

**Triterpene glycosides chemically defend the South African kelp bed sea cucumber,  
*Pseudocnella insolens* (Echinodermata: Holothuroidea), against predation.**

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**A thesis submitted in fulfilment of the requirements for the degree M.Sc. in the  
Botany Department**

**Date: 15.05.97.**

**I declare that**

**“Triterpene glycosides chemically defend the kelp sea cucumber,  
*Pseudocnella insolens* (Echinodermata: Holothuroidea), against predation in  
South Africa.”**

**is my own work and that all the sources I have used or quoted  
have been indicated and acknowledged by means of complete references.**

## TABLE OF CONTENTS

	<b>Page No.</b>
LITERATURE REVIEW	1-21
REFERENCES	22-34
NAME OF JOURNAL	35
TITLE OF PAPER	36
ABSTRACT	37
INTRODUCTION	38-41
MATERIALS AND METHODS	42-47
RESULTS	48-50
DISCUSSION	51-55
ACKNOWLEDGEMENTS	56
REFERENCES	57-61
TABLES	62-63
FIGURE CAPTIONS	64-65
FIGURES	66-74
APPENDIX I	75-76
APPENDIX II	77-81
APPENDIX III	82-86

## LITERATURE REVIEW

### 1. History of chemical ecology

The last three decades have witnessed the growth of a new inter-disciplinary field of study which has developed as an offshoot from natural products chemistry (Manes *et al.*, 1985). This field of study is called ecological biochemistry or chemical ecology and it is concerned with the biochemistry of plant-plant, plant-animal and animal-animal interactions in the natural environment (Harborne, 1988).

Harborne (1988) ascribes the growth of this new discipline to the development and improvement of biochemical techniques. The union between dissimilar disciplines such as ecology and biochemistry may seem a puzzling association at first glance. Ecology is concerned with the interactions between living organisms in their natural habitats (Starr & Targett, 1989). By contrast, biochemistry is essentially experimental and is primarily concerned with interactions at the molecular level (Conn *et al.*, 1987). Despite differences, these two disciplines have in amalgamated with great success (Harborne, 1988).

For over a century the elucidation of the chemistry of natural products has been a dominant theme in organic chemistry (Haslam, 1985) but the emergence of chemical ecology has shifted the focus to investigating the rôle that natural products play in the ecology of living organisms. In natural products chemistry the emphasis of research has been on the structure, chemical properties, and the synthesis of natural products.

The term 'natural product' is easily understood and appreciated by the organic chemists, for the roots of their science lie in the chemistry of natural products. This science has evolved through the study of substances isolated from living organisms (Haslam, 1985). Scientists in the late eighteenth century began to develop theories which suggested possible sources of these natural products. To suggest probable origins from simple precursors, they laid the basis of the biogenetic theory and they brought coherence to groups of apparently unrelated natural products by suggesting biochemical relationships (Haslam, 1985).

From the early twentieth century organic scientists began to separate, purify, and finally analyse those compounds produced in living cells (Torsell, 1983). Separation methods such as various analytical and chromatographic methods were developed, and without a doubt natural products chemistry has led to the development of the refined techniques that are available to chemical ecologists today (Miyamoto *et al.*, 1992). These methods have made it possible to isolate natural products and study their chemical and biological properties.

The success of chemical ecology depends on the co-operation of scientists from different fields of study. It is also apparent that interdisciplinary studies between ecologists and chemists are academically rewarding because they seek the important mechanisms behind ecological principles (Bakus *et al.*, 1986). An example of the co-operation between these scientists from different disciplines is that of Mark Hay, a marine ecologist, and William Fenical, an organic chemist. The success of this association is revealed in numerous publications from the field of marine chemical ecology (eg. Hay *et al.*, 1987; Hay & Fenical, 1988; Hay *et al.*, 1990a,b; Hay & Fenical, 1996). Marine chemical ecology, which is an emerging sub-discipline, seeks to achieve an integrated understanding of chemical biological interactions occurring in the sea.

In recent years, ecologists have demonstrated that naturally occurring products have a significant rôle in the complex interactions occurring between organisms in the natural environment. In animals, naturally occurring products are important in influencing food selection and are also involved in social and reproductive behaviour. Higher plants compete with each other for moisture, light and nutrients in the ecosystem. In the course of this struggle, they have developed various means of defence against their neighbours. Within work done in chemical ecology there are a number of examples of chemically guided behaviours in animals and plants in their environment (Torsell, 1983; Harborne, 1988; Fenical, 1988; 1996).

