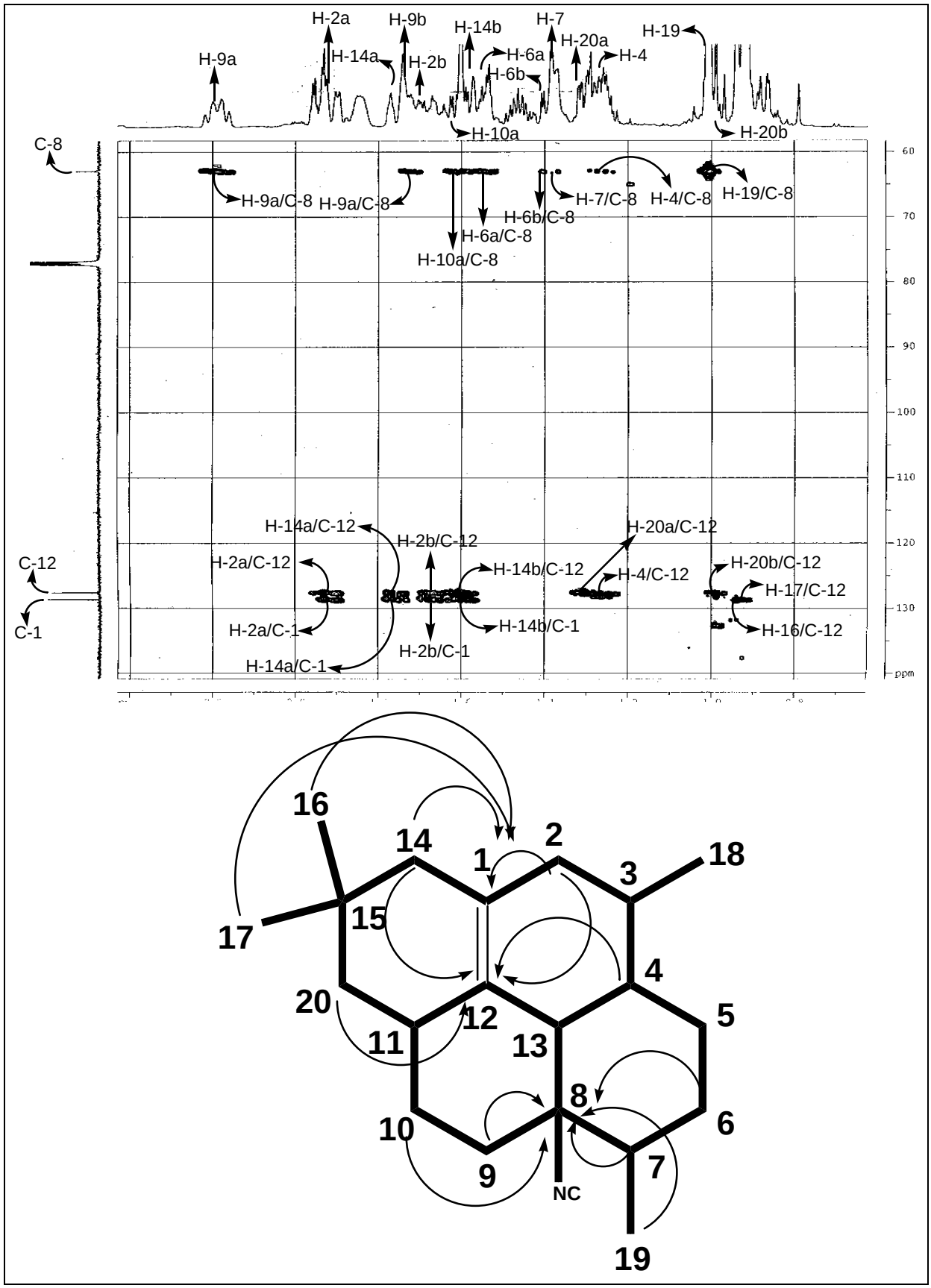


Figure 45. Expansion of HMBC NMR spectrum which confirms the position of unsaturated carbon (C-1 and C12) and carbon bearing –NC functionality in compound **4**.

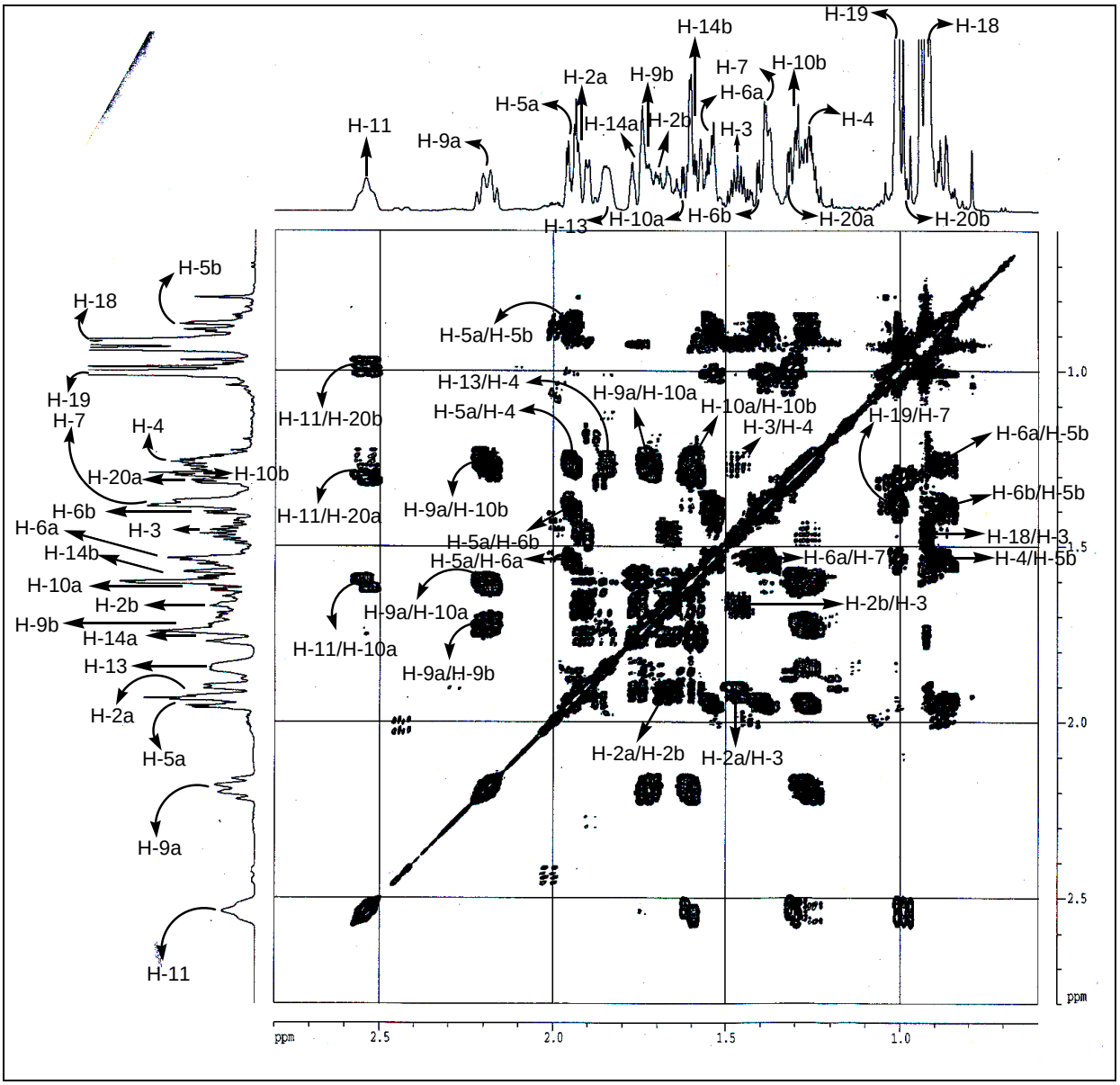


Figure 46. Full COSY spectrum of the isolated compound **4**.

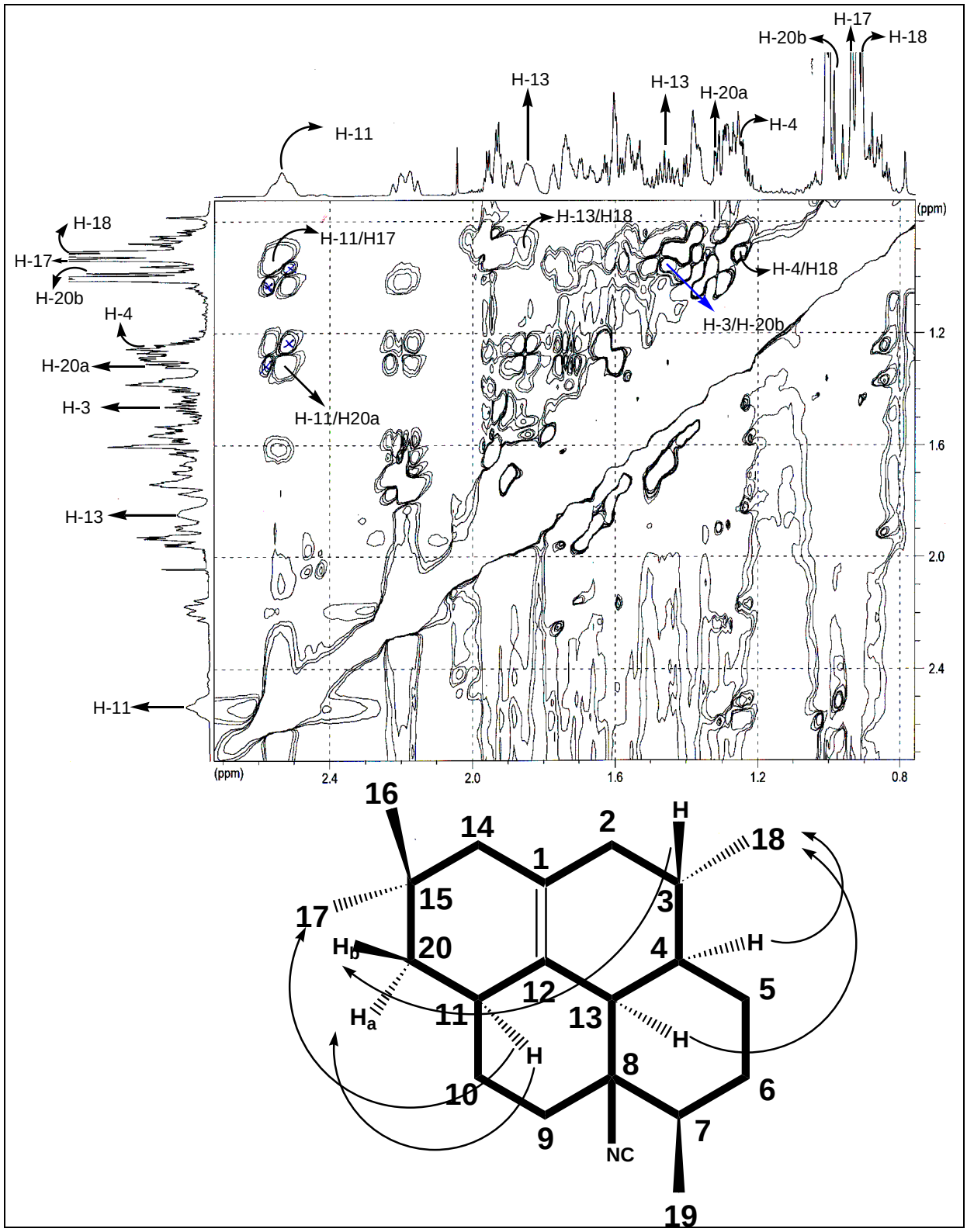
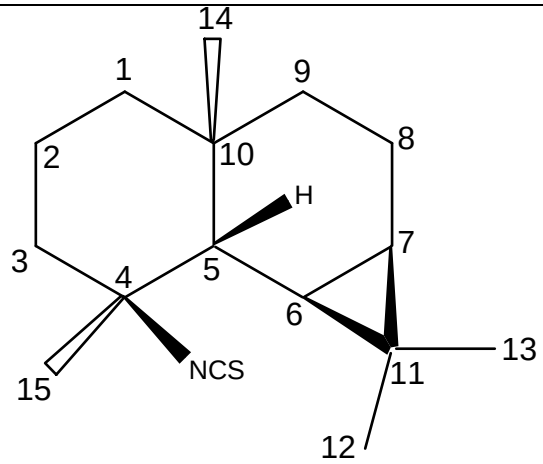


Figure 47. Full ROESY spectrum of the isolated compound **4**.

3.3.4 Isolated compound epipolasin-A (5, known compound)



Compound **5**

Characteristic : colorless oil
Molecular Formula : $C_{16}H_{25}NS$
Molecular weight : 263

Compound **5** was isolated as a colorless oil. It was first isolated from the sponge-prey of phyllidiid *P*. *krempfi*, *Axinyssa* sp. In this study, compound **5** was also found in the sponge *Axinyssa* spp. predated by phyllidiid species in the coral reefs of Thousand Island, Indonesia. The presence of **5** was detected by GC coupled to a FID detector at R.T. 16.4 minute (Figure 48). The EIMS spectrum showed the molecular ion peak at *m/z* 263. The important fragment at m/z 205 showed the backbone of the compound which also indicated the loss of –NCS functional group. The structure of **5** was established as epipolasin-A from its ^{1}H NMR which is comparable to the literature data (Thompson *et al*., 1982; Capon *et al*., 1988; Simpson *et al*., 1997). ^{1}H NMR spectrum showed four singlet signals at δ_H 1.44, 1.14, 1.01, 0.88. The multiplicity of these methyl group indicated that they were attached to a quarternary carbon. The most low field methyl protons (δ_H 1.44) corresponded to C*H*$_3$-15 due to being geminal to the –NCS function, whereas the higher field (δ_H 1.14, 1.01, 0.88) methyl protons corresponded to C*H*$_3$-13, -12, and -14 respectively. The presence of the cyclopropane ring was assigned for H-6 at δ_H 0.56 (1H, dd; 9, 6.5 Hz) and H-7 at δ_H 0.66 (1H, dd; 9, 7.5 Hz). The full ^{1}H NMR data of compound **5** is comparable to the literature data as shown in Table 10. The COSY spectrum clearly showed the two spin systems in compound **5**. The first spin system showed the correlation between

proton H-3a at δ_H 1.97 with H-3b (δ_H 1.70), then coupled to H-2 (δ_H 1.56) further to H-1a (δ_H 1.36). The second spin system commenced at H-9a (δ_H 1.15) which coupled to H-9b (δ_H 1.15), then to H-8b (δ_H 1.56), H-7 (δ_H 0.66), H-6 (δ_H 0.56), and H-5 (δ_H 1.27) (Figure 50). Through the ^{1}H NMR, COSY spectra, and by comparison with literature data, compound **5** was elucidated unambiguously as epipolasin-A. Due to very small yield, no HMBC experiment could be done.

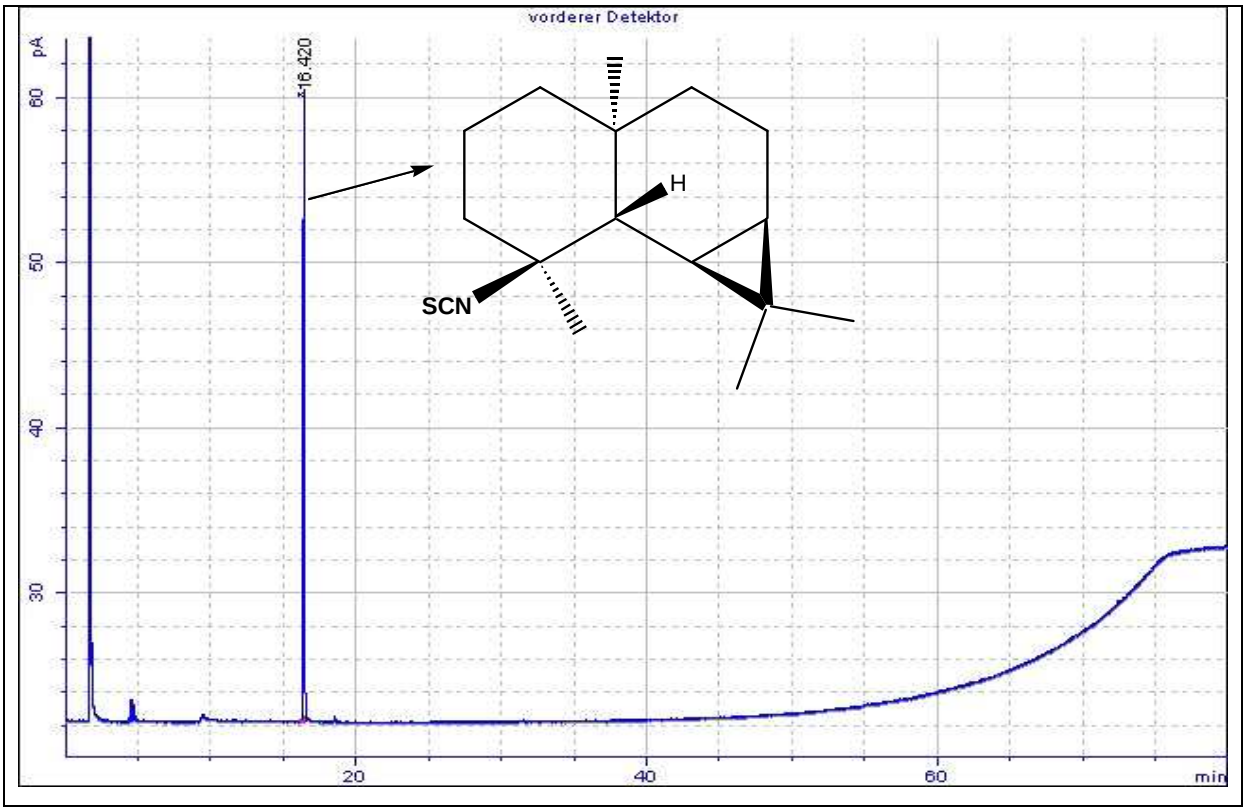


Figure 48. GC profile of the isolated compound **5**.

Table 10. ^{1}H NMR and COSY data of compound **5**.

	^{1}H (δ_H, mult., *J* in Hz)		COSY
	Literature (Simpson *et al*., 1997)	Compound **5**	
1	1.35, ddd, 13.4, 3.2, 3.2	1.36, m	
	0.99, m	1.00, m	H-1a
2	1.57, m	1.56, m	H-1b
	1.57, m	1.56, m	
3	1.94, ddd, 12.6, 3.2, 3.2, 1.6	1.97, m	H-3a
	1.70, m	1.70, m	H-2
4	-	-	-
5	1.25, d, 6.4	1.27, d, 6.5	
6	0.55, dd, 9.0, 6.4	0.56, dd, 9.0, 6.5	H-5
7	0.65, dd, 9.0, 8.3	0.66, dd, 9.0, 7.5	H-6
8	1.81, dddd, 16.6, 12.4, 8.3, 8.3	1.81, m	
	1.55, m	1.58, m	H-7
9	1.15, m	1.15, m	H-9b
	0.78, ddd, 13.0, 13.0, 7.5	0.79, m	H-8b
10	-	-	-
11	-	-	-
12	1.00, s	1.01, s	-
13	1.13, s	1.14, s	-
14	0.87, s	0.88, s	-
15	1.43, s	1.44, s	-
16	-	-	-

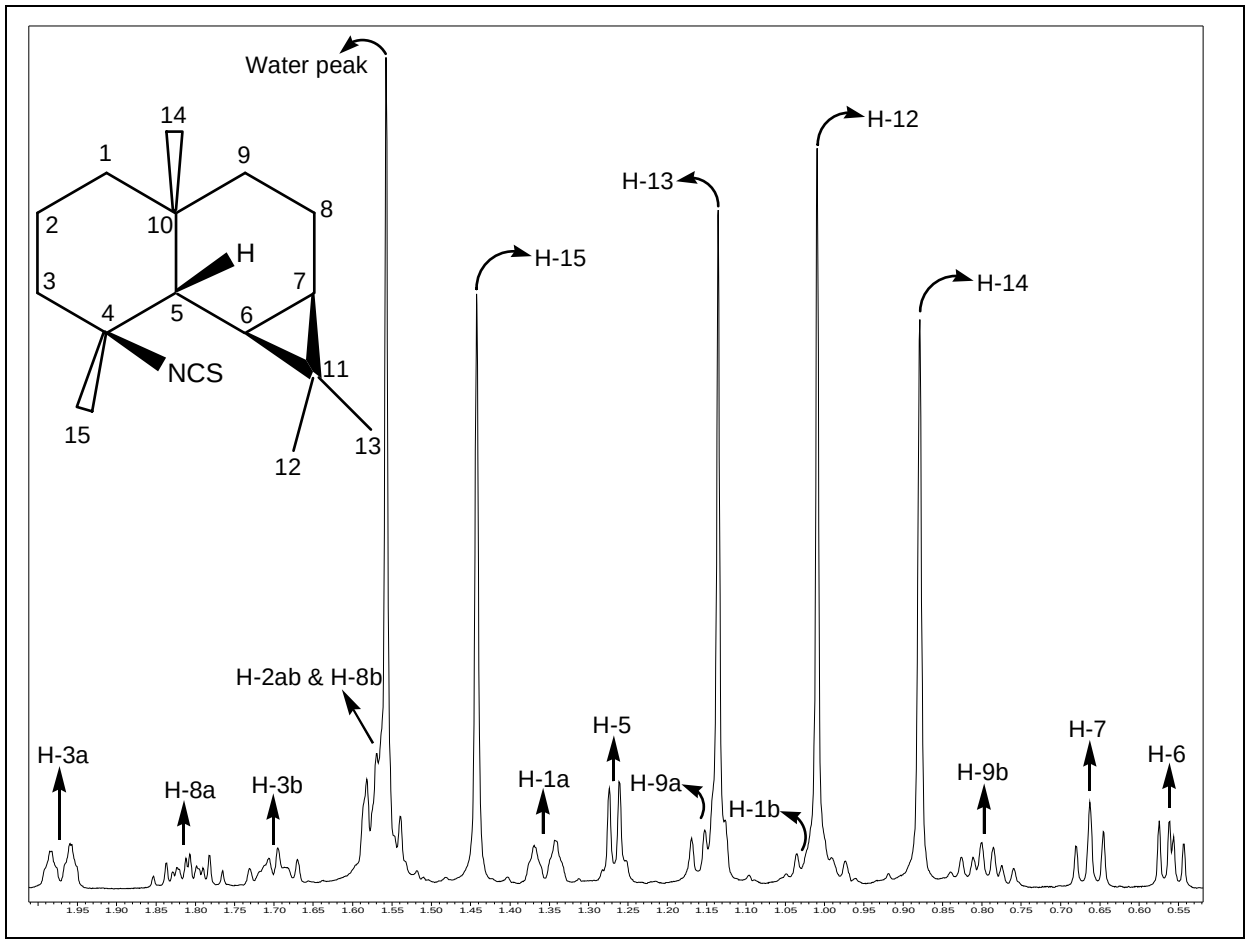


Figure 49. ^{1}H NMR spectrum of the isolated compound **5**.

