Chapter 1

Approaches to natural product research: the importance of understanding the ecological significance of secondary metabolites

1. 0 The ecological and biomedical significance of gastropod natural products

The *de novo* biosynthesis, bio-transformation or sequestration of dietary derived natural products by marine gastropods is a well documented phenomenon (Fenical et al. 1979; Garson and Staunton, 1979; Karuso, 1987; Faulkner, 1992; Cimino and Sodano, 1993; Pawlik, 1993; Avila, 1995, 2006; Garson, 2001; Cimino et al., 2004; Marin and Ros, 2004; Bandaranayake, 2006; Wägele et al., 2006). Although it is generally accepted that secondary metabolites are selected over the course of evolution for the functional advantage they confer (Maplestone et al., 1992), reliable information on their significance to life history strategies is limited (Faulkner, 1992; Avila, 2006). In an effort to identify novel compounds with biomedical potential, research has traditionally focused on the isolation, structure elucidation and *in vitro* bioactivity of natural products from molluscs lacking mechanical protection (Avila, 2006; Wägele et al., 2006). Due to this bioprospecting strategy, defence against predation or functions inferred by *in vitro* activity have been assumed for many secondary metabolites.

Accurate information on the origin and functional role of natural products is not only essential to our understanding of marine chemical ecology, but to the development of pharmaceutical agents. Almost 25% of marine natural products currently in preclinical and clinical trials or commercially available (e.g. dolastatin, ziconotide, aplyronine A, kahalalide F, anti-beta-aminoalcohol) are derived from mollusc secondary metabolites (Proksch et al., 2002; Bioaqua, Marine Bioresources Institute, 2004). Despite this biomedical potential, limited biomass coupled with the

structural complexity and low yields of metabolites obtained from wild stocks or total synthesis often hinders the development and sustainable production of mollusc natural products (Proksch et al., 2002). Although aquaculture and partial synthesis from commercial precursors offers a potential solution for some drug candidates, insight into the biosynthetic pathways and biochemical strategies employed by molluscs may identify novel approaches to the synthesis of analogues, intermediates with preferred therapeutic profiles (Garson, 2001) or novel molecular targets (Carté, 1996). Furthermore, as biosynthesis is likely to be under influence of biotic or abiotic factors (Kubaneck et al., 2000), comprehension of the regulatory process operating at the organism, cellular and molecular level is essential to the development of production strategies, such as the cloning and expression of respective biosynthetic gene clusters (Proksch et al., 2002).

1. 1 An anatomic and ontogenetic approach to chemical ecology

The methodological difficulties associated with demonstrating *in situ* activity and maintaining ecological relevance (e.g. using co-occurring predators) is thought to have contributed to the scarcity of sound evidence on the functional advantage of mollusc secondary metabolites (Wägele et al., 2006). Thus, to better understand the evolutionary significance and realize the commercial potential of these compounds, it is essential that new approaches to natural product research are introduced. The anatomical distribution, incidence and prevalence of secondary metabolites in early and adult life stages has recently been shown to provide valuable insight into the

ecological role of natural products (Lindquist, 2002; Avila, 2006; Wägele et al., 2006). If secondary metabolite function is tailored to factors affecting survivorship at specific life stages by natural selection, monitoring developmental changes in natural products may highlight chemically mediated interactions of ecological importance (Lindquist, 2002). The absence of secondary metabolites from a particular life stage may be due to a trade-off between primary and secondary metabolism, reflect a morphologically vulnerable life stage or a function unrelated to defence (Lindquist, 2002). For example, ichthyotoxic verrucosins (Cimino et al., 1986) and xylosyl-MTA, an analog of the spermine synthase inhibitor MTA (Cimino et al., 1986), are present in the nudibranch *Doris verrucosa*. However, verrucosins are absent from larval stages and xylosyl-MTA only occurs during the first few days of development (unpublished data in Avila, 2006). This suggests verrucosins function in predator defence, a mechanism absent from larvae most likely due to a trade-off between growth and chemical defence, while xylosyl-MTA appears to be associated with embryogenesis and possibly oogenesis in adults. Thus, ontogenetic-dependent changes in natural product composition assessed in combination with *in vitro* activity can aid in determining or confirming the functional significance of secondary metabolites.