## **2. Comparison between primary and secondary metabolites**

Naturally occurring compounds found within living organisms have been categorised as either primary or secondary metabolites. Compounds that are essential to life processes are referred to as primary metabolites (Salisbury & Ross, 1985). These include natural products such as fatty acids, sugars, and nucleic acids. Fatty acids are components of lipid structures. Amino acids are building blocks of proteins, and purine and pyrimidine bases are units of nucleic acid structure which constitute the genetic code. All these products are essential for living organisms and life cannot exist without these basic building blocks (Yudkin & Offord, 1973).

Organisms also contain compounds that are not required for normal growth and therefore have no obvious rôle in the welfare of the organism which produces them (Bullock, 1980). The substances in this group are commonly referred to as secondary metabolites because of their apparent secondary rôle (Manitto, 1981; Salisbury & Ross, 1985). These include compounds such as alkaloids, terpenes, pigments, phenols and myotoxins that occur naturally.

This distinction amongst the chemical constituents of living organisms has long been recognized (Yukin & Offord, 1973), and is useful in explaining the different biological rôles of these compounds (Haslam, 1985). These categories of primary and secondary metabolites are however, merely opposite ends of a continuum of metabolic

function. Because they exist along a continuum, opinions often differ as to the specific functioning and status of a particular metabolite (Yukin & Offord, 1973).

A large portion of research on secondary metabolites has focused on plant-animal interactions, especially herbivory (Rosenthal & Janzen, 1979; Bernays, 1981; Fox, 1981; Denno & McClure, 1983). Rosenthal and Janzen (1979) and Haslam (1985) noted that Stahl, in the late eighteenth century appears to have been the first person to suggest that some of the chemical substances found in plants may have evolved for protection against attack by herbivorous animals. This research served as a starting point in understanding the rôle of secondary metabolites in the life of plants and animals in their natural environment. The secondary metabolites of plants and animals have been recognised to possess biologically important properties and it is now known that organic compounds are responsible for many of these biological properties (Fenical, 1982).

Herbivory in terrestrial communities can be very intense (Rosenthal & Janzen, 1979; Denno & McClure, 1983). Herbivory may reduce the growth and survival of individual plants and can influence interspecific competition and community structure (Rockwood, 1973; Morrow & Fox, 1980; Rausher & Feeny, 1980; Lubchenco & Gaines, 1981; Coley, 1983; Coley *et al.*, 1985). The importance of secondary metabolites in defending terrestrial plants against herbivores is well studied and generally accepted as one of the most effective form of defence mechanism (Sondheimer & Simeone, 1970; Levin, 1976; Harborne, 1978, 1988; Rosenthal & Janzen, 1979; Denno & McClure, 1983).



Studies of chemical ecology in the marine environment were not started until the invention of SCUBA, which allowed scientists to witness directly the biologically diverse and ecologically complex marine environment (Fenical, 1982). Although research on marine secondary metabolites and their ecological rôles has a short history relative to similar investigations in terrestrial systems, more than 1 000 secondary metabolites have been isolated and structurally identified (Elyakov *et al.*, 1973; Fenical & Norris, 1975; Hellou *et al.*, 1982; Faulkner & Ghiselin, 1983; Faulkner, 1984, 1986).

Benthic marine macro-algae contain wide variety of secondary metabolites (Faulkner, 1984, 1986; Hay & Fenical, 1988). Although many of these compounds in algae have been proposed to function as defences against herbivores (Steinberg, 1984; Paul & Van Alstyne, 1988), until recently there was little known about their ecological effects. However, at least some algal secondary metabolites have harmful and deterrent effects against marine herbivores, and thus can function as defences (Targett & McConnell, 1982; Steinberg, 1984; Paul & Fenical, 1986; Hay *et al.*, 1987). Clearly anything which reduces herbivory on a plant can increase the plant's overall fitness and should be strongly selected for. However, it is still not known whether secondary metabolites that convey defensive ability evolved in response to the selection pressure exerted by herbivory, or if secondary metabolites function as antifouling agents of pathogen inhibitors ( Hay & Fenical, 1988; Davis & Wright, 1990; Hay, 1991).