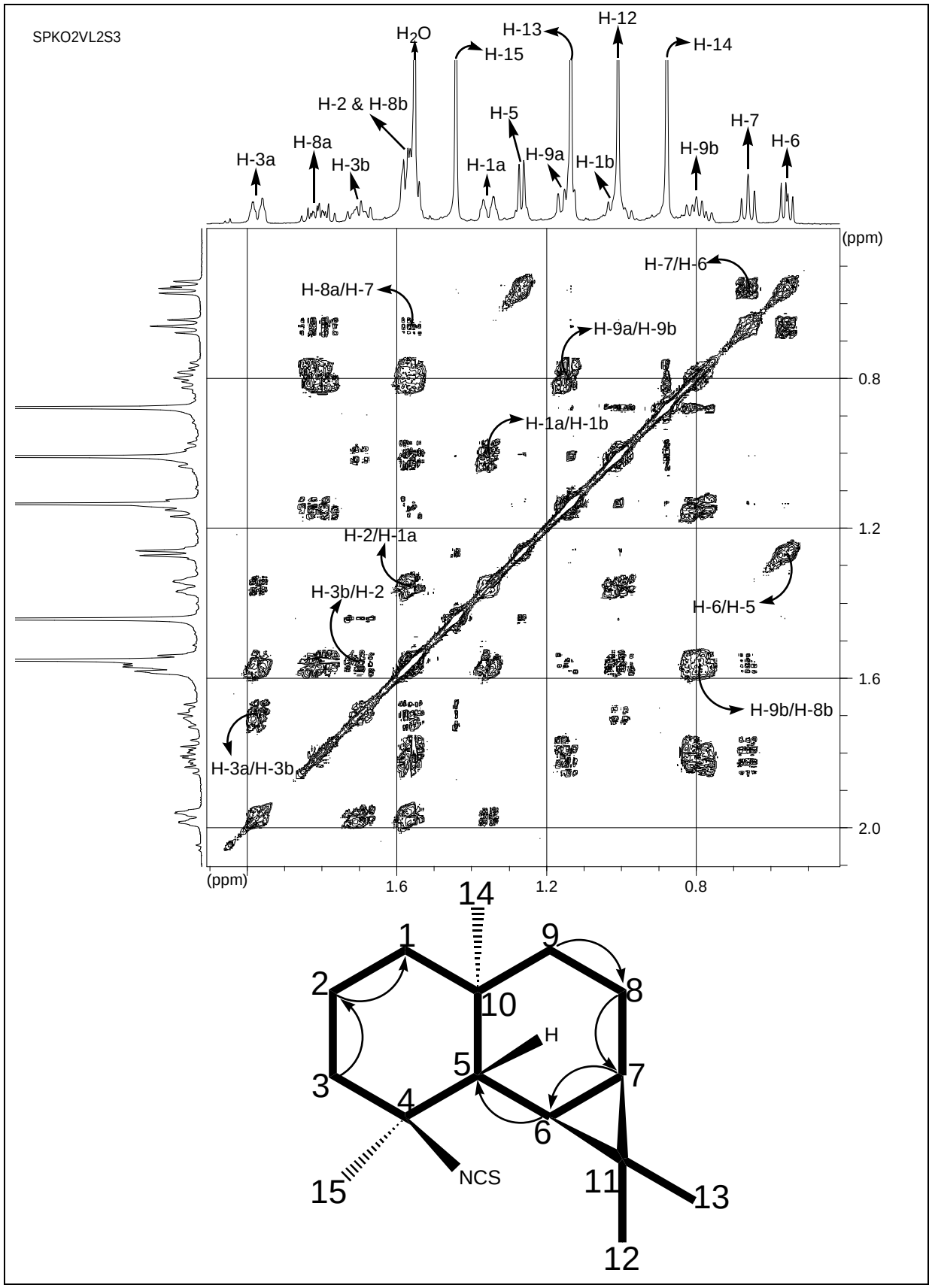


Figure 50. Full COSY spectrum of compound **5**.

3.4 Concentration variations of terpenes in collected sponges

Quantitative analyses of the secondary metabolites particularly the isocyanide terpenes (compounds **1** to **5**) present in the sponge extracts were evaluated. Five mg of each of the total EtOAc extract was diluted with 200 µl EtOAc, of which one µl of each extract was injected into the Agilent 6850 GC system. The concentration of individual cyanide terpenes of each sponge species in ppm (µg/g dw of sponge samples) was quantified using calibration curves obtained for each the respective natural product of interest.

3.4.1 Quantitative analysis of sesquiterpene isocyanides in sponge *Axinyssa* species

3.4.1.1 *Axinyssa 1* n. sp.

Of six sponge specimens collected from four different islands, the relative compositions of each of the terpenes: 9α-thiocyanatopupukeanane (**1**), 9β-thiocyanatopupukeanane (**2**), 2-thiocyanato-neopupukeanane (**3**), and epipolasin-A (**5**) are almost the same. The sponge *Axinyssa 1* n. sp. contained sesquiterpenes **1**, **2**, and **5** as its major component while compound **3** occurred as a minor component. In five sponges which included two samples that were collected from Melinjo Island, compound **2** was the major component. However, one sponge collected from Melinjo Island was an exception because it yielded isothiocyanate **5** as its major compound. This is an interesting finding as it might suggest that such variations in the relative composition of sesquiterpene isocyanides in *Axinyssa 1* n. sp. are not due to the geographical distribution of the species.

In contrast to the sesquiterpenes' relative composition, each *Axinyssa 1* n. sp. sample contained different concentrations of compounds of **1**, **2**, **3**, and **5** (see Table 11). As this variation also occurred within the sponge samples collected from the same location, it might again suggest that such variations in the concentration of iso- and sesquiterpene thiocyanates in *Axinyssa 1* n. sp. are not due to the geographical distribution of the species.

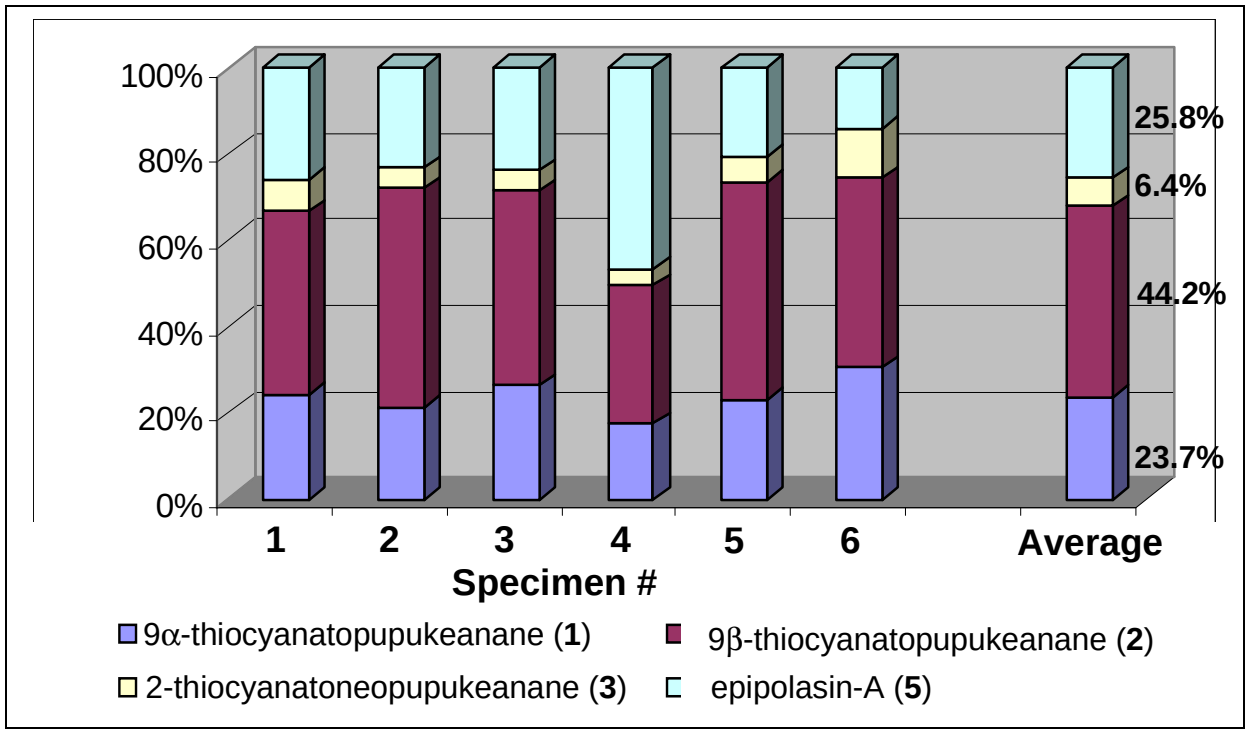


Figure 51. Relative composition of compounds **1**, **2**, **3**, and **5** in different sponge samples of *Axinyssa 1* n. sp.

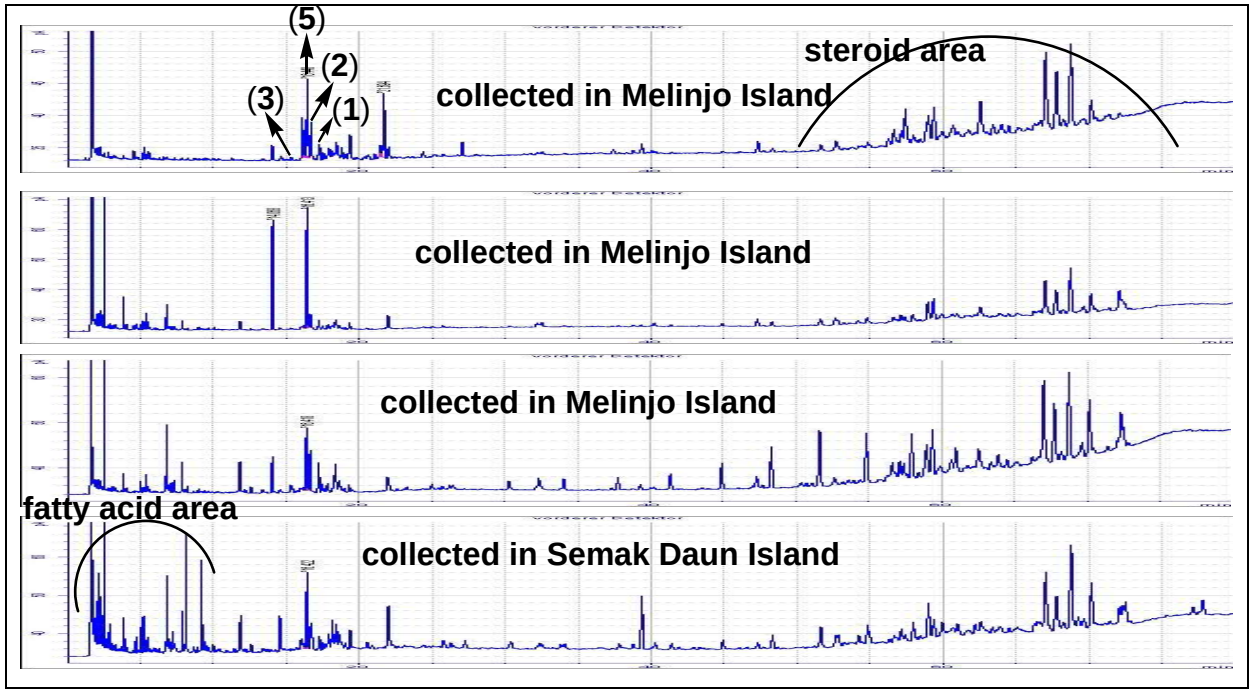


Figure 52. GC profiles of the sponge *Axinyssa 1* n. sp. extract showing the presence of compounds **1**, **2**, **3**, and **5**.

Table 11. Concentration variation in ppm of sesquiterpene isocyanides of sponge samples of *Axinyssa 1* n. sp. collected from different islands in the Thousand Island National Park, Indonesia.

Name of island	9α-thiocyanatopupu-keanane (**1**)	9β-thiocyanatopupu-keanane (**2**)	2-thiocyanatoneopupu-keanane (**3**)	epipolasin (**5**)
Pramuka	116	210	35	125
Semak Daun	116	271	27	112
Kotok Besar	350	603	64	319
Melinjo	131	244	29	355
Melinjo	58	128	15	52
Melinjo	140	201	53	65

3.4.1.2 *Axinyssa 4* n. sp.

The relative composition of terpene isocyanides: 9α-thiocyanatopupukeanane (**1**), 9β-thiocyanatopupukeanane (**2**), 2-thiocyanato-neopupukeanane (**3**), and epipolasin-A (**5**) in the sponge *Axinyssa 4* n. sp. seems not to be constant. Of six specimens of *Axinyssa 4* n. sp. collected from five different islands, four sponge specimens contained the epimeric mixture **1** and **2** as their major compounds; one specimen has compounds **2** and **3** as its major components, while one specimen yielded compound **5** as its major metabolite. But in general when focusing at the average composition of the sesquiterpenes, the trend is comparable as found in sponges of *Axinyssa 1* n. sp. The concentrations of sesquiterpene iso- and thiocyanates **1**, **2**, **3**, **5** in the sponge *Axinyssa 4* n. sp. also did vary (see Table 12).

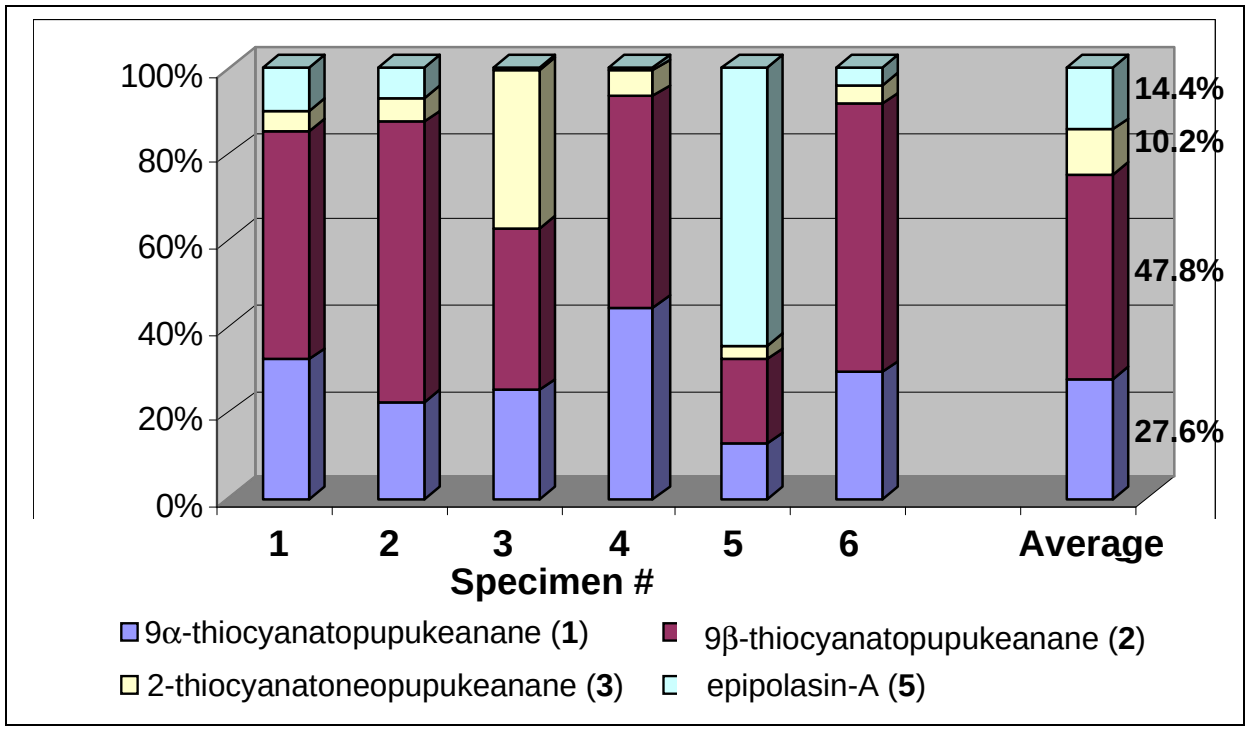


Figure 53. Relative composition of compounds **1**, **2**, **3**, and **5** in different sponge samples of *Axinyssa 4* n. sp.

Table 12. Concentration variation in ppm of sesquiterpene isocyanides of sponge samples of *Axinyssa 4* n. sp. collected from different islands in the Thousand Island National Park, Indonesia.

Name of island	9α-thiocyanatopupu-keanane (**1**)	9β-thiocyanatopupu-keanane (**2**)	2-thiocyanatoneopupu-keanane (**3**)	epipolasin (**5**)
Kotok Besar	1095	1802	164	341
Kotok Besar	856	2508	216	268
Kotok Kecil	1262	1889	1838	31
Pramuka	1916	2116	260	31
Karang Congkak	94	145	25	479
Melinjo	291	623	46	39

3.4.1.3 *Axinyssa 5* n. sp.

In 30 sponge samples of *Axinyssa 5* n. sp. collected from 9 different islands, the relative composition of terpene isocyanides: 9α-thiocyanatopupukeanane (**1**), 9β-thiocyanatopupukeanane (**2**), 2-thiocyanato-neopupukeanane (**3**), and epipolasin-A (**5**) seems to show a similar trend as the two previous *Axinyssa* species, where epimers **1** and **2** were the major compounds. In all sponge samples, 9β-thiocyanatopupukeanane (**2**) was the major metabolite with the exception of two sponges collected from Karang Balik Layar and Melinjo Island where epipolasin-A (**5**) and 2-thiocyanatoneopupukeanane (**3**) were the major compounds, respectively. In contrast with the sponge *Axinyssa 1* n. sp., in the sponge *Axinyssa 5* n. sp. compound **3** was more dominant than **5**. The concentration variations of sesquiterpene isocyanides from sponge samples of *Axinyssa 5* n. sp. are shown in Table 13.

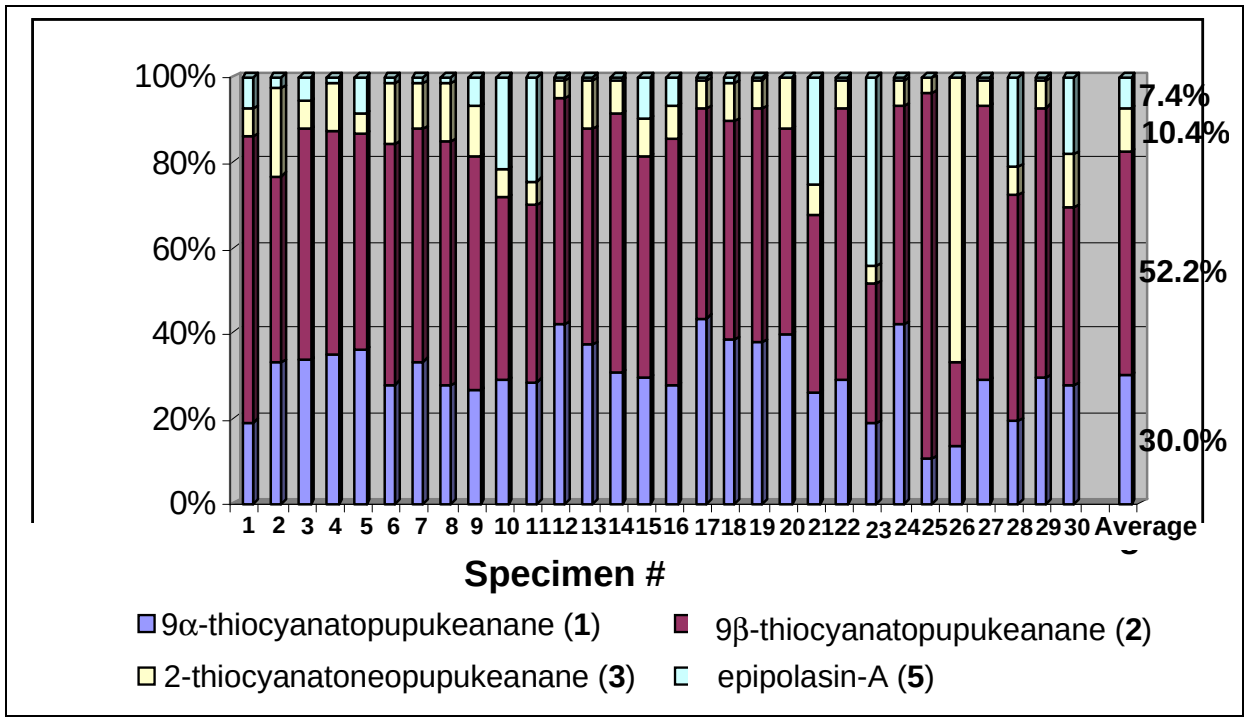


Figure 54. Relative composition of compounds **1**, **2**, **3**, and **5** in different sponge samples of *Axinyssa 5* n. sp.

Table 13. Concentration variation in ppm of sesquiterpene isocyanides of sponge samples of *Axinyssa 5* n. sp. collected from different islands in the Thousand Island National Park, Indonesia.