Plants are richer than animals in the diversity of secondary metabolites which they

produce (Luckner, 1984). Although, secondary metabolism occurs in animals, over four-fifths of all presently known natural products are of plant origin (Swain, 1974; Robinson, 1980). Over 12 000 different natural products of varied biogenetic origins are produced by terrestrial plants (Harborne, 1988). Harborne (1988) ascribes this richness in secondary metabolites to the simple fact that plants are rooted in the soil and cannot move; they cannot respond to the environment in the ways open to mobile animals.

### **3. Defence against consumers**

Consumers are widespread in the natural environment, and play a significant rôle in determining the distribution and abundance of the organisms which are their food (Paine, 1971; Vince *et al.*, 1976; Menge & Lubchenco, 1981; Bingham & Braithwaite, 1986; Hay *et al.*, 1988). In response to the selection pressure of consumption, protective and defensive features evolved that increased the fitness of these organisms. These features include, structural defence, habitat selection and chemical defence.

#### **3.1. Structural Defence**

Plants and animals produce an array of structural defences that reduce their susceptibility to damage from predators (Duffy & Paul, 1992). For example in the marine environment, many sessile organisms produce structural material such as mineral skeletons or organic fibres that are commonly believed to serve a protective function

(Hay, 1984; Paul & Hay, 1986). Some seaweeds may concentrate calcium carbonate which make them very difficult for many marine herbivores to consume (Duffy & Paul, 1992; Schupp & Paul, 1994). All classes in the phylum Echinodermata have spicules or spines in their skin, although this characteristic is developed to different degrees in the various classes (Barnes, 1987). In the Echinoidea their bodies are encased in a hard, calcium carbonate shell or test, covered with spines. In the Holothuroidea the spines are reduced to spicules. In the terrestrial environment the supportive and strengthening function of lignin in plant tissues is well known, but there is also evidence that lignin also gives protection against attack by pathogens and consumption by herbivores, both insect and mammalian (Swain, 1974). Thus there may be a dual structural and defensive function for lignin in plants. Structural defences may not deter all consumers, but they provide some protection against certain consumers.

### **3.2 Habitat and other kinds of defence mechanisms**

Prey species use a variety of mechanisms to escape from their predators. To persist in marine communities, prey must escape, deter or tolerate consumption. The ecological and evolutionary importance of spatial and temporal escapes has been extensively studied for seaweeds (Gaines & Lubchenco, 1982; Hay, 1984, 1985; Lewis, 1986; Hay & Fenical, 1988). Seaweeds may reduce the impact of herbivorous fish by occupying habitats where fish rarely feed or by occurring in habitats so favourable for growth that production exceeds herbivory even if the rates of grazing are high (Hay,

1991). Numerous studies have documented vastly different rates of grazing on different areas of a coral reef system and have noted the importance of these between habitat differences in generating spatial refuges for seaweeds that are highly susceptible to grazing fish (Hay *et al.*, 1983; Lewis, 1986; Morrison, 1988). Spatial patterns of fish grazing can occur on smaller spatial scales within habitats that are otherwise relatively uniform (Hay, 1991).

Interactions between fish and the physical environment are less thoroughly studied but also appear to be important in generating within-habitat refuges for seaweeds. Studies have shown that grazing rates of herbivorous reef fish are correlated with water temperature (Carpenter, 1986; Klumpp & Polunin, 1989). If lowered temperature depresses feeding rates more than rates of algal production, then palatable seaweeds will have a greater probability of escaping herbivorous fish during cooler periods of the year. Other kinds of defence, particularly among invertebrates include: nocturnal activity and parental protection (Fishlyn & Phillips, 1980). It seems likely that an organism may employ a number of these defences to reduce consumption from diverse consumers (Duffy & Paul, 1992).

### 3.3 Chemical defence

Organisms possess a number of mechanisms which reduce the impact of consumers (Hay, 1984; Paul & Hay, 1986; Paul, 1987), many of which may have evolved in response to the intense selection pressure exerted by consumers (Lewis, 1986). One of the most important of these mechanisms is the production of secondary metabolites, which can function as chemical defences against consumers (Hay & Fenical, 1988; Hay *et al.*, 1988; Hay *et al.*, 1990a,b; Steinberg & Paul, 1990; Duffy & Paul, 1992). Secondary metabolites have been shown to have a negative effect on consumer fitness or to deter consumption (Rosenthal & Janzen, 1979). This reduction in consumption increases the chances of survival of the producing organisms thereby increasing their overall fitness.

Metabolites responsible for the chemical defence of an organism are frequently not uniformly distributed within the organism (Van Alstyne *et al.*, 1994). Variation in chemical defence composition and concentration is often seen in marine plants and invertebrates (Mckey, 1979; Paul & Hay, 1986; Hay *et al.*, 1987). Differences in the distributions of chemical defence within marine organisms provide an opportunity for assessing some of the constraints and selective forces that have influenced the evolution of these defences (Harvell *et al.*, 1988). For example, Harvell *et al.* (1988) have proposed a model for the evolution of chemical defences within Caribbean gorgonians. This model is based on the distributions of secondary metabolites within octocoral species. They

suggest that the use of secondary metabolites as defences evolved in response to selective pressures imposed by predators. According to their model, the high concentrations of secondary metabolites in the polyps of some octocorals resulted from selective pressures from fishes. The concentration of secondary metabolites are lower in the bases because predation by fish at the base is less intense. The distribution and concentration of metabolites are therefore related to the extent of consumer pressure.

Organisms may use one of two chemical defence strategies to allocate resources for antipredator defences (Paul & Van Alstyne, 1992). The two strategies are continuous and acquired chemical defence. Continuous chemical defence refers to the use of secondary metabolites as a chemical defence to reduce predation when predation rates are consistent and predictable. This type of strategy describes most kinds of chemical defence. Acquired chemical defence refers to the use of secondary metabolites as a chemical defence to reduce predation when predation rates are temporarily unpredictable. One type of acquired chemical defence that has been described in several marine organisms is the production of predator-induced defences (Schultz, 1989; Adler & Harvell, 1990). Usually this type of defence operates when an attack by a predator acts as a cue for stimulating the synthesis of defensive compounds (Paul & Van Alstyne, 1992). In marine organisms, predator-induced chemical defences have been reported only in the brown seaweed *Fucus* (Van Alstyne, 1988). The different chemical defence strategies allow organisms to deal more effectively with predators.

### **3.3.1. Alternative and additional functions of secondary metabolites**

Seaweed secondary metabolites have been demonstrated to function as herbivore deterrents, but some of these compounds may have additional functions (Habermehl & Krebs, 1990). Secondary metabolites have also shown to play a rôle in microbial and epiphytic deterrence (Targett *et al.*, 1983; Hay & Fenical, 1988; Davis & Wright, 1990; Hay, 1991). Despite the various effects of secondary metabolites it is not known whether the primary function of secondary metabolites is chemical defence. It is likely that many secondary compounds have multiple functions and thus form the basis of a complex chemical defence system (Standing *et al.*, 1982; Rittscof *et al.*, 1985).

### **3.3.2 Cost of chemical defence**

The production of chemical defences against consumers could incur metabolic costs for the defended organism if significant energy or materials have to be diverted from other functions to the production of chemical defence. Thus, defences are hypothesised to be costly and in the absence of consumers less defended individuals will have higher fitness than do heavily defended individuals (Lubchenco & Gaines, 1981; Coley *et al.*, 1985). If this reasoning applies to seaweeds, then habitats that serve as predictable escapes from herbivory should be populated primarily by species that are highly susceptible to herbivore damage. Low susceptibility to herbivory should be characteristic of those species or individuals that occur in habitats where herbivory is predictably high.

This pattern occurs in a number of marine communities (Lubchenco & Gaines, 1981; Hay *et al.*, 1983; Hay, 1984; Paul & Fenical, 1986), and experimental decreases in herbivory result in the more herbivore-susceptible seaweeds dominating the more herbivore-resistant forms (Lubchenco & Gaines, 1981; Lewis, 1986).