Name of Island	9α-thiocyanatopupu-keanane (**1**)	9β-thiocyanatopupu-keanane (**2**)	2-thiocyanatoneopupu-keanane (**3**)	epipolasin (**5**)
Kotok Kecil	684	2398	226	272
Kotok Kecil	741	972	457	61
Kotok Besar	1950	3170	368	342
Kotok Besar	2843	4303	931	125
Kotok Besar	1514	2112	195	365
Kotok Besar	1807	3628	926	102
Kotok Besar	4739	7713	1562	201
Kotok Besar	1824	3725	869	114
Kotok Besar	2224	4525	979	592
Kotok Besar	130	194	30	97
Kotok Besar	137	198	26	116
Kotok Besar	2052	2599	200	48
Pramuka	1530	2111	456	40
Pramuka	1800	3571	456	60
Pramuka	410	718	124	137
Pramuka	941	1994	256	232
Pramuka	1342	1510	206	26
Pramuka	501	670	119	18
Pramuka	1540	2204	279	26
Semak Daun	236	285	71	2
Semak Daun	184	298	52	181
Karang Congkak	334	728	75	13
K. Balik Layar	93	166	22	222
Opak Kecil	703	852	100	13
Opak Kecil	363	2896	120	9
Melinjo	667	933	3250	21
Putri	510	1146	105	18
Putri	91	249	29	99
Putri	1047	2271	219	37
Putri	251	375	112	164

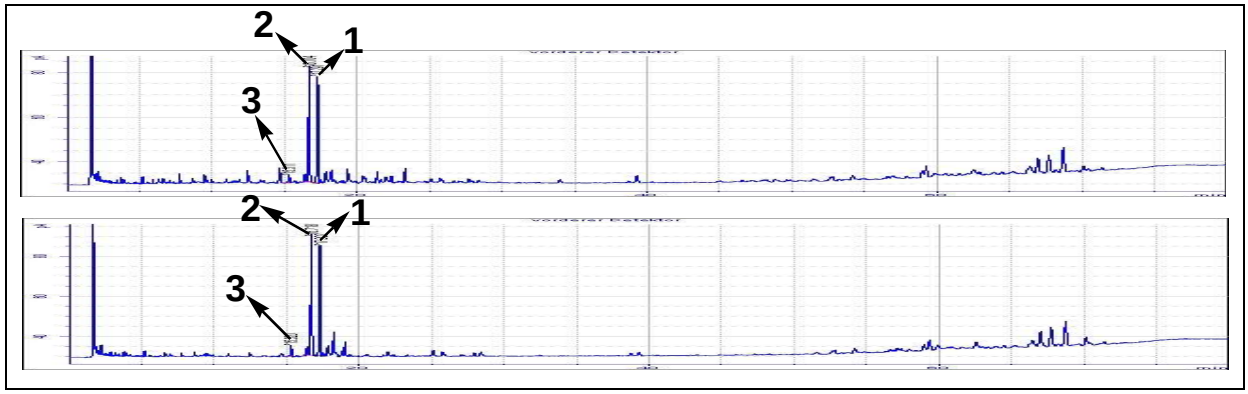


Figure 55. GC profile of EtOAc extracts of *Axinyssa* 5 n. sp. showing compounds **1** & **2** as the major components.

3.4.1.4 *Axinyssa 6* n. sp.

Only two specimens of *Axinyssa 6* n. sp. were collected. Of the two sponge samples collected from two different islands, the relative compositions of compounds 9α-thiocyanatopupukeanane (**1**), 9β-thiocyanatopupukeanane (**2**), 2-thiocyanato-neopupukeanane (**3**), and epipolasin-A (**5**) are different. In the sponge sample collected from Opak Kecil Island, epimers **1** and **2** are the major components. The minor compounds, **3** and **5**, were present at a more or less equal percentage (the percentage of compound **5** is slightly more than that of **3**). In contrast to another sponge sample collected from Karang Congkak Island, **3** twice the concentration of **5**. It is also interesting to note that the sponge sample *Axinyssa 6* n. sp. from Karang Congkak Island contained a higher ratio of compound **1** compared to its epimer **2**. Of a total 51 of sponge specimens of *Axinyssa* spp., this is the only sponge sample that gave this result while among 50 sponge specimens the β epimer is present at a higher ratio than its α counterpart. The absolute concentration variations of sesquiterpene isocyanides from sponge samples of *Axinyssa 6* n. sp. are shown in Table 14.

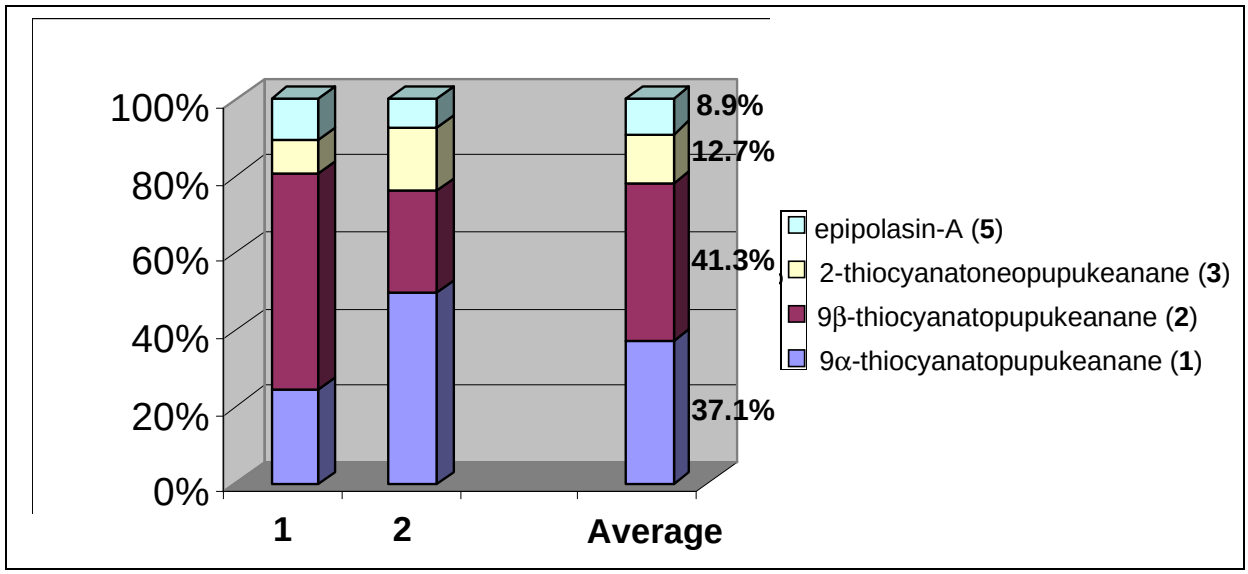


Figure 56. Relative composition of compounds **1**, **2**, **3**, and **5** in two sponge samples of *Axinyssa 6* n. sp.

Table 14. Concentration variation in ppm of sesquiterpene isocyanides of the sponge samples of *Axinyssa 6* n. sp. collected from two different islands in the Thousand Island National Park, Indonesia.

Name of island	9α-thiocyanatopupu-keanane (**1**)	9β-thiocyanatopupu-keanane (**2**)	2-thiocyanatoneopupu-keanane (**3**)	epipolasin (**5**)
Opak Kecil	99	229	37	42
Karang Congkak	33	17	11	5

3.4.2 Quantitative analysis of terpene isocyanides in the sponge *Dragmacidon* sp.

Chemically, the collected sponge of the genus *Dragmacidon* was characterized by the presence of the diterpene amphilectene isocyanide (**4**) in addition to the isolated thio- and isothiocyanate sesquiterpenes **1**, **2**, **3**, and **5**. Metabolite **4** was absent in sponges of the genus *Axinyssa* and in the sponge *Acanthella cavernosa*. Furthermore, the sponge *Dragmacidon* sp. in general has **4** as its major compound (see Figure 57 & Table 15).

Sponge samples of *Dragmacidon* sp. vary in the relative composition of compounds 9α-thiocyanatopupukeanane (**1**), 9β-thiocyanatopupukeanane (**2**), and amphilectene isocyanide (**4**) but seem to show a similar trend in the relative composition of compounds 2-thiocyanatoneopupukeanane (**3**) and epipolasin-A (**5**).

Of five specimens of *Dragmacidon* sp. collected from three different islands, three sponge specimens contained compounds **2** and **4** as their major compounds while two specimens have **1** and **2** as their major metabolites of which the percentage of compound **4** is only slightly less than that of **1**. The concentration variations of sesquiterpene isocyanides from sponge samples of *Dragmacidon* sp. are shown in Table 15.

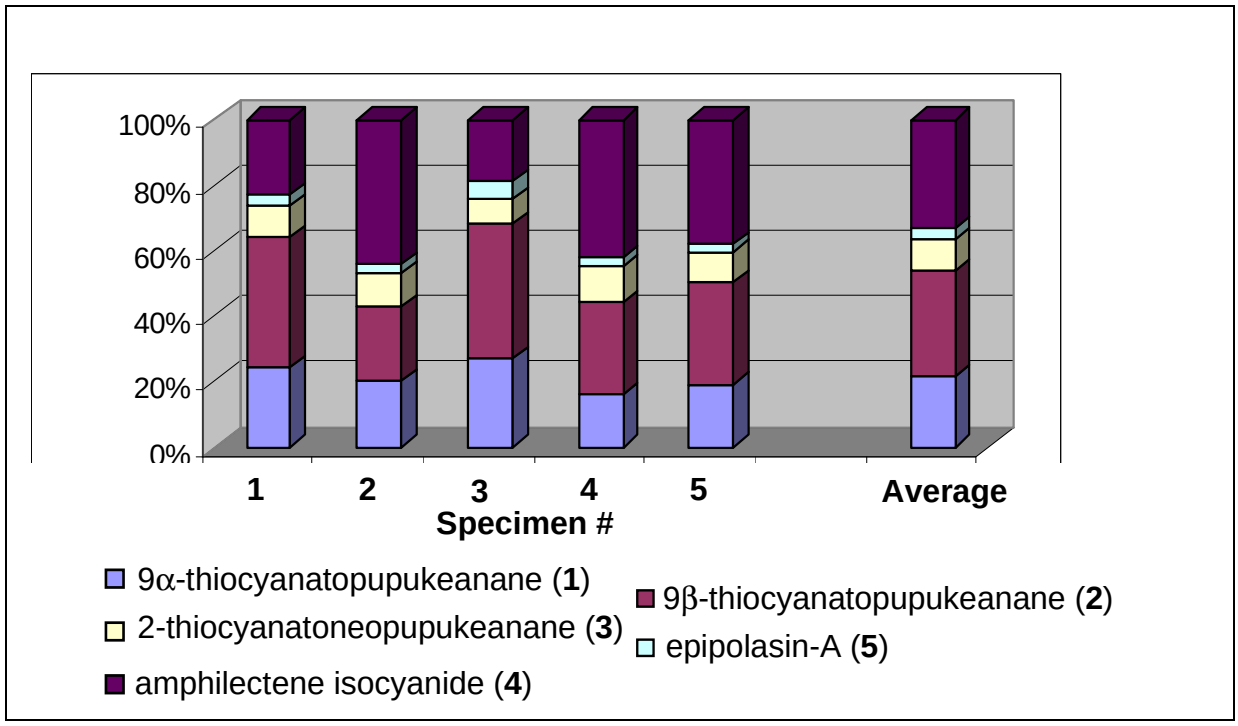


Figure 57. Relative composition of terpene isocyanides in *Dragmacidon* sp.

Table 15. Concentration variation in ppm of terpene isocyanides of sponge samples of *Dragmacidon* sp. collected from different islands in the Thousand Island National Park, Indonesia.

Name of island	9α-thiocyanatopupu-keanane (**1**)	9β-thiocyanatopupu-keanane (**2**)	2-thiocyanatoneo-pupukeanane (**3**)	amphilectene isocyanide (**4**)	epi-polasin (**5**)
Karang Lebar reefs	131	218	51	126	18
Karang Congkak	180	198	89	291	21
Karang lebar reefs	106	159	30	73	21
K. Balik Layar	341	592	232	884	61
Karang Lebar reefs	207	334	102	409	29

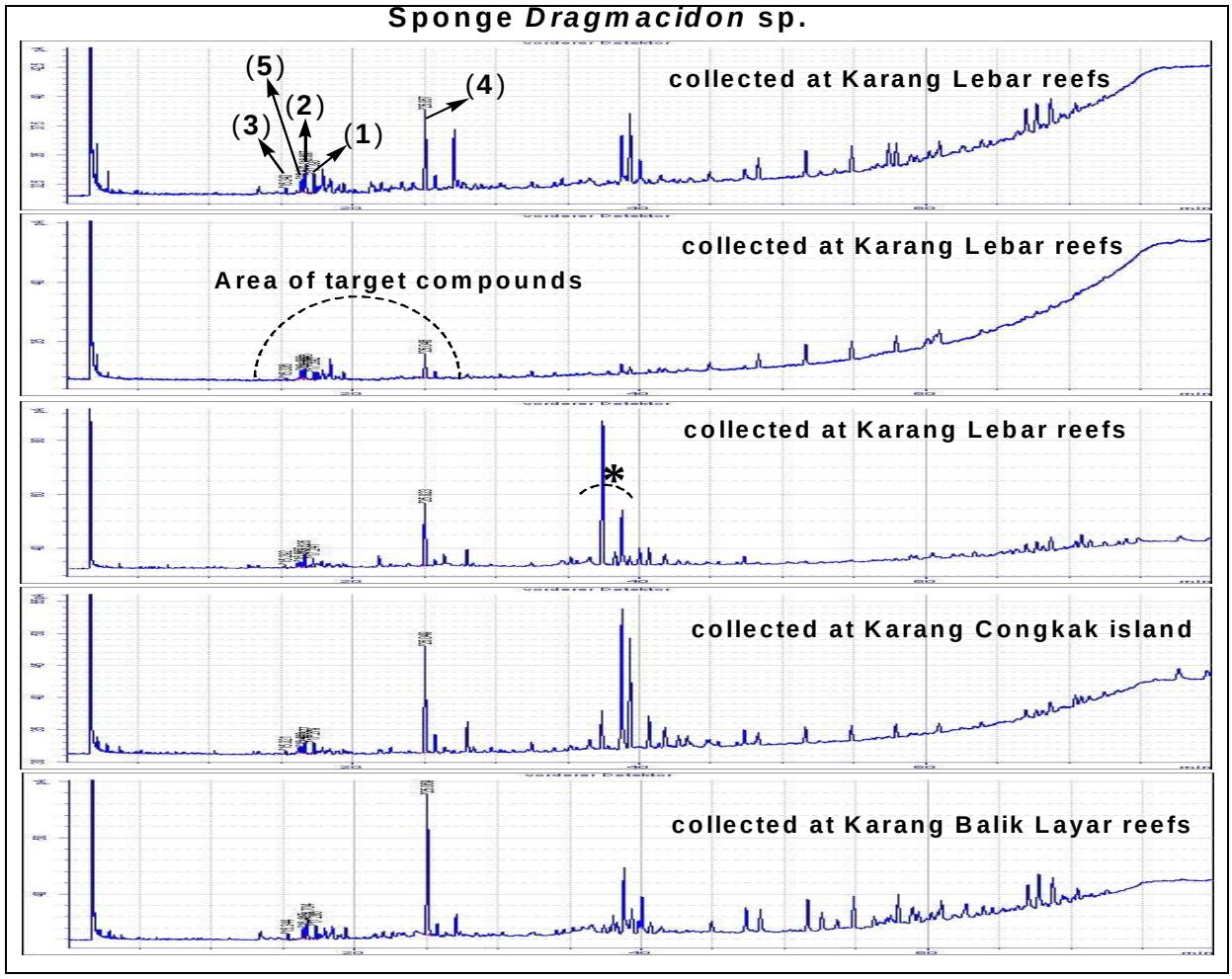


Figure 58. GC chromatogram of EtOAc extracs of the sponge *Dragmacidon* sp.
[* Due to very small amounts of sample collected, these peaks can not be isolated and further elucidated]

3.4.3 Quantitative variation of compounds in the sponge *Acanthella cavernosa*

Sponge samples of *A. cavernosa* vary in the relative composition of 9α-thiocyanatopupukeanane (**1**), 9β-thiocyanatopupukeanane (**2**), and 2-thiocyanatoneopupukeanane (**3**). Compound **3** was the dominanting metabolite in two sponge samples collected from the coral reefs of Putri and Karang Congkak Islands, respectively. In both sponges, compound **1** also occurred at a higher ratio compared to **2**. Of the total 61 collected sponge samples, this is the second and third records where the α epimer **1** was more dominant than its β form **2**. This phenomenon was first observed in a sponge specimen of *Axinyssa 6* n. sp., collected from Karang Congkak Island.

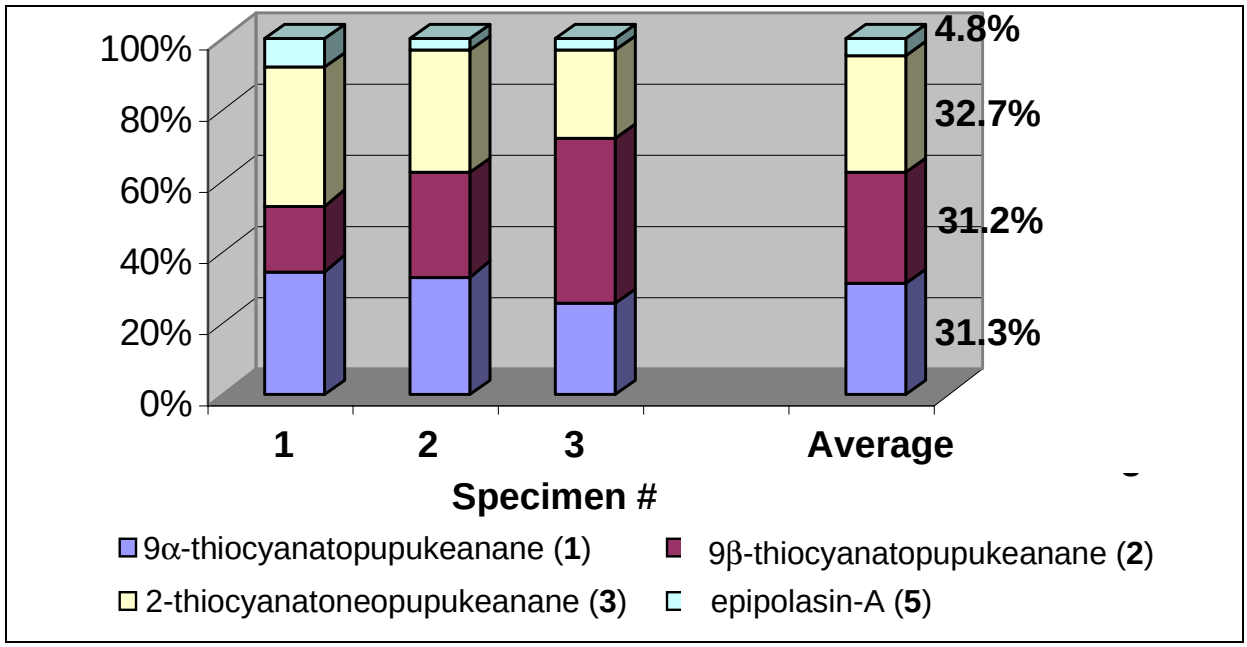


Figure 59. Relative composition of sesquiterpene isocyanides in *A. cavernosa*.

Table 16. Concentration variation in ppm of sesquiterpene isocyanides of sponge samples of *Acanthella cavernosa* collected from different islands in the Thousand Island National Park, Indonesia.

Name of island	9α-thiocyanatopupu-keanane (**1**)	9β-thiocyanatopupu-keanane (**2**)	2-thiocyanatoneopupu-keanane (**3**)	epipolasin (**5**)
Putri	33	17	37	7
Karang Congkak	140	122	145	14.3
Opak Kecil	20	35	19	3

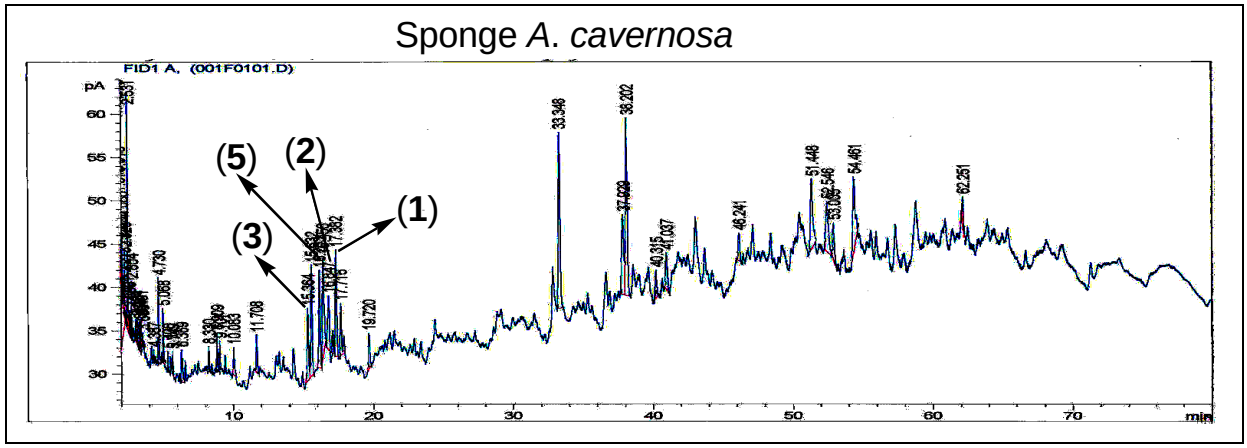


Figure 60. GC chromatogram of EtOAc extract of sponge *A. cavernosa*.

3.5 Analysis of isocyanide compounds between phyllidiid species and their sponge-preys

Phyllidiid species have been reported to sequester isocyanide compounds from their sponge-preys. The content ratio of the terpene isocyanides present in both pyhyllidiid species and their sponge-preys was clearly evidenced by analysis of the GC chromatogram of their crude organic extracts. From this analysis, unknown peaks were also observed to occur in both phyllidiid and sponge extracts or in only one of them (Figure 62). Due to very small quantity of samples collected, it was not possible to isolate and elucidate these peaks. However, it was shown that the presence of terpene isocyanides in both predator and prey were comparable in their content ratio (Figure 61).

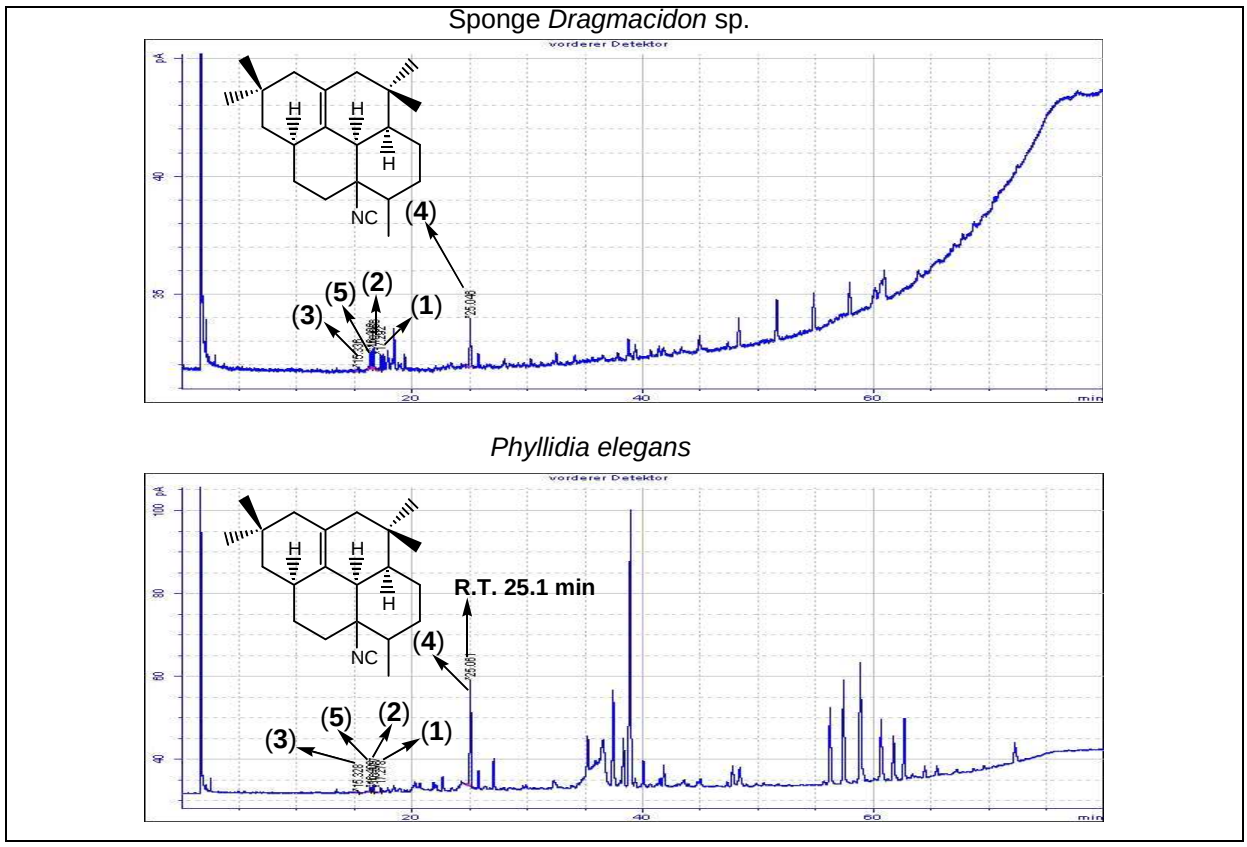


Figure 61. Comparison of GC chromatograms of EtOAc extract of phylidiid *P. elegans* and its sponge-prey to show terpene isocyanide derivatives in both extracts.

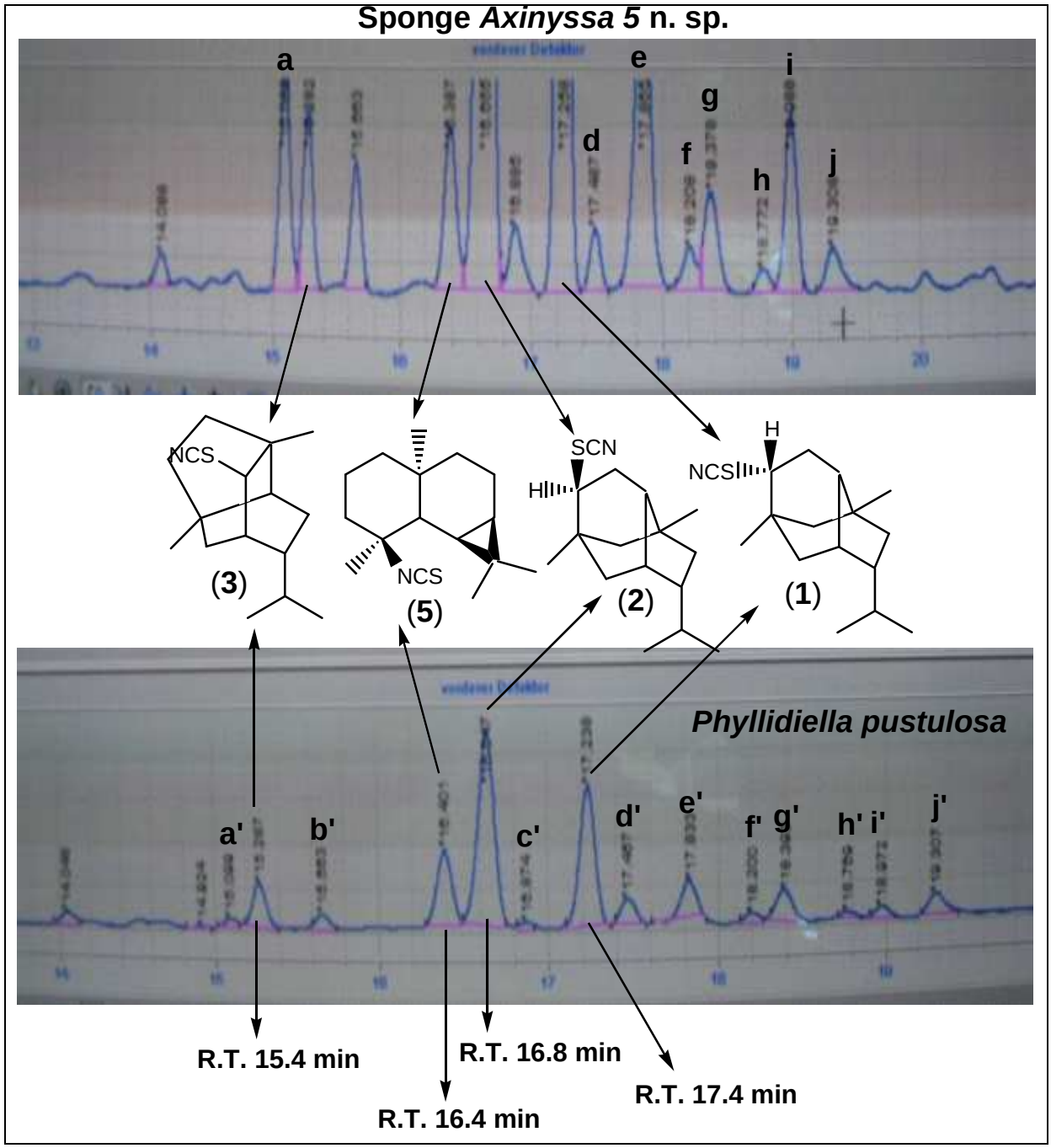


Figure 62. Expansion of selected region of the GC chromatograms of EtOAc extracts of the phyllidiid and its sponge-prey to show sesquiterpene isocyanide derivatives in both extracts.

It was also sometimes shown that the content ratio of terpene isocyanides found in phyllidid species seems not to be always comparable with their sponge-preys. A quantitative comparison of the presence of sesquiterpene isocyanides in the phyllidiid *F. menindie* and its sponge-prey showed that phyllidiids tend to selectively accumulate the metabolites from their diet. *F. menindie* seems to accumulate 2-

thiocyanatoneopupukeanane (**3**) from its sponge-prey that has the metabolite 9α-thiocyanatopupukeanane (**1**) as its major component (Figure 63).

Table 17. Concentration in ppm of terpene isocyanides of the phyllidiid *F. menindie* and its sponge-prey *Axinyssa 6* n. sp. collected from Karang Congkak islands, Thousand Island National Park, Indonesia.

Name of animals	9α-thiocyanatopupu-keanane (**1**)	9β-thiocyanatopupu-keanane (**2**)	2-thiocyanatoneopupu-keanane (**3**)	epipolasin (**5**)
F. menindie	55	105	321	Trace amounts
Axinyssa 6 n. sp.	33	17	11	5

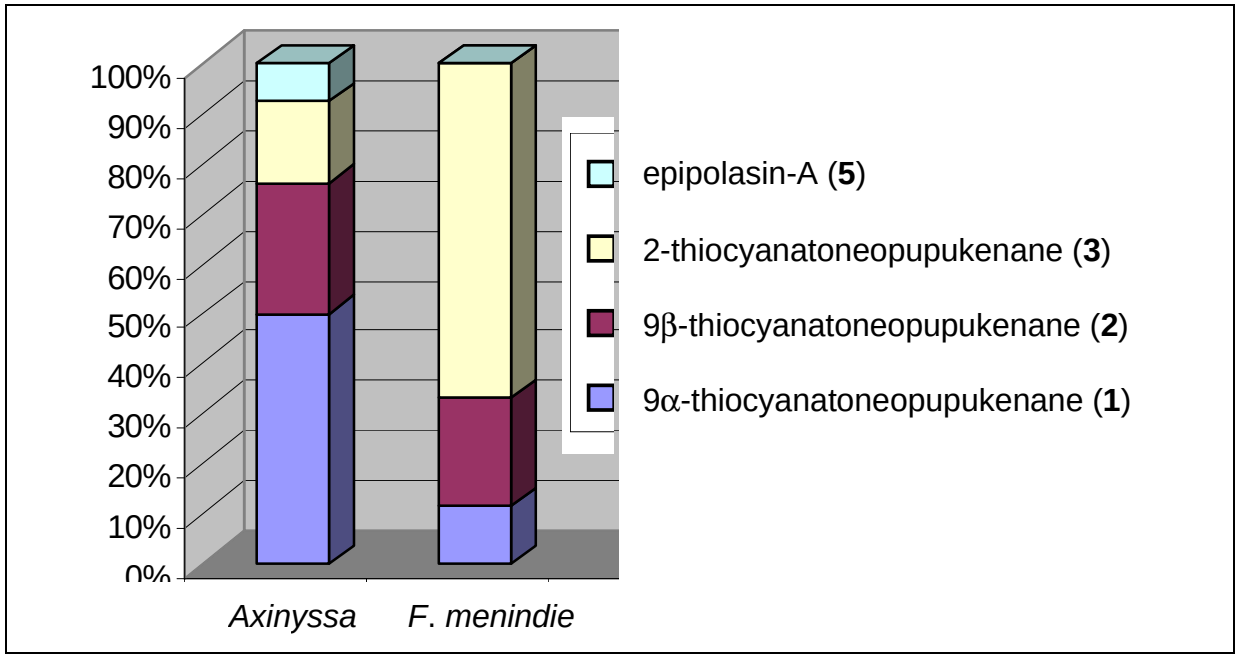


Figure 63. Comparison of the relative composition of sesquiterpene isocyanides of phyllidiid *F. menindie* and its sponge-prey *Axinyssa 6* n. sp.

TLC chromatogram analysis of the EtOAc extract both from phyllidiids and sponge-preys can only clearly show the presence of the epimers **1** and **2** as well as compound **4** which were present as their major compounds. However, compounds **3** and **4** that were present in minor quantities can only be detected after fractionation.

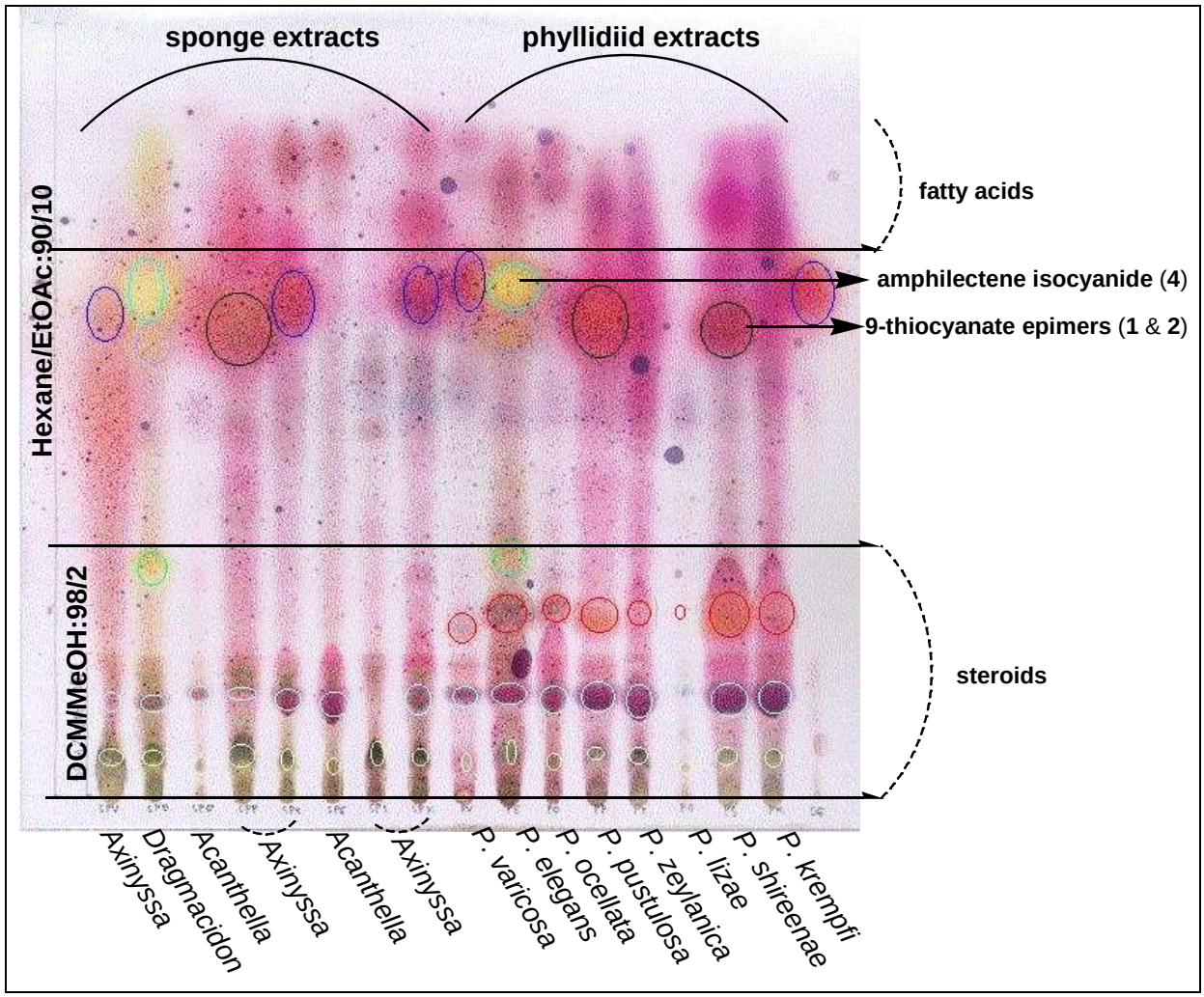


Figure 64. TLC chromatogram of phyllidiids and their sponge-preys.

The presence of sesquiterpene thiocyanates **1**, **2**, and **3** could be also evidenced by their ^{1}H NMR spectra through the resonances of the proton attached to carbon bearing the thiocyanate functionality which was observed between 3.20-3.50 ppm. They were observed in the ^{1}H NMR spectra of sesquiterpene isocyanides containing fractions from both the EtOAc extracts of the phyllidiids and their sponge-preys. The different intensity of the proton signal indirectly showed the content ratio of the respective sesquiterpenes in the fraction being analyzed. This information was very useful in comparing the ratios of the epimeric mixture, 9-thiocyanatopupukeanane (**1** and **2**) present in a purified fraction since it was not possible to separate these two isomers. Their content ratio was also supported by GC analysis and such information was again necessary when testing the mixture in biological assays.

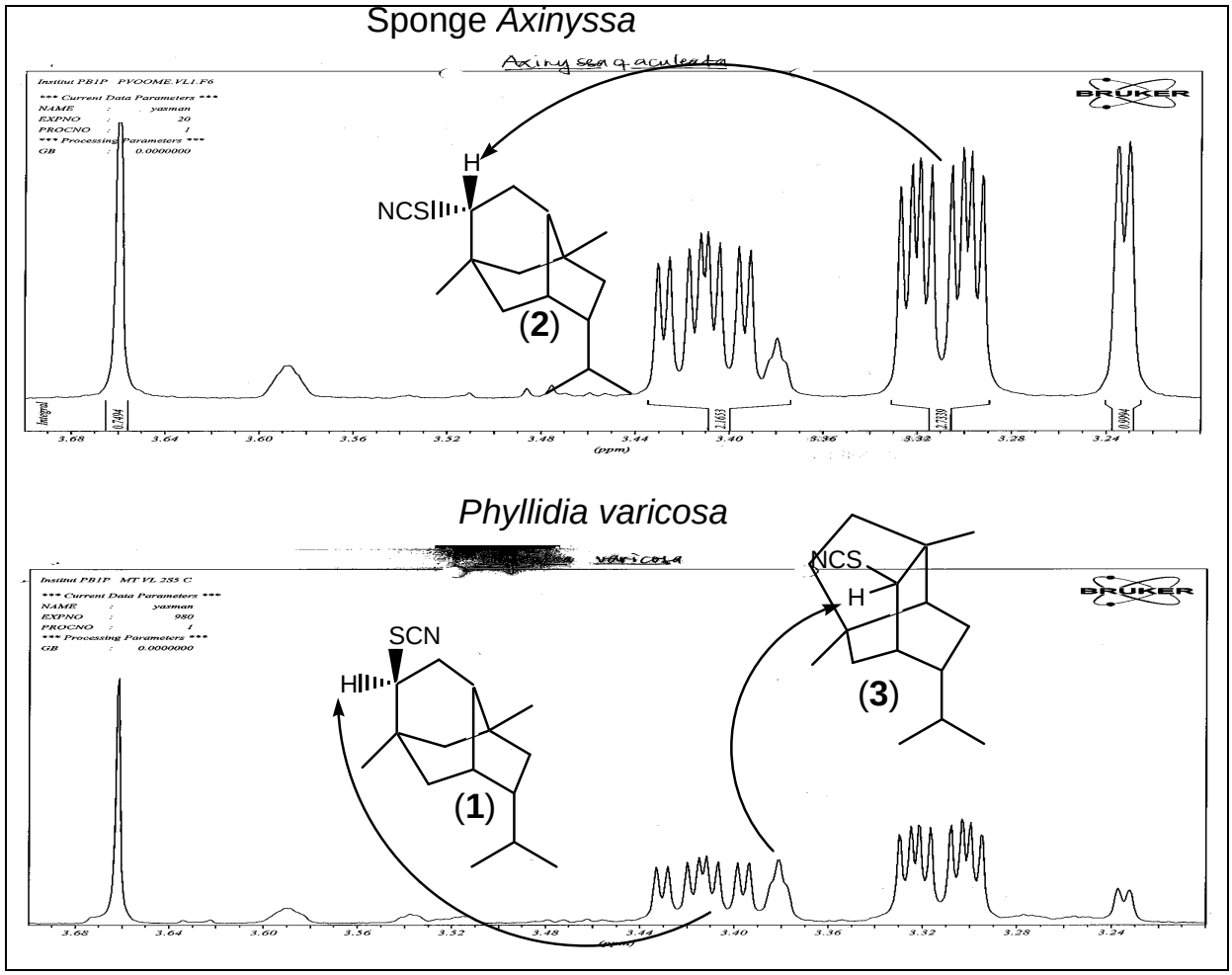


Figure 65. ^{1}H NMR spectra of the sesquiterpene thiocyanates containing fractions of phyllidiid and its sponge-prey.

With regard to their terpene metabolites, most of the collected phyllidiids species in the Thousand Islands contained the epimers 9α- and 9β-thiocyanatopupukeanane (**1**, **2**) as their major compounds. Only *P*. *elegans* and *F*. *menindie* yielded amphilectene isocyanide (**4**) and 2-thiocyanatoneopupukeane (**3**) respectively, in addition to the epimers **1** and **2** as their major component.

3.6 Organ specific distribution of terpene isocyanides in phyllidiids

Three selected species (*P*. *pustulosa*, *P*. *shireenae*, *P*. *krempfi*) tend to sequester 9α- and 9β-thiocyanatopupukeanane (**1**, **2**) as the major compounds and phyllidiid *P*. *elegans* which accumulated amphilectene isocyanide **4** showed a similar distribution strategy of the sequestered isocyanide derivatives. The concentration of terpene isocyanides in the digestive gland is the highest compared to the mantle and foot part. This is supporting the idea that the digestive gland should be the first

responsible organ of storing the nudibranch's metabolites of dietary origin (Faulkner & Ghiselin, 1983; Cimino & Sodano, 1994). It has been assumed that the nudibranchs transport the metabolites to the mantle which is more vulnerable to predation (Garcia-Gomez *et al*., 1990). As observed, the mantle part contains a higher concentration of sequestered terpene isocyanide compared to the foot (Table 18-21). The mechanism of transporting the defensive chemicals to the mantle through the digestive gland is unfortunately not known (Avila, 1995; Avila & Paul, 1997).

Table 18. Organ specific distribution of terpene isocyanides in the phyllidiid *P. pustulosa*.

Compounds	*Phyllidiella pustulosa* (1 ind. ~ 0.6 g dw)		
	Digest. gland (in ppm)	Mantle (in ppm)	Foot (in ppm)
9α-thiocyanatopupukeanane (**1**)	315.5	145.2	52.2
9β-thiocyanatopupukeanane (**2**)	466.7	263.8	100.2
2-thiocyanatoneopupukeanane (**3**)	140.8	72.5	21.2
8-isocyano-1(12)-cycloamphilectene (**4**)	-	-	-
Epipolasin-A (**5**)	37.0	34.3	10.4

Table 19. Organ specific distribution of terpene isocyanides in the phyllidiid *P. shireenae*.

Compounds	*Phyllidiopsis shireenae* (1 ind. ~ 0.9 g dw)		
	Digest. gland (in ppm)	Mantle (in ppm)	Foot (in ppm)
9α-thiocyanatopupukeanane (**1**)	265.3	78.4	12.4
9β-thiocyanatopupukeanane (**2**)	357.8	116.6	18.9
2-thiocyanatoneopupukeanane (**3**)	435.6	78.3	10.2
8-isocyano-1(12)-cycloamphilectene (**4**)	-	-	-
Epipolasin-A (**5**)	23.0	13.4	3.4

Table 20. Organ specific distribution of terpene isocyanides in the phyllidiid *P. krempfi*.

Compounds	*Phyllidiopsis krempfi* (1 ind. ~ 1.4 g dw)		
	Digest. gland (in ppm)	Mantle (in ppm)	Foot (in ppm)
9α-thiocyanatopupukeanane (**1**)	29.5	33.4	19.0
9β-thiocyanatopupukeanane (**2**)	57.3	51.6	32.4
2-thiocyanatoneopupukeanane (**3**)	23.1	19.2	13.7
8-isocyano-1(12)-cycloamphilectene (**4**)	-	-	-
Epipolasin-A (**5**)	65.9	27.2	11.9

Table 21. Organ specific distribution of terpene isocyanides in the phyllidiid *P. elegans*.