There are no direct experimental assessments of the costs of chemical defences in seaweeds. The currencies in which to measure the costs associated with chemical defences are difficult to separate and quantify, but probably involve more than the chemical energy stored in the molecular bonds of secondary metabolites (Hay & Fenical, 1988; Fagerstrom, 1989; Cronin & Hay, 1996). Since seaweeds are often nitrogen limited but seldom carbon limited (Hay & Fenical, 1988), it is unclear that allocation of energy (i.e. carbon bonds) will entail any direct costs. However, substantial costs could be involved in the synthesis and storage of compounds. It is interesting to note that seaweeds rarely produce the nitrogen containing compounds that are defences in some terrestrial plants. Nitrogen limitation may make nitrogen-based defences too costly, especially when carbon-based defences work well. With few exceptions, the only seaweed secondary metabolites that contain nitrogen are produced by the blue-green algae (Moore; 1981; Faulkner, 1984), many of which are nitrogen fixers. However, substantial costs could still be involved in synthesis, and storage. The allocation of chemical defences to different organism parts (Paul & Fenical, 1986; Hay *et al.*, 1988), the activation of chemical defence following attack (Van Alstyne, 1988), and the competitive interactions between chemically defended and undefended organisms in the absence of



consumers (Lewis, 1986) all suggest that chemical defences are costly.

#### **4. Categories of compounds, their functional and evolutionary significance**

The major classes of secondary metabolites have been assigned various rôles in the complex interactions occurring between organisms in the natural environment (Harborne, 1988). The major classes and their physiological activity include:

1. Nitrogenous compounds: many toxic and bitter tasting;
2. Terpenoids: many toxic, bitter and haemolyse blood;
3. Phenolic compounds: anti-microbial, anti-digestive agents and pigments;
4. Miscellaneous compounds: some toxic.

These compounds have served as a starting point for many studies of chemical-ecological interactions.

Marine metabolites in general structure do not differ from those produced in terrestrial organisms and there are many biosynthetic pathways which are common to organisms from both realms. The general classes of compounds are outlined below.

**Nitrogenous compounds.** Nitrogenous compounds have nitrogen as a basic element, and many are known for their toxicity. The simplest nitrogen-based toxins are the non-protein amino acids. These are widely present in plants and may be directly toxic to the organism eating them. In the simplest case, azetidine 2-carboxylic acid (Fig. 1),

may be mistakenly incorporated into proteins during synthesis so that the organism produces unnatural proteins which cannot function properly so the organism dies (Harborne, 1988). Although, the production of nitrogen containing compounds are hypothesized to be costly in the marine environment, a wide variety of nitrogenous compounds have been isolated from marine organisms, ranging from simple compounds such as tetramine to complex ones such as tetrodotoxin (Woodward, 1964; Shimizu, 1978). The latter are strong neurotoxins which inhibit sodium passage through membranes (Chevolet, 1981). Other simpler nitrogenous compounds play important rôles in marine biological interactions. For example, gamma-amino-butyric acid, produced by encrusting coralline algae, influences settlement and metamorphosis in abalone larvae (Morse & Morse, 1984). The most familiar class of nitrogen-based toxins are the alkaloids. Alkaloids isolated from marine acorn worms function as a chemical defence against micro- and macro-organisms (Higa *et al.*, 1980). Nitrogen-based compounds are significant feeding deterrents to grazing animals, particularly when their presence is often associated with a bitter taste.

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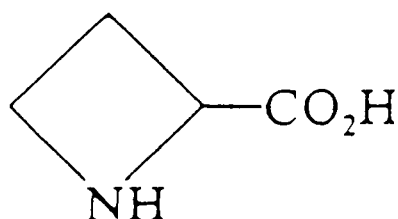


Fig. 1. azetidine 2-carboxylic acid (Harborne, 1988)

**Terpenoids.** Terpenoids represent the largest and biologically most important class of natural products (Rosenthal & Janzen, 1979). These compounds are multiples of C<sub>5</sub> units linked together 'head to tail' (Fig. 2). They have been found in all living organisms so far examined for them. Although most terpenoids occur free in nature, often being accumulated in specific tissues, many are conjugates of organic acids, sugars, and many other constituents (Rosenthal & Janzen, 1979). Some researchers call these conjugates terpenes, but this term should be reserved for the terpenoids that are true hydrocarbons (Robinson, 1980).