Compounds	*Phyllidia elegans* (1 ind. ~ 0.6 g dw)		
	Digest. gland (in ppm)	Mantle (in ppm)	Foot (in ppm)
9α-thiocyanatopupukeanane (**1**)	98.2	35.2	2.2
9β-thiocyanatopupukeanane (**2**)	177.3	58.0	6.8
2-thiocyanatoneopupukeanane (**3**)	95.7	13.0	Trace amounts
8-isocyano-1(12)-cycloamphilectene (**4**)	21.2	199.8	2.7
Epipolasin-A (**5**)	36.8	5.3	0.8

3.7 Fish feeding experiments

3.7.1 Qualitative feeding deterrent experiments with intact phyllidiid species

At the site of the experiment, many small and later bigger fishes of different species were observed swimming nearby and attacked a piece of fresh octopus's arm which was used as a bait to initiate the feeding experiment. Once a big fish came to swallow the food, this initiation process was placed to an end and phyllidiid species

were then offered as food. One individual of *P*. *varicosa* was allowed to pass through the water column and it was observed that the marine fishes also came nearby but then they let the phyllidiid free to swim down to the bottom of the column. The same reaction was also observed for other three samples of phyllidiid species (2 ind. *P*. *pustulosa*, and 1 ind. *P*. *krempfi*, respectively). The next six phyllidiid species (1 ind. *P*. *varicosa*, 1 ind. *P*. *pustulosa*, 2 ind. *P*. *zeylanica*, 1 ind. *P*. *ocellata*, and 1 ind. *P*. *krempfi*, respectively) were not even attacked by the fishes. Interestingly, once a piece of octopus's arm was again passed through the water column, a similar situation as seen in the initiation process was again observed even though this has already occurred for some minutes since the initiation process. Qualitative feeding experiments in three different locations along the reefs of Pramuka Island showed a similar result as the first experiment.

3.7.2 Quantitative feeding deterrent experiments

Food pellets were incorporated with the respective isolated terpene isocyanides. Percentage of pellets eaten by fishes in comparison with the blank of each terpene compounds was very high in this fish antifeedant experiment: 64% for the epimeric mixture (1:2) 9α- and 9β-thiocyanatopupukeanane (**1**, **2**) at a concentration of 2000 ppm, 84% for the 2-thiocyanatoneopupukeanane (**3**) at 1000 ppm, and even 111% for the amphilectene isocyanide **4** at 2000 ppm. In comparison with the control of each isolated compounds, the percentage of pellets eaten by fish was also very high: 82%, 84%, 70%, and 76% respectively. From this result, it is apparent that all isolated compounds did not show a significant antifeedant activity.

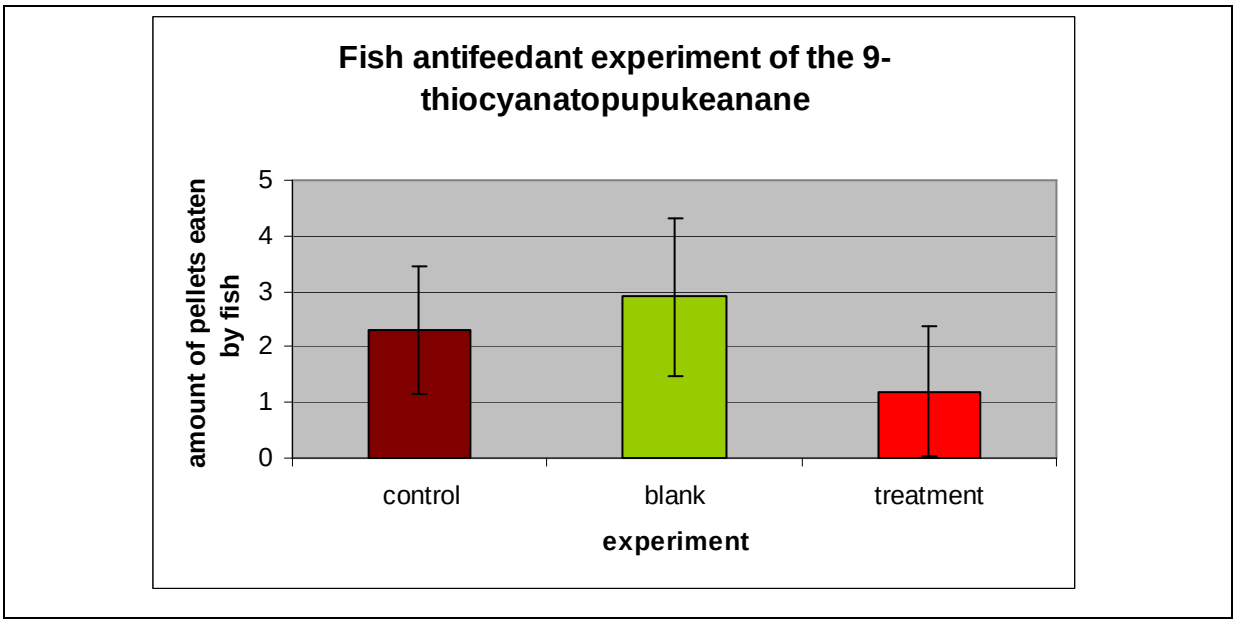


Figure 66. Amount of EtOAc and blank control as well as treated pellets eaten by fishes in the fish antifeedant experiment of the epimeric mixture **1** and **2**.

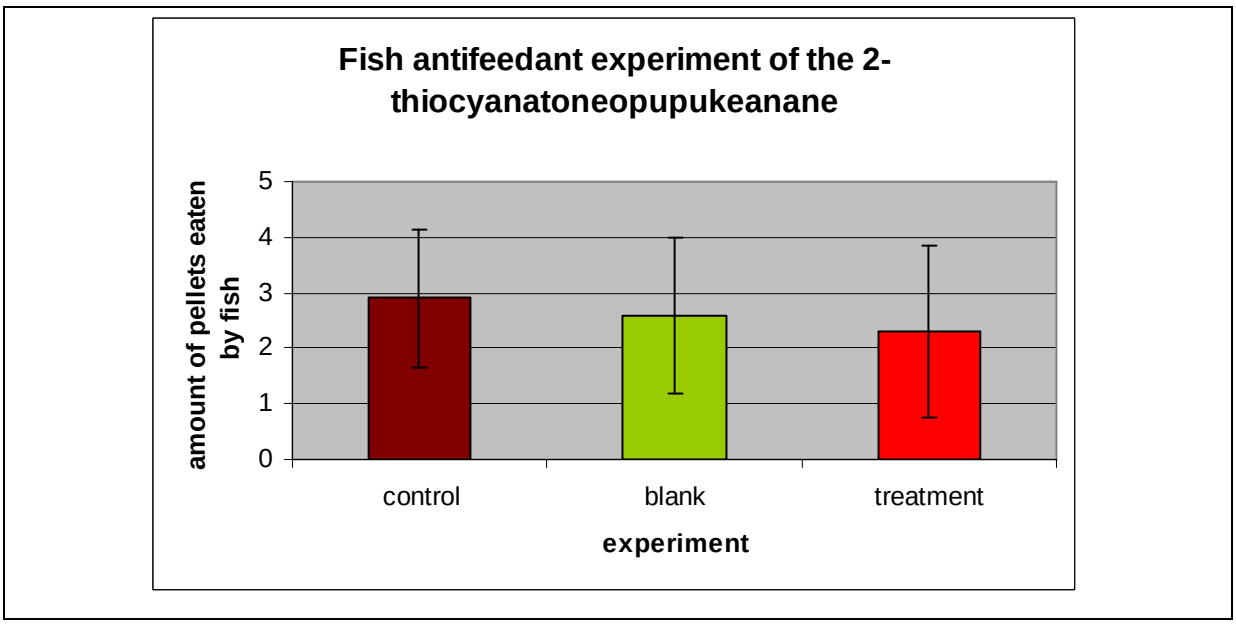


Figure 67. Amount of EtOAc and blank control as well as treated pellets eaten by fishes in the fish antifeedant experiment of 2-thiocyanatoneopupukeanane.

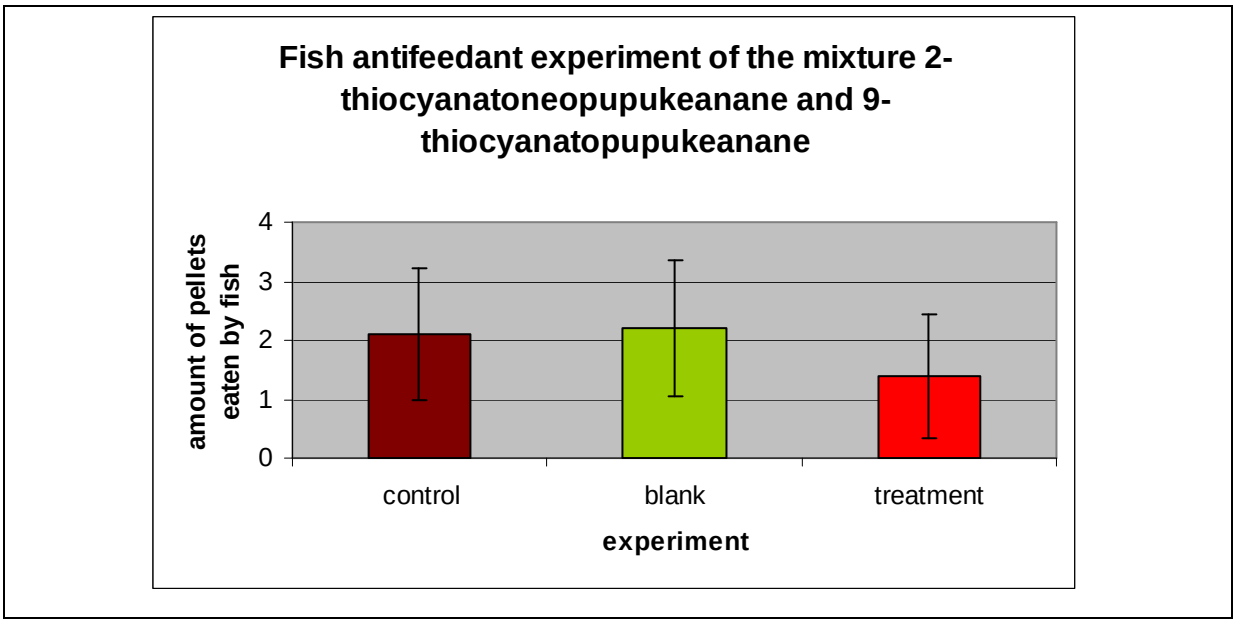


Figure 68. Amount of EtOAc and blank control as well as treated pellets eaten by fishes in the fish antifeedant experiment of the mixtures 9- and 2-thiocyanate.

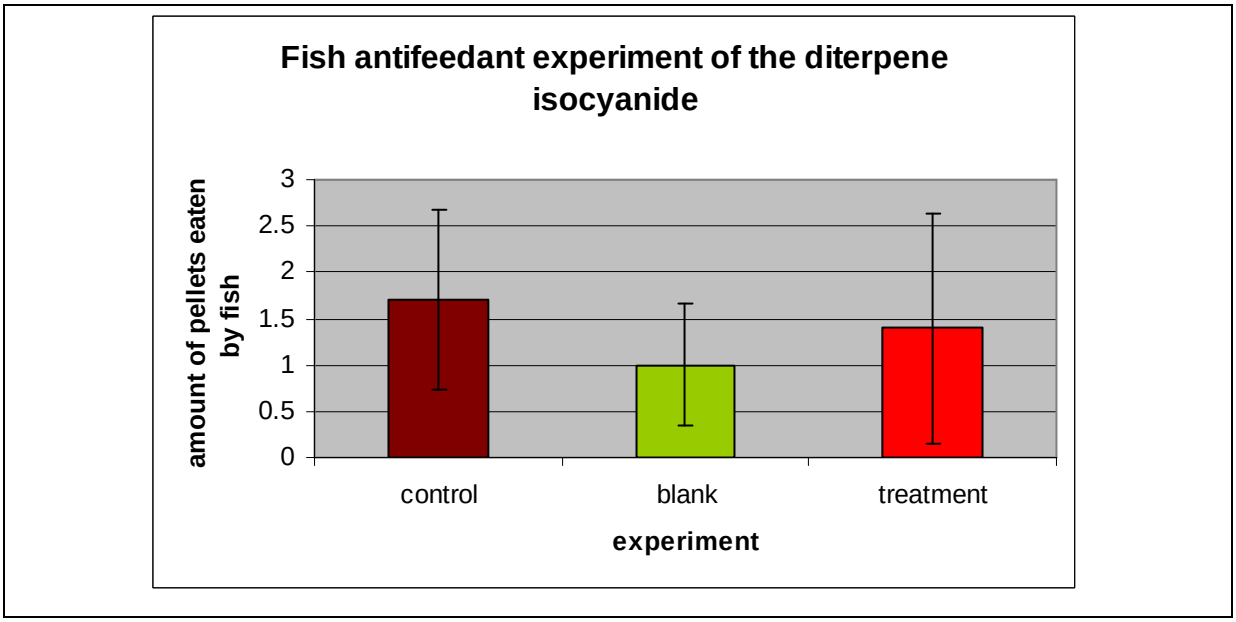


Figure 69. Amount of EtOAc and blank control as well as treated pellets eaten by fishes in the fish antifeedant experiment of amphilectene isocyanide.

3.8 Brine-shrimp and antimicrobial assays

Due to the limited amount of compounds **3**, **4**, and **5** only 9α- and 9β-thiocyanatopupukeanane (**1** & **2**) which was the major compound and new compounds were subjected to brine shrimp and antimicrobial assay. Since separation of compounds **1** and **2** was not successful, both compounds were bioassayed as an epimeric mixture. The ratios of epimers **1** and **2** present in the mixture were quantified by GC analysis from aliquots of the test samples used for the bioassays. At a dose of 200 ppm, different ratios of the epimeric mixture of compounds **1** and **2** were subjected to a brine shrimp assay. A mixture of 30:70 for epimer **1** versus epimer **2** showed a mortality of 90%, while a ratio 50:50 resulted in a 65% mortality rate. A series of concentration of the ratio 30:70 **1** to **2** showed a LD_{50} at 5 ppm (Figure 70). At a dose level of 20 µg, the mixture of 30:70 for epimer **1** versus epimer **2** was found to be weakly and moderately active against *B*. *subtilis* and *C*. *albicans*, respectively (Table 22).

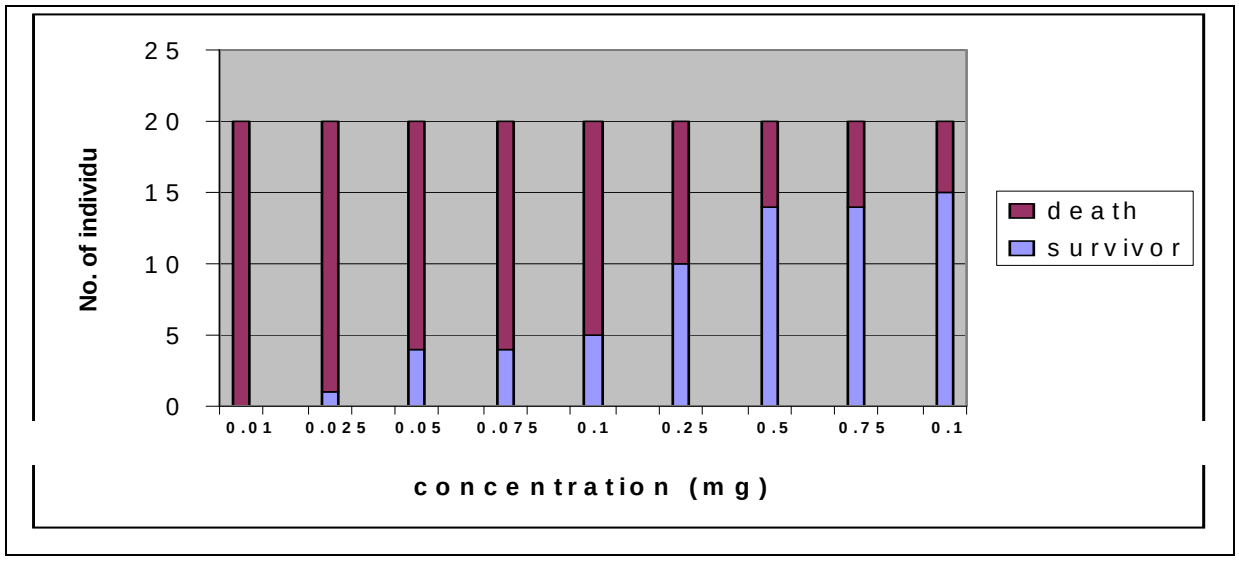


Figure 70. Brine shrimp assay of the epimers **1** and **2**.

Table 22. Antimicrobial activities of the epimeric mixture of **1** and **2**.

	S. *aureus*	*B*. *substilis*	*E*. *coli*	*C*. *albican*
Comp. **1** & **2**	-	12	-	9

4 Discussion

4.1 Feeding and behaviour of phyllidiid species

4.1.1 Feeding mechanism

Biological feeding process of phyllidiids species has been very well described by Brunckhorst (1993), but in general, reported feeding observations of phyllidiids are rare to date. The lack of radula, a special feeding tool for gastropods, makes the feeding strategy of phyllidiid species distinct from other rasping nudibranch species. Brunckhorst (1993) has proposed a possible feeding *modus operandi* for these phyllidiid species. The evertion of the pharyngeal bulb of phyllidiid species secreted both strong and weak acids which function to externally digest the sponge tissue. Phyllidiids are known as suctorial sponge feeders because the muscular pharyngeal bulb also produces the suction necessary to take up the products of predigestion. In agreement with Brunckhorst, it was found that *Phyllidia varicosa* is a good evidence of this proposed feeding *modus operandi* as shown in the present study. Strong and weak acids secreted by the pharyngeal bulb diluted the surface of the organic layer of the epidermis of the sponge to access the mesohyl which is gelatinous and collagenous but contain amorphous, amoeboid cells called archaeocytes. This process produced traces of predation on its sponge-preys which was documented for the first time in the present study (Figure 18 and 71)

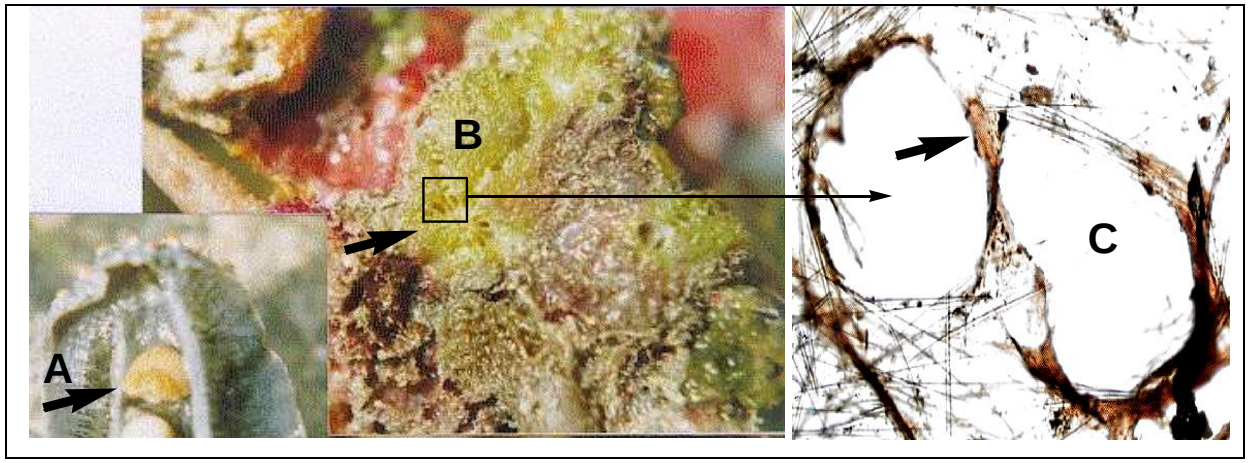


Figure 71. A. Dissected *P. varicosa* to expose the pharyngeal bulb; **B**. Trace of predation on the sponge-prey; **C**. Microscope slide of tangential section of predation trace on the sponge.

4.1.2 Morphological correlations between phyllidiids and their sponge-preys

Diets are the result of complex interactions between predator's abilities and preferences and prey availability (Emlen, 1966, 1968; McClintock, 1987). The current concept of optimal food selection involved the process of evolution acting on the predator, the food which maximizes their fitness in their environment then becomes the preferred prey (Emlen, 1968). Regarding the feeding morphology of nudibranchs, it was assumed that the most preferred preys are those to which the predator is most morphologically adapted.

Morphological correlations between rasping dorid nudibranch predators and their sponge-preys had been examined by Bloom in 1976. It was found that nudibranch species having less robust radulae preferrably consume non-reticulate sponges (e.g. sponge species of Halichondrida) while nudibranch species with more robust radulae would prefer to consume more reticulate sponges. In that study, Bloom also reported two species of radulae-less dorid, *Dendrodoris* nigra and *Doriopsilla albopunctata*, feeding on non-reticulate sponges (e.g. *Halichondria dura*, *cliona* sp., and *suberites ficus*) which followed the feeding preference trend of dorids having less robust radulae.

In the present study, all phyllidiid species were also observed feeding exclusively on the sponge species of the order Halichondrida. Of ten observed phyllidiid species, six of them fed on the sponge *Axinyssa* spp. (fam. Halichondriidae) that has a random type of spiculation, while three species on *Acanthella cavernosa* (fam. Dictyonellidae) whose skeletal spiculation is condensed choanosomal, and only one species fed on the sponge *Dragmacidon* sp. (fam. Axinellidae) which has the plumose reticulate type of spiculation. The latter two sponge families were characterized by the lack of ectosomal skeleton. In contrast, the sponge species of family Halichondriidae have a specialized ectosomal skeleton but the genus *Axinyssa* is an exception. This might suggest that phyllidiid species prefer to feed on certain sponge species of the order Halichondrida, those of which that have no ectosomal skeleton. Because of the phyllidiids's lack of radula, this preference could be understood. The possible reason for this choice is that phyllidiids need less effort to predigest the sponge's epidermis, contact with the mesohyl of sponges becomes faster, and therefore optimizes their predation. This assumption is apparently also supported by other chemistry-based studies on phyllidiid-sponge-prey relationships.

Phyllidiids have been reported to feed on non-reticulated sponges belonging to the order Halichondrida: *Ciocalypta* sp. (formerly know as *Hymeniacidon* sp.), *Axinyssa* sp., *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). With an exception for the sponge *Ciocalypta* sp., all those reported phyllidiids' sponge-preys are halichondrids that possess a non-ectosomal skeleton.

Brunckhorst (1993) found that each genus in the family of Phyllidiidae has a specific pharyngeal bulb morphology that distinct them to each other. But these differences apparently seem not to play a biological role for phyllidiid species being a specialist predator on a specific sponge. *Phyllidia varicosa* seems to feed exclusively on the sponge *Axinyssa* spp. that has a random type of spiculation. In contrast, *P*. *ocellata* seems to feed exclusively on the sponge *Acanthella cavernosa* whose skeletal spiculation is condensed choanosomal, meanwhile *P*. *elegans* seems to feed exclusively on the sponge *Dragmacidon* sp. which has the plumose reticulate type of spiculation. It was also found that *P*. *pustulosa* and *P*. *zeylanica* seem to be a specialist predator against the sponge *Axinyssa* spp. while *P*. *lizae* and *P*. *rudmani* seem to feed exclusively on the sponge *A*. *cavernosa* (see Figure 20).

Table 23. Morphological variations of the pharyngeal bulb of phyllidiid nudibranchs

	Phyllidia	*Fryeria*	*Phyllidiella*	*Phyllidiopsis*
Pharyngeal bulb	large, ovate	small, broad	very large	very elongated

4.1.3 Phyllidiids behaviours in aquarium

Ex situ observation of phyllidiids showed that the commencement of feeding activities took a longer time compared to the observations made by Garson and co-workers. Garson reported that *P*. *pustulosa* used in their observation settled and started to feed on the sponge *A*. *cavernosa* within 24 hours (Dumdei *et al*., 1997). In contrast, there were no feeding activities observed during the 24-hours observation period in the present study. Two feeding activities were just shown by phyllidiid *P*. *zeylanica* and *P*. *varicosa* in the 11th day of observation. Both phyllidiids were observed feeding on the sponge *Axinyssa*. Phyllidiid species, including *P*. *pustulosa*,

were never observed feeding on the sponge *A*. *cavernosa*, even after a month of *ex situ* feeding observation. In contrast with Garson's *in situ* feeding observation, the sponge *A*. *cavernosa* was observed to be predated by the phyllidiid *P*. *rudmani*, *P*. *lizae*, and *P*. *ocellata* but was never observed to be predated by *P*. *pustulosa*.

Unusual behaviour of phyllidiid species recorded in the present study showed how difficult it is to keep them naturally in an aquarium (see Figure 22). This difficulty has been discussed by Brunckhorst (1993) and Rudman (2001, *see* www.seaslugfroum.net). Even though the circulation of sea water in the aquarium was very well maintained, the phenomenon of anoxic condition behaviour, (e.g. lifting up the mantle edge to expose the gills and laying up side down to the water surface) were demonstrated by the observed phyllidiid species.

The observed mating processes between different phyllidiid species could not be explained (e.g. between *Phyllidiella pustulosa* and *P*. *zeylanica* as well as between *Phyllidia elegans* and *P*. *ocellata*; see Figure 21). Even if there is a possibility that *Phyllidiella pustulosa* and *P*. *zeylanica* in the present study were taxonomically not properly identified, this would not be possible for *Phyllidia elegans* and *P*. *ocellata*. Since *Phyllidiella pustulosa* and *P*. *zeylanica* were reported to be sympatric species then it could be plausible that they actually belong to one valid species but again this will be absurd for *Phyllidia elegans* and *P*. *ocellata*. This possibility can be actually assessed from egg masses produced by *P*. *pustulosa* and *P*. *ocellata* by proving their fertility. But this will consume a longer period of time and is beyond the aims of the present study.

4.2 Taxonomy and chemo-taxonomy of the collected sponge-preys of phyllidiid species

The order Halichondrida is characterized by their non-functional localized spicules; lack of microscleres and if present are rather microoxeas and/or trichodragmas; skeleton is peripherally tangential or undifferentiated; main skeleton composed of a criss-cross arrangement of spicules, or compressed into a distinct axis, or with plumose, plumo-reticulate or dendritic mineral skeleton; fibre system is poorly developed or absent. All these characteristics that would identify the sponge species to belong to the order Halichondrida are quite difficult (van Soest, 2005: pers.

comm.). This taxonomic difficulty also influenced the taxonomic identification of collected sponges in the present study.

4.2.1 *Dragmacidon* sp.

Dragmacidon is identified as an Axinellidae sponge (order Halichondrida) possessing plumoreticulate choanosomal skeleton and undifferentiated axial and extra-axial region; megascleres are oxeas and styles (Alvarez & Hooper, 2002). The type species of this genus, *Dragmacidon agariciformis* (Dendy, 1905) has a characteristic spiculation of styles and oxeas arranged in plumoreticulate skeleton with ascending plumose tract. Megascleres were generally found in similar proportions and dimension (Alvarez & Hooper, 2002).

The specimen under the registration no. ZMA POR 17172 which was identified as *Dragmacidon* sp. has also a similar characteristic as defined above except that the proportion of oxeot and stylote spicules is different. Stylote spicules were more commonly found compared to the oxeot spicules. The same case was also found in the specimens under the reg. no. 18177-81 which were observed to have exclusively stylote spicules; in fact oxeot spicules were just occasionally found, and were therefore primarily identified as *Stylissa* sp. According to Dr. van Soest (pers. comm., 2005), with regard to stylote spicules which were more commonly found, its identification as a *Dragmacidon* species could be re-examined.

Published reports on the chemistry of *Dragmacidon* sponges are rare. A literature survey showed that only three papers on this genus had been published (MarinLit version September 2004; Pedpradap *et al*., 2004). To date, *Dragmacidon* sponges were only reported to yield bis(indole) piperazine alkaloids (Kohmoto, *et al*., 1988; Mancini *et al*., 1996) and β-carboline alkaloids (Pedpradap *et al*., 2004). In the present study, however, all collected sponge of the genus *Dragmacidon* showed to contain the amphilectene isocyanide **4** and the sesquiterpene epimers **1** and **2** as their major compounds as well as 2-thiocyanatoneopupukeanane (**3**) and epipolasin-A (**5**) which were present as minor components. These chemical analyses may support the suggestion that the taxonomic identification of *Dragmacidon* sp. in the present study is still questionable and could be re-examined.

From a chemo-taxonomical point of view, the taxonomy of the collected *Dragmacidon* sponge is more acceptable than the other three previous studies.

Isocyanide compounds were commonly found in sponges of the order Halichondrida (Chang, 2000; Garson *et al*., 2000; Garson & Simpson, 2004) and were later proposed as a chemical marker of the order Halichondrida (Erpenbeck, 2004). It is therefore not surprising if the sponge *Dragmacidon* sp. were reported to contain a set of isocyanide compounds. Besides, the collected sponges were also observed to be predated by the phyllidiid *Phyllidia elegans* which also contain the same set of isocyanide compounds. With regards to its plumoreticulate choanosomal skeleton, the sponge *Dragmacidon* is actually not a very good prey candidate for phyllidiid species (*see* section 4.1.1). If sponges of the genus *Dragmacidon* contain no isocyanide compounds, it is likely that phyllidiid species might not feed on them. It has been known, that phyllidiid species metabolize their food, those which only contain compounds with the isocyanide functionality (Garson & Simpson, 2004 and related literatures therein; Manzo *et al*., 2004).

β-carboline alkaloid derivatives were previously isolated from the New Caledonian sponge *Hyrtios erecta* (Dictyoceratida) (Bourguet-Kondracki *et al*., 1996). Currently, a derivative of bis(indole) piperazine alkaloid (dragmacidin F) has been isolated from the sponge *Halichortex* sp. (Cutignano *et al.*, 2000). These data may suggest that chemo-taxonomically, the three former sponges studied by Kohmoto, *et al*. (1988), Mancini *et al*. (1996), and Pedpradap *et al*. (2004) are more closely related to the sponge *Halichortex* sp. and *Hyrtios erecta*, respectively, rather than the sponge *Dragmacidon*. Nevertheles, further study on the spicules character will be needed to consider the revision of their taxonomical position.

4.2.2 *Axinyssa* spp.

Ten sponge vouchers were identified as *A*. *aplysinoides* while two were referred to as *A*. cf. *aculeata* and *A*. aff. *aplysinoides*, respectively. When these sponge vouchers were re-examined, it was observed that none of them have spicules characteristic to either *A*. *aplysinoides* or *A*. *aculeata*. *A*. *aplysinoides* has a characteristic spiculation of long oxeas (maximal size 1.08 mm by 0.1175) which come out slightly on the surface to make a hispid condition (Dendy, 1922) while *A*. *aculeata* has a characteristic spiculation of long oxeas (size range 1.00-1.50 mm) arranged in a separated radial tract so that the conulus contains very few spicules, usually only two or three and sometimes even only one (Wilson, 1925).

Of the total 51 collected sponge vouchers of the genus *Axinyssa*, only two specimens have a characteristic spiculation which could be referred to the species *A*. *aplysinoides* (PK04-I and PZ02; predated by *P*. *krempfi* and *P*. *zeylanica*, respectively). The sponge voucher PZ02 was primarily identified as *Topsentia* aff. *dura* based on its hard, stony consistency. Following microscopical re-examination of the spicules, it was observed that that the specimen has no detachable surface skeleton which should be typical for sponges of the genus *Topsentia*. (Eppenberg & van Soest, 2002).

The sponge *Axinyssa 6* n.sp. consists of two specimens coded as FM04 and PP04-XIV. Sponges FM04 (ZMA POR 18286) and PP04-XIV (ZMA POR 18283) were identified by Dr. van Soest as *A*. aff. *aplysinoides* and *A*. *aplysinoides*, respectively. These two specimens were then excluded from those previously identified as *A*. *aplysinoides* because of their bundled microconulus spiculation and were proposed as a new species *Axinyssa 6* n. sp. due to their fully grown spicule size which was the shortest (0.78 and 0.80 mm) among the other sponge specimens. Regarding the sponge TM55 (reg. no. ZMA POR 17054) collected from Ujung Pandang, it has more or less the same spiculation and spicule size range (0.72 mm) as *Axinyssa 6* n. sp. but it was previously identified as *A*. *aplysinoides* (see Hassan 2004).

The same problem also occured with *Axinyssa 5* n. sp., a taxonomical identification assigned to 34 sponge specimens. Six of them were identified as *A*. cf. *aculeata* (PV00; ZMA POR 10885), *A*. aff. *aplysinoides* (PP02 and PS02; ZMA POR 17362 and 17363 respectively), *Axinyssa aplysinoides* (PV04-XIII and PZ04-II; ZMA POR 18285 and 18282), respectively. With regards to their fully grown spicule size, *Axinyssa 5* n. sp. is actually comparable with *A*. *aplysinoides* but showed differences in speculation form and arrangement. These 34 sponge specimens were proposed as *Axinyssa 5* n. sp. Unexpectedly the sponge ROK-K0 2 (reg. no. ZMA POR 17287) collected from Andaman Sea, Thailand, has more or less the same spiculation and spicule size (1.06 mm) as *Axinyssa 5* n. sp. In fact this sponge had been also identified as *A*. aff. *aplysinoides* (Pedpradap, 2005; pers. comm.).

Some variations in the relative chemical composition were observed within the different individuals' sponge species identified as *Axinyssa*. One specimen in the sponge *Axinyssa 6* n. sp. has 9β-thiocyanatopupukeanane (**2**) as the major compound and has 2-thiocyanatoneopupukeanane (**3**) more dominant than

epipolasin-A (**5**). Another specimen has 9α-thiocyanatopupukeanane (**1**) as its major component and isothiocyanate **5** was more dominant than **3**. From a total of 51 collected sponge specimens identified as *Axinyssa*, this is the only account that 9α-thiocyanatopupukeanane (**1**) was more dominant than 9β-thiocyanatopupukeanane (**2**).

Sponge specimens *Axinyssa 1* n. sp., *Axinyssa 4* n. sp., and *Axinyssa 5* n. sp. have epipolasin-A (**5**) as their major compound which was in contrary to the other specimens within these species that have 9β-thiocyanatopupukeanane (**2**) as their major compound. Another chemical variation was also found in the sponge species *Axinyssa 5* n. sp. One sponge specimen has 2-thiocyanatoneopupukeanane (**3**) as its major compound. Among the 51 sponge specimens collected, this is the first account that thiocyanate **3** more dominant than **2**.

Sponge specimens within the genus *Axinyssa* collected in this study have more or less the same set of sesquiterpene isocyanide metabolites. All the sponge specimens have 9β-thiocyanatopupukeanane (**2**) as their major compound while the other sesquiterpenes **1**, **3**, and **5** varied in their concentrations which were rather dependent on the species itself. *Axinyssa 6* n. sp. and *Axinyssa 5* n. sp. have **3** as a more dominant metabolite than **5** but less dominant than compound **1**. In contrast *Axinyssa 2* n. sp., *Axinyssa 4* n. sp., *Axinyssa 3* n. sp., *Axinyssa 1* n. sp., and *A. aplysinoides* have **5** to be a more dominant metabolite than **3** (Figure 72).

Such chemical variations in the sponge *Axinyssa* spp. are merely due to variation in sponge cells as similarly shown in the sponge *Aplysina aerophoba* (Thoms, 2004). It has been also reported that marine terpene isocyanides might function as a structural component of sponge cell membranes (Garson & Simpson, 2004). This might suggest that chemical analyses are not strongly recommended to decide sponge taxonomy. The sponge *A. aplysinoides* for instance has an identical chemical composition with that of *Axinyssa 3* n. sp. (Figure 72) but taxonomically they have different forms of spiculation. On the other hand, the two specimens of sponge *Axinyssa 6* n. sp. are totally different in their chemical composition (Figure 56), but taxonomically their spiculations are identical.

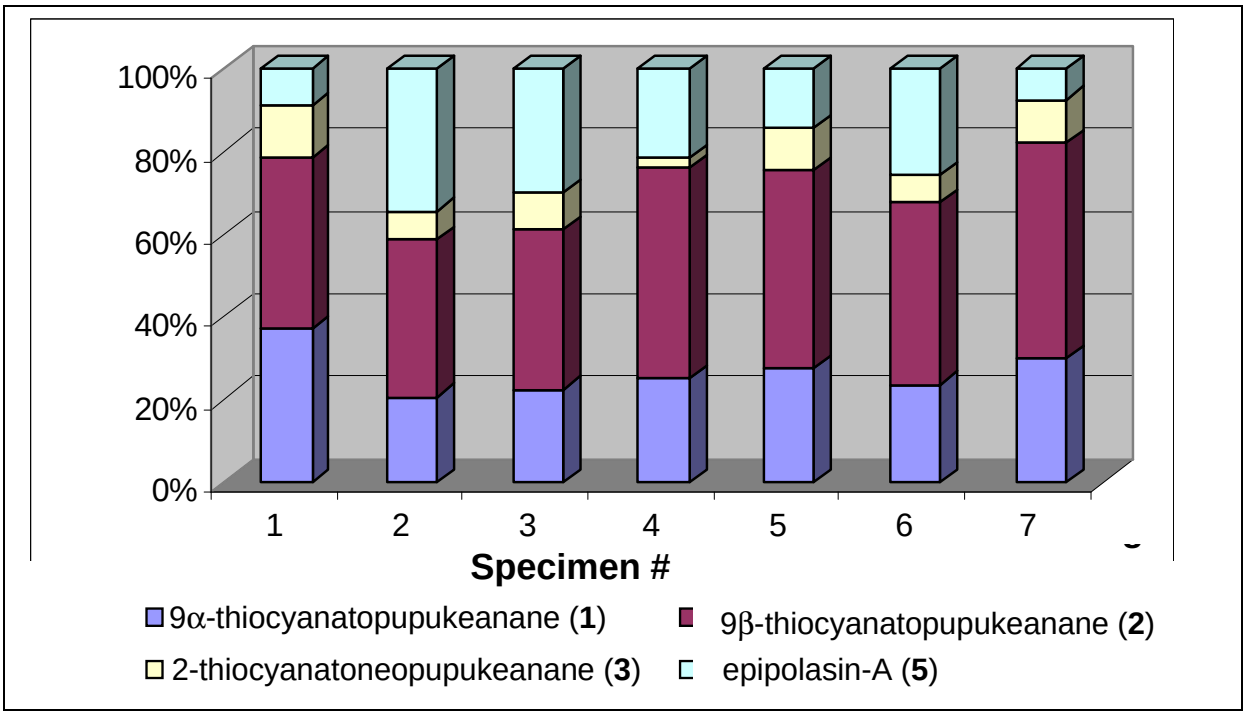


Figure 72. Relative composition of sesquiterpene isocyanides **1**, **2**, **3**, and **5** in the collected sponge of the genus *Axinyssa*: (1) *Axinyssa 6* n. sp.; (2) *Axinyssa aplysinoides*; (3) *Axinyssa 3* n. sp.; (4) *Axinyssa 2* n. sp.; (5) *Axinyssa 4* n. sp.; (6) *Axinyssa 1* n. sp.; (7) *Axinyssa 5* n. sp.

4.3 Isolated compounds of diterpene isocyanide, thio- and isothiocyanate sesquiterpenes

Compounds containing an isocyano group have fascinated natural products chemists ever since the first isocyanide xanthocillin was isolated from the fungus *Penicillium notatum* Westling (Rothe, 1950). To date isocyanide compounds were only found in bacteria, fungi, blue-green algae (cyanobacteria), and marine sponges and their predator (nudibranchs) (Chang, 2000; Garson & Simpson, 2004). Nowadays, marine isocyanides represent the largest group of naturally occuring isocyanide in nature (Garson & Simpson, 2004).

Most sesquiterpenoid marine isocyanides and isothiocyanates as well as thiocyanates belong to eight major skeletal types whereas diterpene marine isocyanides belong to two major skeleton, i.e. kalihinane and amphilectene (Figure 73). In common with their diterpene counterparts, the C_{15} sponge constituents are cyclic but unlike the diterpenes, the sesquiterpenes contain only a single nitrogenous function (Chang, 2000).

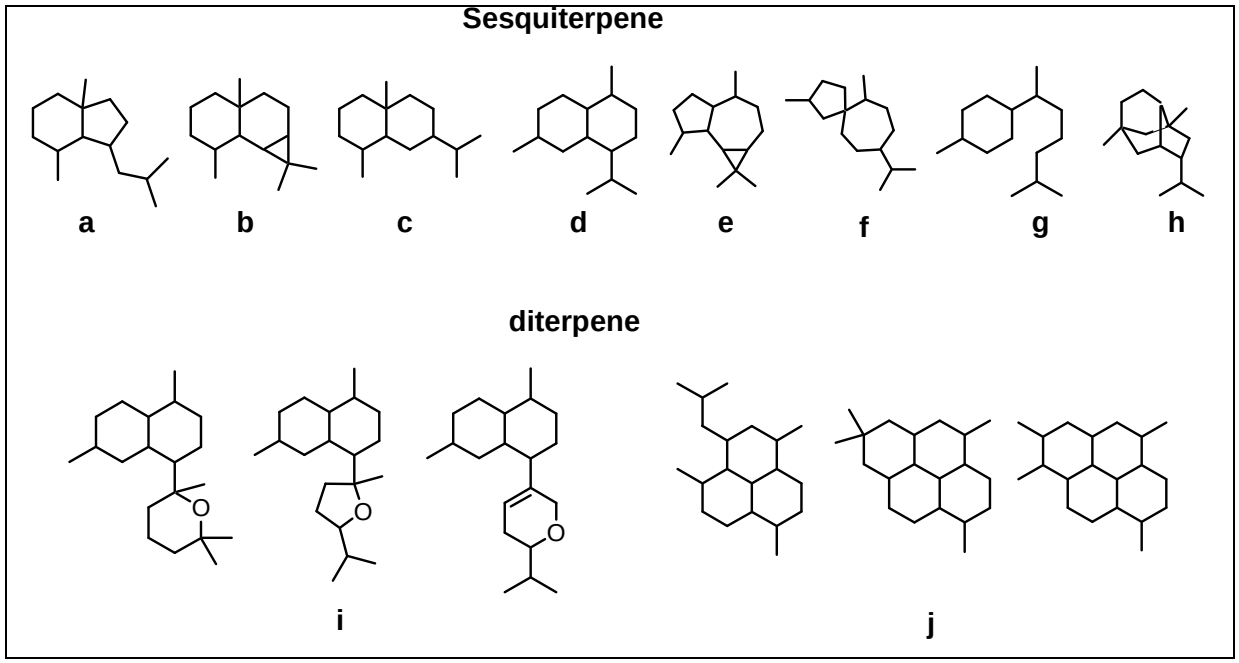


Figure 73. Major skeletal types of marine isocyanide: a. axane; b.epimaaliane; c. cadinane; d. aromadendrene; e. spiroaxane; f. bisabolene; g. pupukeanane; h. kalihinane; i. amphilectene. [Reproduced from Chang, 2000].

In the present study, four thio- and isothiocyanate sesquiterpenes (**1**, **2**, **3**, **5**) and one diterpene isocyanide (**4**) were successfully isolated. Thiocyanates **1** and **2** have the pupukeanane skeleton and thiocyanate **3** has the modified backbone of the pupukeanane which was named neopupukeanane, whereas isothiocyanate **5** has the epimaaliane skeleton.

4.3.1 Compounds containing the thiocyanato function (compounds 1, 2, & 3)

The complex tricyclic sesquiterpenes, pupukeanane, and neopupukeanane, were initially elucidated by Scheuer's group through X-ray analysis of 9- and 2-isocyanopupukeanane (Burreson *et al*., 1975a; Hagadone *et al*., 1979) and through NMR data analysis for 9-isocyanoneopupukeanane (Karuso *et al*., 1989). Owing to their unique molecular structure, those natural tricyclic sesquiterpenes offer an interesting challenge to synthetic chemists (Srikrishna & Gharpure 2002). Three selected analog synthetic pathways of 9-isocyanopupukeanane which is comparable to compounds **1** and **2** are shown in Figure 74; whereas synthetic pathways of 4-thiocyanatoneopupukeanane as to that of compound **3** is shown in Figure 75. Biosynthetically, the origin of pupukeananes and neopupukeananes can be

explained by a common pathway via cyclization and rearrangement of cadinanes as shown in Figure 77 (Karuso, 1989).

The possibly natural origin of the thiocyanate functionality has been investigated by Garson's group (Simpson & Garson, 1998). The results of their experiment strongly suggested the conversion of inorganic cyanide into inorganic thiocyanate in a sponge (*see* the complete scheme in Figure 76). The incorporation of cyanide into a terpene, followed by insertion of sulphur (by an enzyme functionally equivalent to rhodanese) remains a possibility, however equilibrium usually favors an isothiocyanate over a thiocyanate. The lack of neither 9-isocyanide nor 9-isothiocyanatopupukeanane in the present study also suggested that direct conversion of inorganic cyanide into inorganic thiocyanate in the sponges was more possible than the insertion of sulphur into a cyanide sesquiterpene.