Terpenoid compounds have been isolated from algae, sponges, molluscs, coelenterates, and especially the holothurians (Bakus *et al.*, 1986). Triterpene glycosides are known to be vital for numerous plants with a broad range of biological activity. Nigrelli *et al.* (1955) and Yamanouchi (1955) were the first to discover that marine animals, sea cucumbers in particular were the source of triterpene glycosides. More than 60 sesquiterpenes have been identified from algae (Bakus *et al.*, 1986). Most typically, halogen containing terpenes are found in algae in the genus *Laurencia* where they are thought to function in part as herbivore feeding deterrents (Fenical, 1975; Erikson, 1983). The sea slugs *Aplysia* spp. are one of the few herbivores adapted to grazing on *Laurencia* spp. The slug sequesters this halogenated metabolite from the plant and is thought to use it as a means of defence against its own predators (Fenical, 1975). In general, terpenoid compounds are thought to function in antipredation, competition for space and possible antifouling (Rosenthal & Janzen, 1979 Bakus *et al.*, 1986).

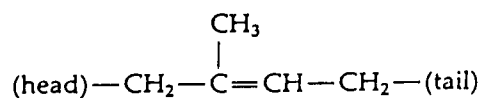


Fig. 2. Terpenoids, multiples of C<sub>3</sub> units linked together head to tail

(Salisbury & Ross, 1985).

**Phenolics.** All phenolic compounds have an aromatic ring that contains various attached substituent groups, such as hydroxyl, carboxyl, and methoxyl groups (Fig. 3). They arise via a variety of biosynthetic pathways. Their most obvious characteristic is the presence and abundance of halogenated substituents. Bromine is the halogen most frequently incorporated. Halogenated and non-halogenated phenolics have been shown to have a multiplicity of potential ecological rôles. Phenolic compounds occur in algae, sponges, and annelids. The well known ones occur in brown and red algae, and sponges (Higa *et al.*, 1980). More than 200 phenolic compounds have been described from marine organisms (Steinberg, 1984; Van Alstyne, 1988; Steinberg & Paul, 1990). Phenolic compounds are known to function as feeding deterrents in certain marine macrophytes (Phillips & Towers, 1982; Steinberg, 1984; 1985) and they also function as antifouling and antimicrobial agents in sessile organisms (Davis & Wright, 1990; Hay, 1991).

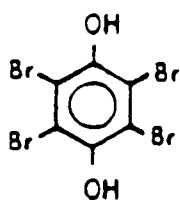


Fig. 3. Phenolic compound with an aromatic ring and characteristic substituents

(Bakus *et al.*, 1986).

**Miscellaneous compounds and those of mixed origins.** Many compounds are derived from the condensation of several products each arising from a different biogenetic pathway. An example of a compound of mixed origin is asterosaponin A (Fig. 4), which is an oligosaccharide with a steroidal aglycone (Ikegami *et al.*, 1972). It is thought to be responsible for the obvious avoidance behaviour of a number of marine species when placed in close proximity with a starfish (Burnell & Apsimon, 1983). Miscellaneous compounds include acetylenes and lipids. A variety of rôles have been attributed to these compounds, including chemical defence and species recognition (Muller, 1979; Gerhart, 1984).

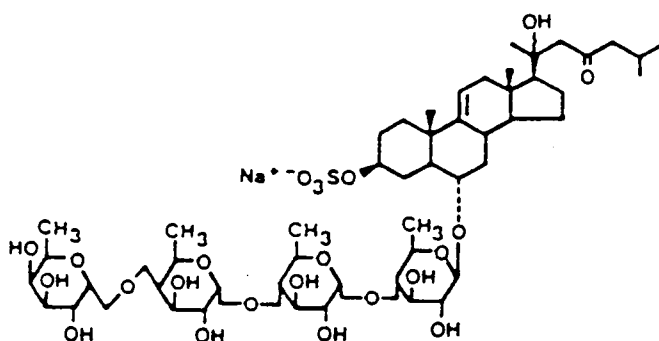


Fig. 4. Compound of mixed origin: asterosaponin A (Ikegami *et al.*, 1972)

## 5. Defence mechanisms in holothurians

Holothurians have developed a number of antipredator defence mechanisms, including thick integument, toxicity, evisceration, and unpalatability (de Vore & Brodie, 1982). The presence of a thick and/or tough integument may discourage most predation