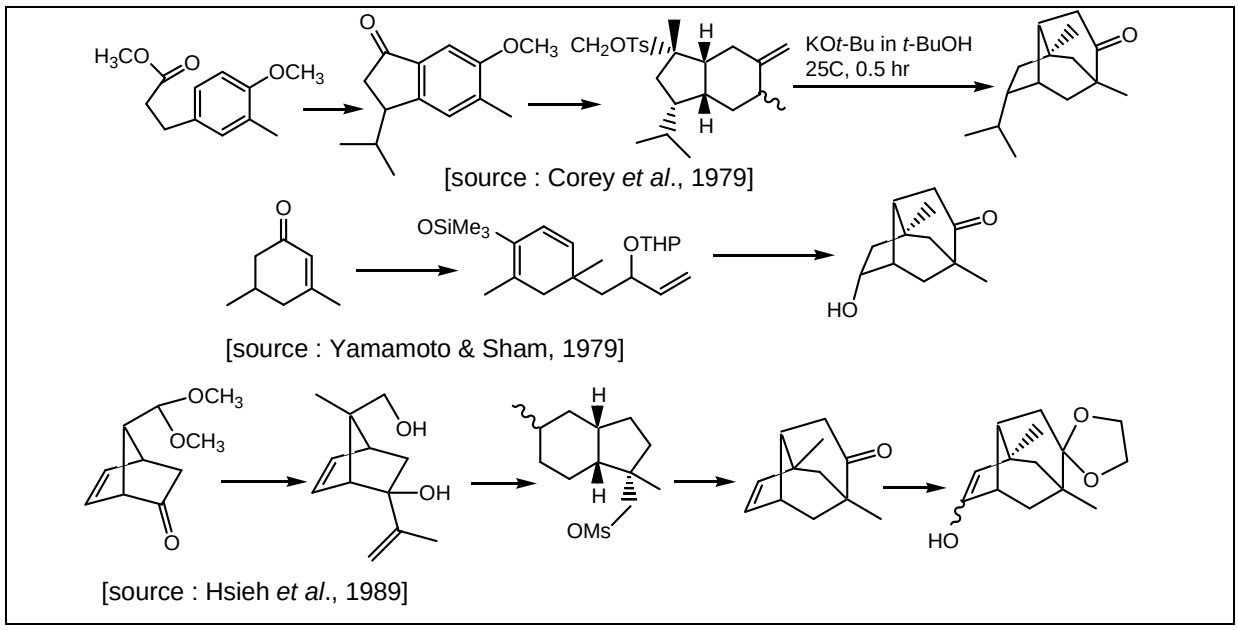


Figure 74. Three selected analogs synthetic pathways of 9-isocyanopupukeanane.

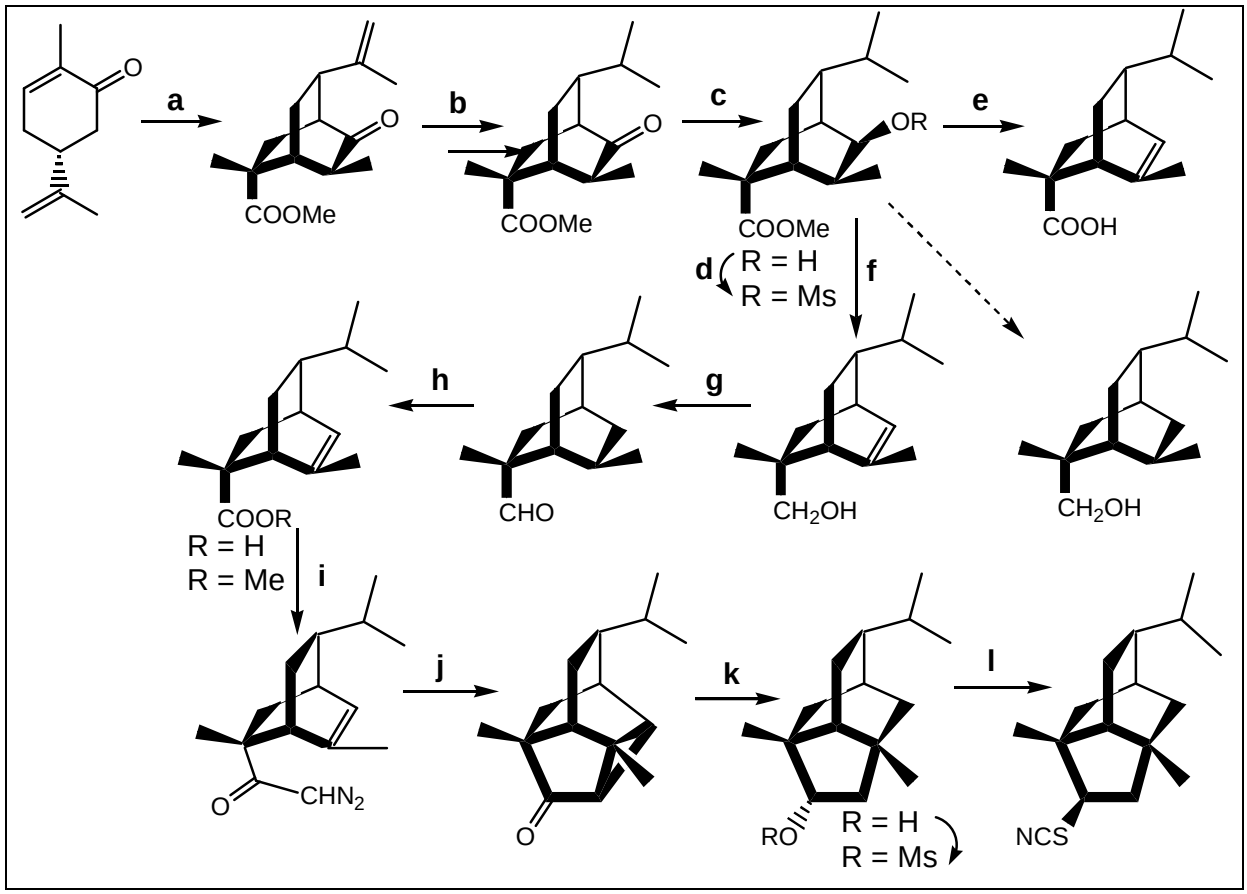


Figure 75. Synthesis of 4-thiocyanatoneopupukeanane. Reagents: (a) LiHMDS, CH_2=C(Me)COOMe; (b) H_2, 10%Pt/C; (c) $NaBH_4$; (d) MsCl, py, DMAP; (e) NaOEt; (f) LAH; (g) PCC, NaOAc; (h) Jones' reagent; (i) $(COCl)_2$, CH_2N_2; (j) $CuSO_4$; (k) Li, liq. NH_3; (l) KSCN

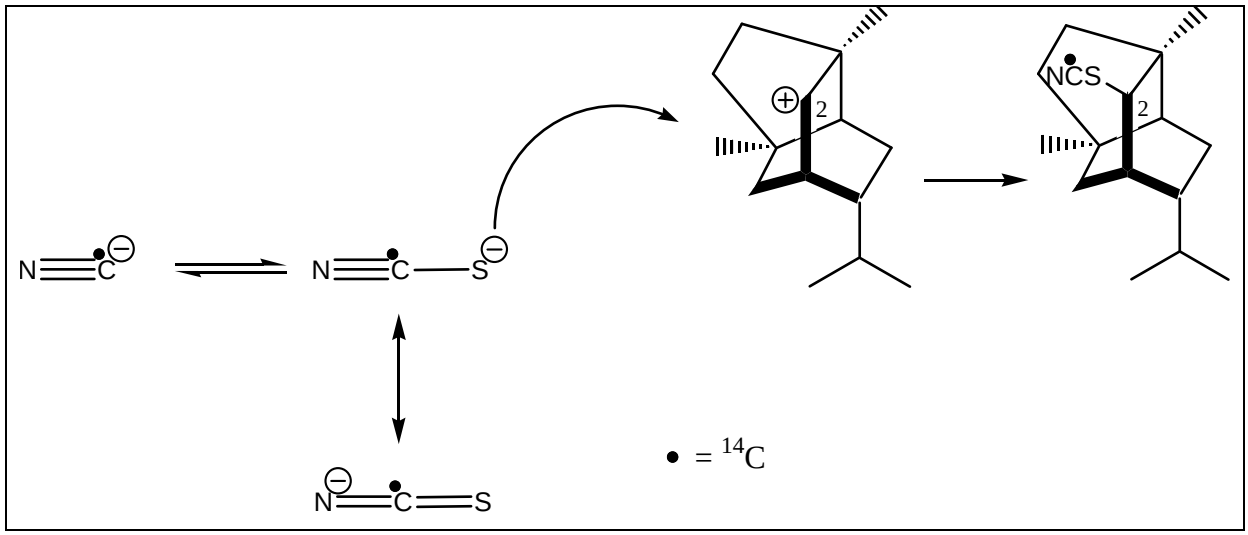


Figure 76. Biosynthetic origin of thiocyanate group in marine sponges. [Simpson & Garson, 1998]

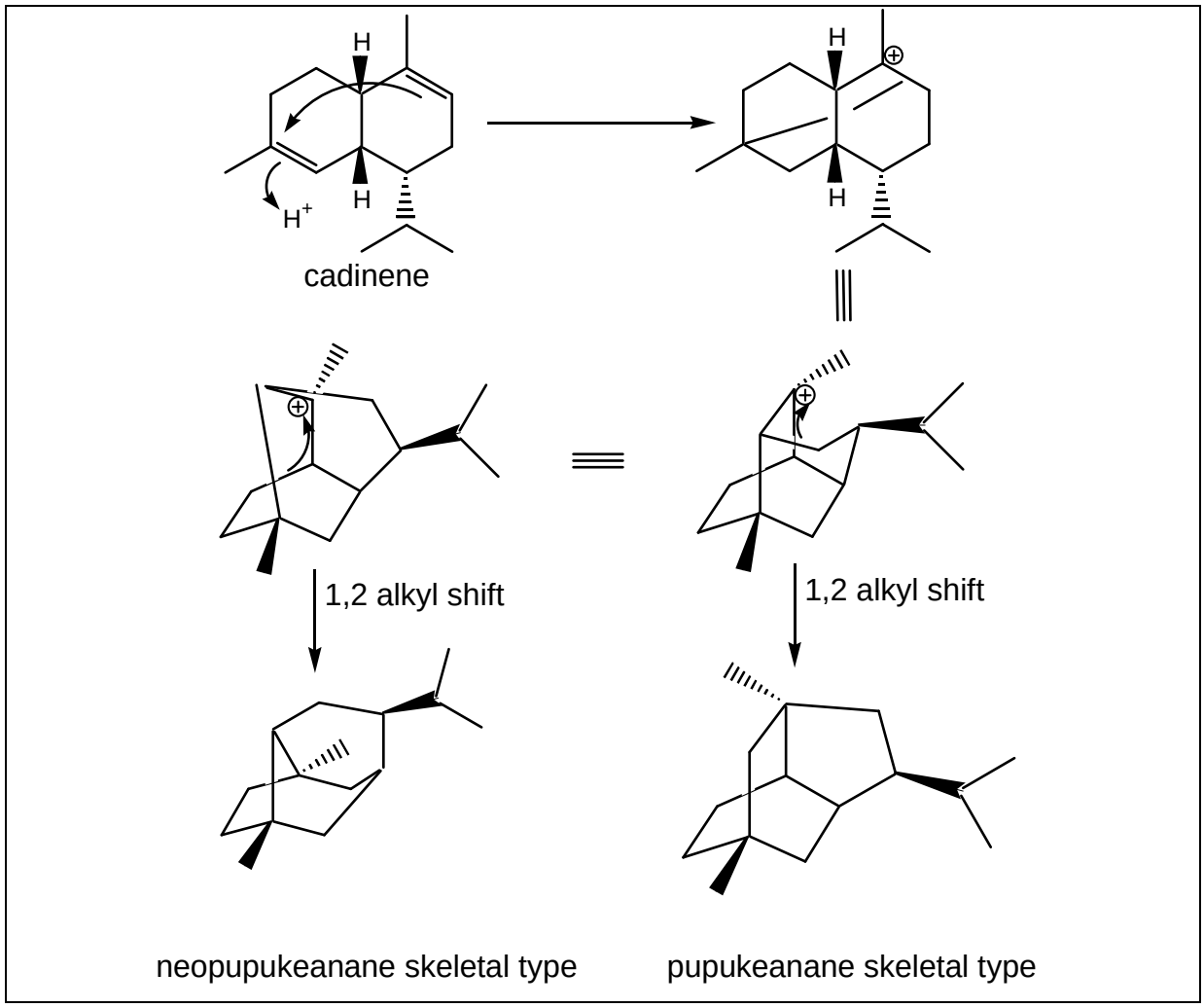


Figure 77. Biosynthetic pathways of pupukeanane and neopupukeanane sesquiterpenes. [Karuso *et al*., 1989]

Natural compounds containing a thiocyanato functionality are very rare to find. Garson (2004) in her review covering the literature up to mid 2003 found that from hundreds isolated marine isocyanide compounds, only five thiocyanato-substituted terpenes have been reported from marine sponges and phyllidiid nudibranchs (Figure 78). In contrast with this rarity, compounds **1**, **2**, and **3** which contain the thiocyanate functionality were present in all collected sponges. Even compounds **1** and **2** were found as major metabolites in all collected sponges and in all phyllidiid species that fed on them. Subsequently, the presence of **1**, **2**, and **3** in the sponge *A*. *cavernosa* (fam. Dictyonellidae) has also extended the chemo-taxonomical status of pupukeananes and neopupukeananes that currently were reported to be exclusively found in sponges of the family Halichondriidae and Axinellidae (Eppenberg, 2004).

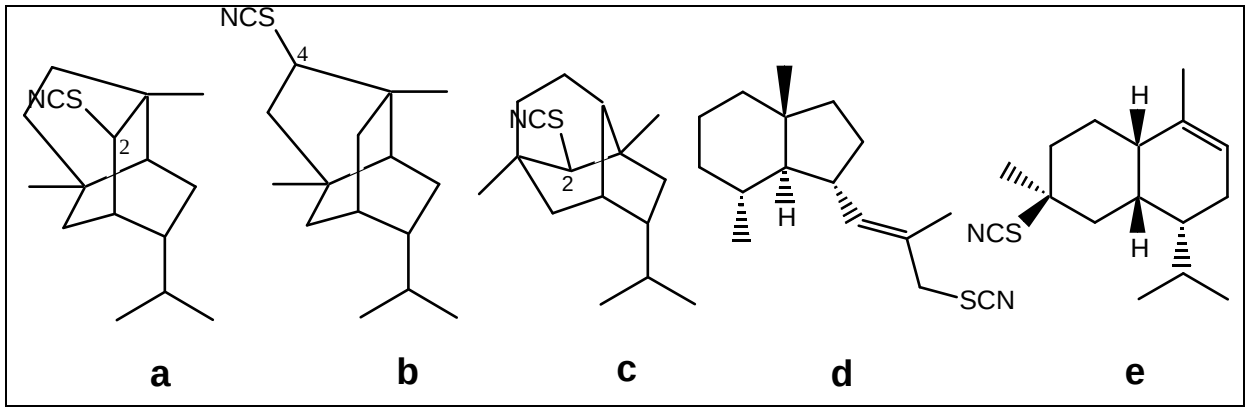


Figure 78. Compounds containing thiocyanato group (literature dates up mid 2003): a. 2-thiocyanatoneopupukeanane; b. 4-thiocyanatoneopupukeanane; c. 2-thiopupukeanane; d. cavernothiocyanate; e. 4-thiocyanato-9-cadinene.

4.3.2 Compounds containing the isothiocyanato function (5; epipolasin-A)

Epipolasin-A (**5**) has an epimaaliane skeleton. This carbon framework was synthetically derived from aromadendrene (Figure 80). In ecological studies involving the dorid *Cadlina luetomarginata*, Thomson *et al*. (1982) reported the first examples of isocyanides incorporated in an epimaaliane skeleton. Compound **5** has an isothiocyanato function which biosynthetically originated from isocyanide by means of the rhodanese enzyme, which is widespread in nature from sources such as bacteria, molluscs, echinoderms, and mammals, or an equivalent enzyme that may be present in sponges (Dumdei, 1997). Currently, the possibility of an interconversion process between isocyanide and isothiocyanate due to the presence of a peroxidase and rhodanese enzyme has been reported (Simpson & Garson, 2001). Isocyanide congeners have always been therefore isolated together with their isothiocyanate derivatives. However, in the present study, the isocyanide congener of compound **5** was not found in any of the collected sponge or phyllidiid samples.

Epipolasin-A (**5**) was first isolated from the sponge *Epipolasis kushimotoensis* (fam. Halichondriidae) by Tada & Yasuda (1985). Later it was also isolated from *Acanthella pulcherrima* (fam. Dictyonellidae), *Axinyssa* n. sp., and *A*. *aplysinoides* (fam. Halichondriidae) (Capon & Macleod, 1988; Simpson *et al*., 1997, Hassan, 2004). Compound **5** was found in all collected sponges. From the total 51 *Axinyssa* spp., three sponge samples contain **5** as the major compound. More specifically, compound **5** was commonly found in the sponge *Axinyssa 1* n. sp. Epipolasin-A (**5**) was a minor component in the sponge *Dragmacidon* sp. (fam. Axinellidae) and

Acanthella cavernosa (fam. Dictyonellidae). This suggested that compound **5** was commonly found in Indonesian sponges of the order Halichondrida as Hassan (2004) also isolated isothiocyanate **5** from the sponge *Axinyssa aplysinoides* (ZMA POR 17054) collected from Ujung Pandang, Indonesia. In contrast, Murti (in preparation) and Hertiani (2006, pers. comm.) did not isolate **5** from the sponge *A. aculeata* (ZMA POR 18289) and *Axinyssa* sp., respectively, which were collected from the same geographical location. Hertiani has just isolated two sesquiterpenes, curcuphenol and curcudiol, containing no isocyanide functionality (Figure 79). Subsequently, Pedpradap (2005, pers. comm.) isolated only steroids from the sponge *A. aplysinoides* (ZMA POR 17287) collected from the Andaman Sea. This might suggest that the composition of metabolites of *Axinyssa* spp. could vary geographically. This also might further suggest that any specific terpene isocyanide can not be used as a chemical marker of the order Halichondrida.

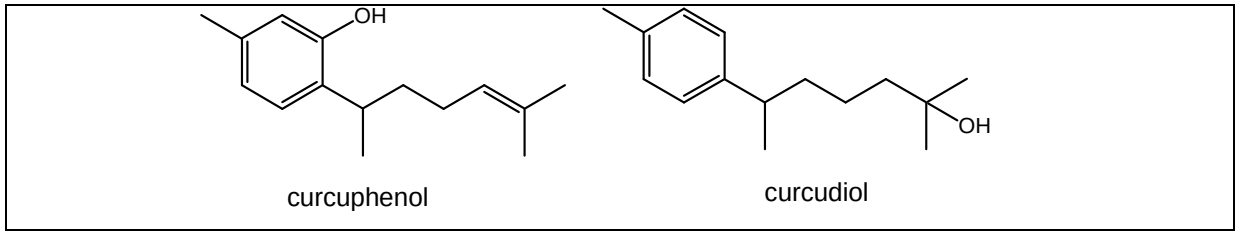


Figure 79. Sesquiterpenes isolated from the sponge *Axinyssa* sp. collected from Ujung Pandang, Indonesia.

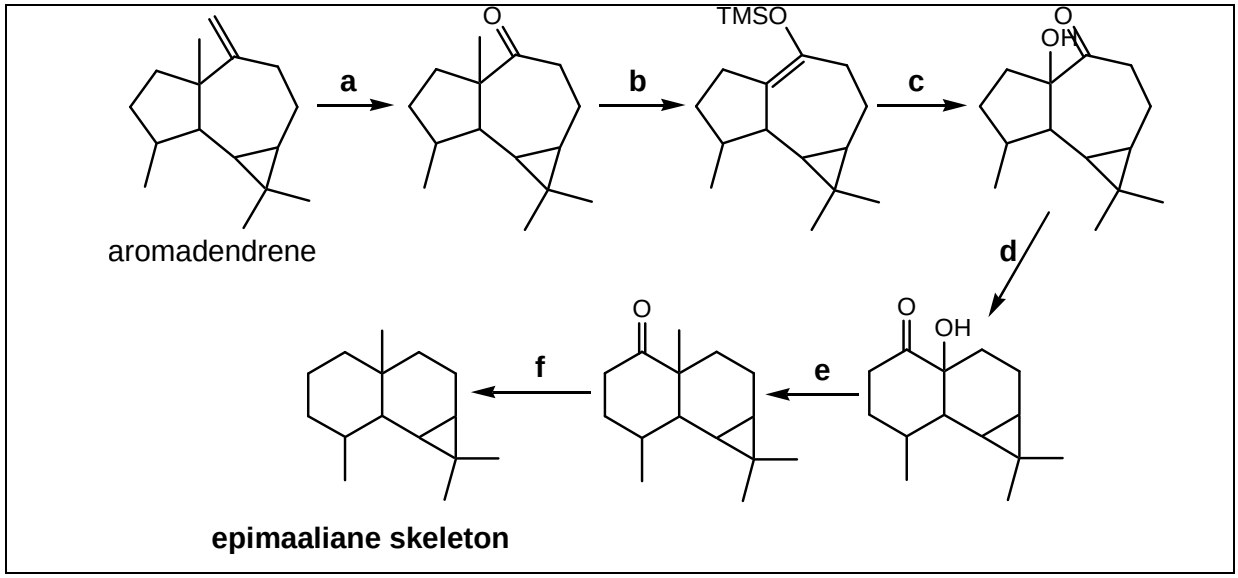


Figure 80. Synthetic pathway of epimaalianes: a. ozonolysis; b. TMSCl, Et_3N, DMF, 130C; c. dimethyldioxirane; SiO_2; e. Li, NH_3, *t*BuOH, MeI; f. N_2H_4, diethylene glycol, 100-200C. [Source: Gijsen *et al*., 1994]

4.3.3 Compound containing isocyano function (4; known compound)

Compound **4** has a cycloamphilectene skeleton. This unique tetracyclic diterpene was first proposed by Kazlauskas and co-workers (1980) for 8-isocyano-10-cycloamphilectene isolated from the sponge *Amphimedon* sp. (formerly known as *Adocia* sp.). Later Faulkner group isolated 8-isocyano-1(12)-cycloamphilectene (**4**) from the sponge *Halichondria* sp. and revised the structure previously proposed by Kazlauskas *et al*. (1980) as 8-isocyano-11(12)-cycloamphilectene (Molinski *et al*., 1987). Since these two studies, compound **4** was never found in later literatures (Chang, 2000; Garson & Simpson, 2004). In the present study, compound **4** was found as a major compound in the sponge *Dragmacidon* sp. and its predator, *P*. *elegans*. A literature survey showed that this is the first report of a cycloamphilectene found in the sponge *Dragmacidon* and the phyllidiid nudibranch, *P*. *elegans* (MarinLit version September 2004).

4.4 Quantitative variation of compounds in the collected sponges

A quantitative variation in sponge secondary metabolites has been observed in sponges *Aplysina aerophoba* and *A*. *cavernicola* which were collected from the same and/or different locations (Thoms, 2004). It was shown that the relative composition of a certain group of compounds in the sponge *A*. *aerophoba* was constant but their absolute amounts varied due to the variation of the presence and distribution of so-called roset cell in the sponge body.

Axinyssa sponges collected in the present study seem to have 9β-thiocyanatopupukeanane (**2**) as the major compound. There was always an exception of one or more sponge samples where either 9α-thiocyanatopupukeanane (**1**), 2-thiocyanatoneopupu-keanane (**3**), or epipolasin-A (**5**) could be the major component. The relative composition of the terpene isocyanides in *Acanthella cavernosa* showed that thiocyanates **1**, **2**, and **3** were present as major compounds in comparable concentrations. However, collected sponges of the genus *Dragmacidon* exhibited interesting variation. This sponge group was dominated by the presence of diterpene amphilectene isocyanide (**4**) which was absent in *Axinyssa* spp. and *A*. *cavernosa*. From a total of six collected sponges, only two samples have 9β-thiocyanatopupukeanane (**2**) as their major compound.

The concentration of isolated sesquiterpene isocyanide compounds among collected sponge samples of the genus *Axinyssa* spp. also varied. *Axinyssa 6* n. sp. has the lowest concentration (less then 407 ppm) while *Axinyssa 5* n. sp. has the highest concentration of sesquiterpene isocyanides (up to 14,116 ppm).

One possibility to explain this chemical variation was provided by Garson (2004). Garson's group found that *Axinyssa* sp. contains cyanobacteria in its surface tissue plus high bacterial populations in the core tissue while *A*. *cavernosa* contain few bacterial symbionts. But the different symbiont profiles of biosynthetically-related sponges are entirely consistent with the terpenes being products of metabolism of the sponge rather than of the symbiont. These lead to a speculation that marine terpene isocyanides might function as a structural component of sponge cell membranes (Garson & Simpson, 2004). This suggests that such variation of isocyanide compounds in the present study might be due to the variation in structural component of sponge cell membranes.

4.5 Chemical correlations between phyllidiids and sponge-preys

Chemically based studies of phyllidiids-sponge-preys relationship showed that phylidiids' isocyanide compounds were also found in their sponge-preys (Burreson *et al*., 1975b; Hagadone *et al*., 1979; Cimino *et al*., 1982; Fusetani *et al*., 1992; Dumdei *et al*., 1997; Simpson *et al*., 1997; Wright, 2003). Through an experiment using the phyllidiid *P*. *pustulosa* and halichondrid sponge *A*. *cavernosa*, Garson's group then was able to obtain definitive evidence for this dietary transfer of sponge metabolites (Dumdei *et al*., 1997). Further experiments of direct injection of either ^{14}C cyanide or ^{14}C thiocyanate into the digestive gland of phyllidiid *P*. *pustulosa* implied that phyllidiid species are unable to use cyanide/thiocyanide directly in their biosynthetic processes (Dumdei *et al*., 1997). Those results lead to a conclusion that phylidiids' terpene isocyanides are metabolites of dietary origin.

All collected phyllidiid species in the present study were observed feeding upon halichondrid sponges. GC analysis of these sponges and phyllidiids extracts showed that the set of terpene isocyanides in phyllidiid species were also found in their sponge-preys which supported the conclusion that phylidiids' terpene isocyanides are metabolites of dietary origin. It is interesting to note that phyllidiid species not merely sequetered but also tend to selectively accumulate terpene

isocyanide in their diet. This was clearly shown in *F*. *menindie* and *P*. *elegans* feeding on the sponge *Axinyssa 6* n. sp. and *Dragmacidon* sp. respectively.

The sponge *Axinyssa 6* n. sp. tends to yield the epimers **1** and **2** as the major compounds rather than 2-thiocyanatoneopupukeanane (**3**). In contrast, its predator *F*. *menindie* (1 individual) tends to accumulate compound **3** rather then the epimeric mixtures **1** and **2** (see Figure 63). Another interesting finding is that *F*. *menindie* tends to have a higher concentration of the thiocyanate metabolite **2** compared to its isomer **1** in a ratio of 2:1. In the sponge-prey, however, the epimeric mixture was found to be at a ratio of 1:2. The higher concentration of compound **2** compared to **1** in the phyllidiid *F*. *menindie* is very important in terms of chemical defense as it is known that epimer **2** is more toxic than **1** in the brine shrimp experiment (Yasman *et al*., 2003).

The sponge *Dragmacidon* sp. tends to have amphilectene isocyanide (**4**), which was absent in other sponges, as the major compound (see Figure 57 & Table 15). One sponge extract of *Dragmacidon* sp. collected in Karang Lebar reefs, however, showed that it has a mixture of sesquiterpene thiocyanate **1** and **2** which was more dominant than compound **4**. In contrast, its predator *P*. *elegans* (1 individual) yielded a major quantity of **4** (Figure 81 & Table 24).

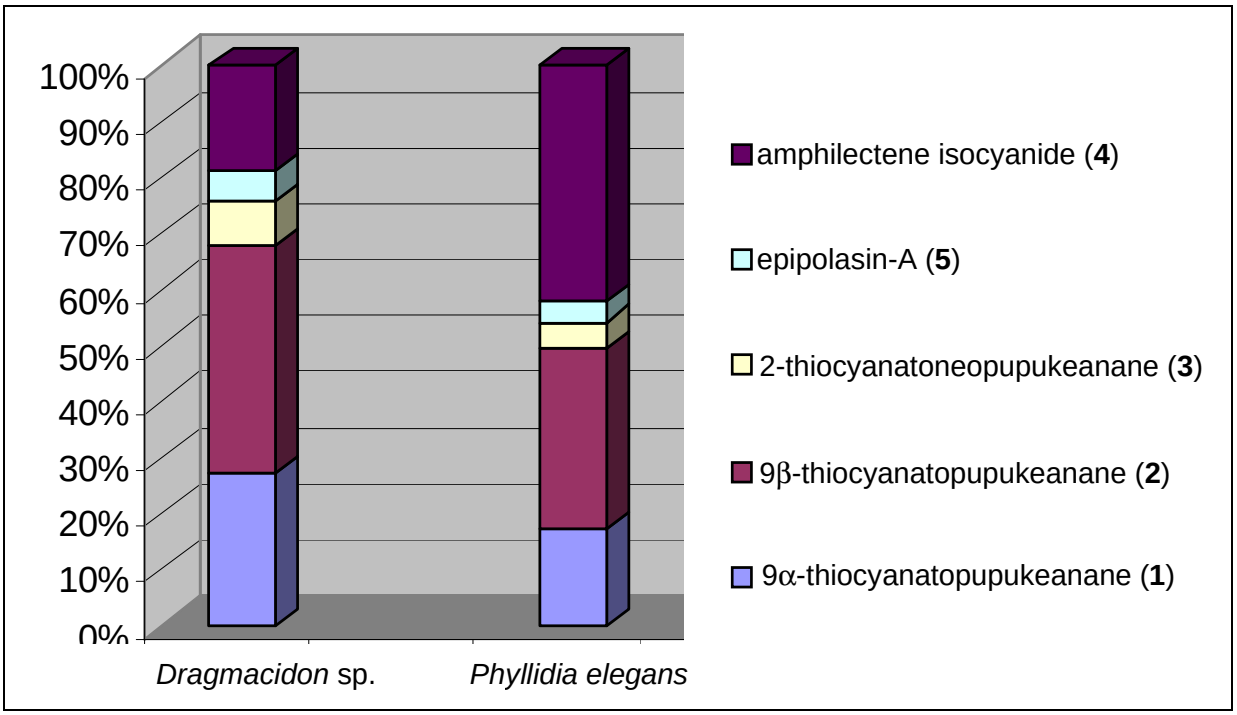


Figure 81. Comparison of relative composition of isolated **1**, **2**, **3**, **4**, and **5** between the *P*. *elegans* and its sponge-preys, *Dragmacidon* sp.

Table 24. Concentration variation in ppm of terpene isocyanides in the EtOAc extracts of *P*. *elegans* and its sponge-prey, *Dragmacidon* sp.

Species	Comp. **1**	Comp. **2**	Comp. **3**	Comp. **4**	Comp. **5**
Dragmacidon sp	106	159	30	73	21
P. *elegans*	61	114	17	149	13

4.6 Phyllidiids's organ specific distribution of isolated compounds

The optimal defense theory and plant apparency model originally developed for terrestrial plants suggest that the most valuable plant parts should be preferentially defended and predict a positive correlation between chemical defense and the risk of being discovered by herbivores, respectively (Feeny, 1976; Rhoades & Cates, 1976). Proksch's group has suggested that terrestrial plants defense theory can also be applied in the marine environment. They were able to prove that the distribution of the alkaloids in the sponge *Oceanapia* sp. followed the plant apparency model as well as the optimal defense theory (Schupp *et al*. 1999b; Proksch *et al*., 2003). Marine sponges are likely analogs of plants in the terrestrial ecosystem with regard to their habitat (i.e. sessile) and their ecological position (i.e. suffering from being eaten by other organisms). In order to prove if this theory is also relevant to marine predator invertebrates, four representative phyllidiid species were dissected into three parts: digestive gland, foot, and mantle. The mantle part usually covers the whole body of phyllidids and therefore is visually recognizable and more vulnerable to suffer any attacks from potential predators. It was then hypothesized that the mantle part has to accumulate a larger proportion of the terpene isocyanide compared to the foot part. GC analysis of *P*. *shireenae*, *P*. *pustulosa*, and *P*. *krempfi* extracts showed that the concentration of terpene isocyanides in the mantle part was two to six folds higher than that found in the foot part which supported the given hypothesis. Extracts obtained from phyllidiid *P*. *elegans* even strongly supported the above hypothesis where the mantle extract contained a higher concentration of terpene isocyanides which is 25 times larger than found in the foot extract. With regard to amphilectene isocyanide, the major metabolite found in *P*. *elegans*, it was 75 times higher in the mantle than in the foot part. This may suggest that the optimal defense theory and plant apparency model in marine ecosystems may not only be applied to ecological studies of organisms in the "first level of the food chain" (with regard to the

assumption that marine sponges are analogs of terrestrial plants) but also in the secondary level, where nudibranchs are the predators.

The application of the optimal defense theory and plant apparency model in the nudibranchs as marine predators was even more advancely exhibited by some dorid nudibranchs. Cimino's group found that the dorid *Hypselodoris* spp. was able to selectively accumulate its dietary defense allomones in the mantle dermal formations (MDF's) that are localized in a strategic position near the gill and the rhinophores which are not only visually recognizable to attract potential predators but are also the vital part of the nudibranchs (Gracia-Gomez et al. 1990; Cimino & Sodano, 1994).

4.7 Ecological role of the isolated terpene isocyanides

Phyllidiid species have been reported to be specialist predators of certain marine sponges (Brunckhorst, 1993). In the present study, phyllidiids show the tendency to choose halichondrid sponge species that have no ectosomal skeleton. The differences in their pharyngeal bulb among the genus of phyllidiids however seem not to play a biological role for phyllidiid species their being a specialist predator on a specific sponge-prey (*see* discussion in **4.1**). This finding might suggest that there is a tendency to regard the relationship within phyllidiids and the sponge-preys only as tool for the predator to acquire a chemical defense (chemical correlations) rather then morphological feeding adaptation of the phyllidiid against the sponge's morphology.

Secondary metabolites produced by halichondrid sponges seem to repel a generalist predator but on the other side attract a specialist predator. The amphilectene isocyanide (**4**) that was present only in the sponge *Dragmacidon* sp. seems to act as attractant compound for the phyllidiid *P*. *elegans*. During the field feeding observation, the sponge *Dragmacidon* sp. was observed only to be predated by *P*. *elegans*. In contrast, compound **4** also seems to act as a repellent compound for other phyllidiid species. Of five collected sponges, there were two sponge specimens that have the group of sesquiterpenes **1**, **2**, **3**, and **5** dominating rather than the amphilectene isocyanide (**4**) and therefore suggested that compound **4** was not always present as a major compound. In fact six phyllidiid species that tend to deal more with sesquiterpenes **1**, **2**, **3**, and **5** were never observed feeding on the sponge *Dragmacidon* sp. Because maintaining such phyllidiids in a laboratory

aquarium is very difficult, a precise laboratory experiment is still needed to further prove this assumption.

To date, terpene isocyanides were isolated mainly from sponges of the order Halichondrida and phyllidiid nudibranchs (Garson & Simpson, 2004; Mitome *et al*., 2004; Manzo *et al*., 2004). Marine isocyanide compounds have been well-known to be ichthyotoxic, presumably to protect the host organisms (Thompson *et al*., 1982; Cimino *et al*., 1982; Braekman *et al*., 1987; Gunthorpe & Cameron, 1987; Fusetani *et al*., 1990; Fusetani *et al*., 1991).

In the tropical reef ecosystems, fishes are considered to be the most potential predators for many invertebrate organisms (Green, 1977; McClintock, 1987). In order to assess if the ichthyotoxic marine isocyanides are effective to protect the host organisms from fish predation, a qualitative fish feeding experiment using intact phyllidiids was performed. Due to the phyllidiids' naked soft-bodied, they were assumed to be more vulnerable to suffer predations in comparison to their sponge-preys. Even though the results obtained from the experiment can statistically not be proven, this is a quick and easy test that can be done in the field. Of 37 phyllidids (consisting of 6 species) used in the experiments, all of them were free from fish predation. Coral reef fishes seem to show a learning behaviour against distasteful preys. Fishes were observed to come nearby but then they allowed the first four intact phyllidiids used in the experiment to swim down to the bottom. The next six individuals of phyllidiid species coming through the water column were even totally ignored by the fishes. This suggested that phyllidiids seem to be visually not accepted as potential prey for fishes. As phylidiids have and can actively secret ichthyotoxins which have an unusual odor, this apparently disturbed fishes.

The result of fish feeding experiments showed that some isocyanide compounds were active as antifeedant (Thomson *et al*. 1982; Garson *et al*., 2000). In the present study, however, terpene isocyanides **1**, **2**, **3**, and **4** apparently did not show any significant antifeedant activity. This is an unexpected result even though some other former experiments showed the same results (see Cimino *et al*., 1982; Marcus *et al*., 1989; Dumdei *et al*., 1997; Pawlik *et al*., 2002). Even Paul and Rogers (1991) had speculated that isocyanides were not effective feeding deterrents. This is a premature conclusion, as to date, feeding experiments of terpene isocyanides for chemical defense purposes has not yet been adequately performed (Garson and Simpson, 2004). Even if that conclusion was true, it has been assumed that phylidiids

are protected from fish predation due to the combination of the toxicity of the isocyanides which are of dietary origin and the low nutritional quality of phyllidids (Penney, 2002). Phyllidiids have numerous large calcareous and chitinous spicules that distinguish them from other dorid nudibranchs (Brunckhorst, 1993). Following the concept of optimal food selection, this combination might reduce the optimum fitness of phyllidiids to being predated by fishes or other marine animals.

The negative result of the feeding experiments in the present study might be due to some idiosyncracies in the experimental methodology. The use of blank control was unusual even though it can be allowed and even may useful in some circumstances (Hurlbert, 1984). From the result of the feeding habituation, each fish needed 8-10 food pellets. To avoid treatment of pellets being eaten due to a starving condition, the blank control pellets then were used. Another alternative reason was the use of different pellet colors to differentiate the blank control from the solvent control and treatment. The results of the feeding habituation showed that pellet color did not influence the fishes feeding behaviour. Nevertheless, fishes apparently show color preference during the experiments. This behaviour might be responsible for the unexpected result in the fish antifeedant experiment on compound **4** where the treated pellets were far more eaten than the blank control pellets. The last possibility to explain the negative result of the feeding experiment is the fish learning behaviour. During the experiments, it was observed that fishes were eating again the treated pellets after they were spit out. After several times, the treatment pellets were then finally consumed. This feeding behaviour has been shown by coral reef fishes in the field. The reef fish *Abudefduf* spp. invariably reattacked aeolid nudibranch *Phyllodesmium guamensis* to reduce distasteful cerata of *P*. *guamensis* following several mouthing events in the field (Slattery *et al*., 1998).

The epimeric mixture of 9-thiocyanatopupukeanane **1** and **2** were found to be toxic toward brine shrimp at LC_{50} of 5 ppm. At a dose level of 20 µg, they were found to be weakly and moderately active against *B*. *subtilis* and *C*. *albicans*, respectively (Yasman *et al*., 2003). Amphilectene isocyanide (**4**) has been reported to show antimicrobial activity (Molinski *et al*., 1987). This suggested that isolated terpene isocyanides may provide some other alternative ecological role.

5 Summary

Phyllidiids field feeding observations and isolation of isocyanide compounds both from phyllidiid species and from their sponge-preys have been done in order to assess the predator-prey relationships between phyllidiid species and their sponge-preys. Field studies and sample collections were done in the coral reefs of Thousand Islands, Indonesia while chemical studies were done in the Institute of Pharmaceutical Biology and Biotechnology, Heinrich-Heine University of Düsseldorf.

In this study, phyllidiid species were found to feed exclusively on sponges from the order Halichondrida, especially the species that have no ectosomal skeleton. A possible reason for this choice is that phyllidiids need less effort to predigest the sponge's epidermis, contact with the mesohyl of sponges becomes easier, resulting in an easier predation. Interestingly there is no specific correlation between pharyngeal bulb morphology of phyllidiid species with their specific sponge-preys. Of ten collected phyllidiids, six species (*P*. *varicosa*, *P*. *zeylanica*, *P*. *pustulosa*, *P*. *krempfi*, *P*. *shireenae*, and *F*. *menindie*) tend to be specialist predators on the sponge *Axinyssa* spp. (fam. Halichondriidae) while three species (*P*. *lizae*, *P*. *rudmani*, and *P*. *ocellata*) and one species (*P*. *elegans*) tend to feed exclusively on the sponges *Acanthella cavernosa* (fam. Dictyonellidae) and *Dragmacidon* sp. (fam. Axinellidae) respectively. The identification of collected sponges was done by the author himself under the supervision of Dr. van Soest (Zoological Museum of Amsterdam). The identification was based on the spiculation types and the size of fully grown spicules as well as on morphological data.

Four thio- and isothiocyanate sesquiterpenes (**1**, **2**, **3**, **5**) and one diterpene isocyanide, 8-isocyano-1(12)-cycloamphilectene (**4**) were successfully isolated. The new compounds, 9α-thiocyanatopupukeanane (**1)** and 9β-thiocyanatopupukeanane (**2**) have the pupukeanane skeleton while the known congener, 2-thiocyanatoneopupukeanane (**3**) has a modified backbone of the pupukeanane, whereas epipolasin-A (**5**) has the epimaaliane skeleton (Figure 82).

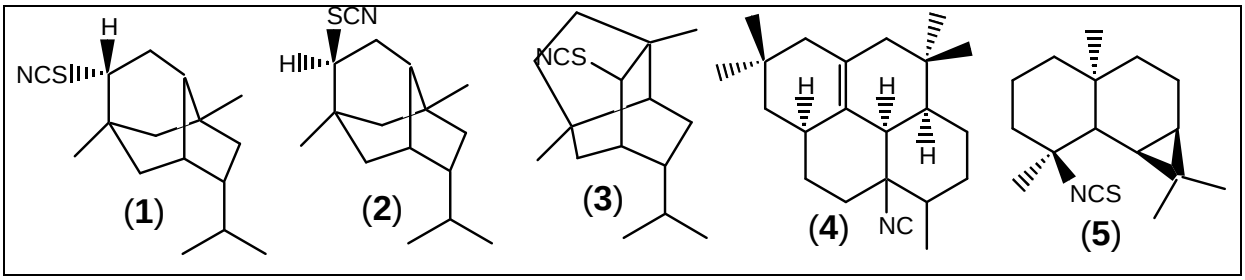


Figure 82. Isolated terpene isocyanides from phyllidiids and their sponge-preys collected from the Thousand Islands, Indonesia.

Extraction and analysis of the collected sponges showed that sponge metabolites varied qualitatively and quantitatively. This variation might be due to the variation of sponge cell. It is known that marine terpene isocyanides might function as a structural component of sponge cell membranes (Garson & Simpson, 2004). Extraction of phyllidiids and their sponge-preys showed that the presence of terpene isocyanides in both predator and prey were not always comparable in their content ratio. This suggests that phyllidiids not only sequestered but also selectively accumulated the metabolites from their diets.

Analysis of organ specific distribution of isolated compounds in phyllidiids showed that the concentration of terpene isocyanides in the digestive gland is the highest compared to the mantle and foot part. This supports the idea that the digestive gland should be the first responsible organ of storing the nudibranch's metabolites of dietary origin. It has been assumed that the nudibranchs later transport the metabolites to the mantle which is more vulnerable to predation. As observed, the mantle part contains a higher concentration of sequestered terpene isocyanide compared to the foot.

In the present study, terpene isocyanides **1**, **2**, **3**, and **4** apparently did not show any significant antifeedant activity. The epimeric mixture of 9-thiocyanatopupukeananes (**1** and **2**) was found to be toxic toward brine shrimp at LC_{50} of 5 ppm. At a dose level of 20 µg, they were found to be weakly or moderately active against *B*. *subtilis* and *C*. *albicans*, respectively. Amphilectene isocyanide (**4**) has been reported to show antimicrobial activity. This suggested that isolated terpene isocyanides may provide some other alternative ecological roles.

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List of Abbreviations

$[\alpha]_D$	: specific rotation at the soldium D-line
BuOH	: butanol
br	: broad signal
CD	: circular dichroism
CI	: chemical ionization
COSY	: correlation spectroscopy
d	: doublett
dd	: double doublett
ddd	: double double doublett
DEPT	: distortionless enhancement by polarization transfer
DMSO	: dimethylsulfoxide
DNA	: deoxyribonucleic acid
ED	: effective dose
EI	: electron impact
ESI	: electron spray ionization
EtOAc	: ethyl acetate
eV	: electronvolt
Fig	: Figure
g	: gram
h	: hour
HMBC	: heteronuclear multiple bond connectivity
HMQC	: heteronuclear multiple quantum coherence
HPLC	: high performace liquid chromatography
Hz	: herz
IR	: infrared spectroscopy
LC	: lethal concentration
lit.	: literature
m	: multiplett
MeOD	: deuterated methanol
MeOH	: methanol
mg	: milligram
mL	: milliliter

μg	: microgram
μL	: microliter
mRNA	: messenger-RNA
MS	: mass spectrometry
m/z	: mass per charge
n.a	: no activity
ng	: nanogram
nm	: nanometer
NMR	: nuclear magnetic resonance
NOE	: nuclear Overhauser effect
ppm	: parts per million
q	: quartett
RNA	: ribonucleic acid
RP-18	: reversed phase C-18
s	: singlett
t	: triplett
TFA	: trifluoroacetic acid
TLC	: thin layedr chromatography
UV	: ultra-violet
n. sp.	: new species

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