Establishing the anatomical distribution of secondary metabolites is another approach to mollusc chemical ecology which can provide valuable information on the biosynthetic origin, storage and hence the ecological role of natural products (Avila, 2006). Although few attempts had been made to locate storage structures in molluscs, relating secondary metabolite incidence to anatomical features known from histology has proved useful in identifying such structures (Wägele et al., 2006). This

histological approach can also reveal correlations between specific glands and the biosynthetic origin of natural products. For instance, it has tentatively been suggested that dietary derived metabolites accumulate in specialized glands (e.g. mantle dermal formations), while biosynthesized compounds are stored in single gland cell layers (e.g. hypobranchial gland) (Wägele et al., 2006). Localization of transport pathways from digestive to storage glands and structures involved in biosynthesis or bio-transformation would also greatly aid in deciphering the ecological role of natural products. Although directly labeling metabolites with oligonucleotide aptamers or various immunocytological probes has thus far proven unsuccessful (see Wägele et al., 2006), more traditional histochemical techniques may offer an alternative.

1. 2 A histochemical approach to natural product biosynthesis

A histochemical approach to natural product research can not only provide information on the biosynthetic origin and functional significance of secondary metabolites, but may be advantageous over traditional methods for establishing biosynthesis. The *de novo* biosynthesis of natural products is typically examined using stable isotopes in conjunction with nuclear magnetic resonance (N.M.R.) and mass spectrometry (Cimino et al., 2004). However, low incorporation rates often render questionable results (Cimino et al., 2004) and labeled precursor integration into extraneous metabolic pathways (Garson, 1993) can be misleading. For example, ^{14}C labeled mevalonic acid and glycerol injected into the hepatopancreas of *D. verrucosa,* failed to demonstrate the *de novo* biosynthesis of diterpene glycerides

(Avila et al., 1990) due to the diversion of precursors during passage to mantle storage structures (Cimino and Sodano, 1993). The high levels of precursor incorporation required to afford detection by N.M.R. can also alter the metabolic pathway under investigation (Garson, 1993). Furthermore, stable isotopes methodologies are insensitive to the increasing incidence of natural product biosynthesis by symbiotic microorganisms (Cimino et al., 2004).

A rarely employed, but highly informative alternative to radioisotopes is the application of carefully selected histochemical techniques. The application of staining reactions for proposed precursors and enzymes, can not only establish the primary metabolic origin of natural products, but identify sites of active biosynthesis and qualitatively define the comparative concentration and activity of precursors and enzymes, respectively. The biochemical and morphological properties of associated biosynthetic tissues can also provide information on the transport, storage and secretion of biosynthetic constituents and highlight potential regulatory mechanisms. Marine natural products represent numerous chemical classes, including terpenes, alkaloids, polyketides, peptides, shikimate-derived metabolites and compounds of mixed biogenesis (Garson, 2001). Many alkaloids and peptides are generated through the elaboration and condensation of amino acids (Cordell, 1981), while terpenes are derived from mevalonate (Gustafson and Anderson, 1985) and polyketides from the condensation of acetate units (Simpson, 1987). Histochemical techniques are available for many proteins, glycoproteins, carbohydrates, lipids, functional groups and enzymes (e.g. Thompson 1966).

A comprehensive understanding of the genetic processes governing secondary metabolite biosynthesis is becoming an increasingly important aim of natural product

research. Investigations into the genetic organization of biosynthetic pathways and hence the number and sequences of enzymes, can reveal the relative position of gene clusters where regulatory (repressors and activators) and resistance genes occur (Maplestone et al., 1992). As natural products are often stored in a different cellular environment to where they are synthesized (Garson, 1993), differentiating between these tissues is an essential prerequisite to genetic analyses. Thus, adopting a histochemical approach to natural product biosynthesis would not only increase the efficiency of subsequent investigations into biomedically important genetic processes, but expand our understanding of chemically mediated phylogenetic and ecological relationships.

1. 3 Muricids as a model for gastropod natural product research

Although shelled molluscs are usually assumed to be absent of chemical defenses in lieu of mechanical protection (Faulkner, 1992; Wägele et al., 2006), many possess natural products of biomedical importance. For example, ES-285 from the bivalve *Spisula polynyma*, is currently in clinical trials as a promising anticancer agent (Jimeno, 2002). The Muricoidea is an exceptionally biodiverse superfamily of predominantly carnivorous neogastropods (Kay et al., 1998), which has already yielded compounds of biotechnological and pharmaceutical value. Conotoxins utilized during prey capture by *Conus* species, have provided neuroscientists and molecular pharmacologists with a suite of chemical probes for the examination of ion channels and receptors (Myers et al., 1993). Furthermore, ziconotide (Prialt®) from

Conus magus (McIntosh et al., 1982) has recently been approved for sale as an intrathecal analgesic (Elan Pharmaceuticals Inc., 2004). These examples highlight shelled molluscs, in particular predatory families, as a virtually untapped biomedical resource.

Species of the family Muricidae are renowned for one of the most historically important marine natural products, Tyrian purple. Tyrian purple precursors constitute a suite of bioactive secondary metabolites (Westley et al., 2006). Tyrian purple prochromogens are choline ester salts of 6-bromoindoxyl and indoxyl, unsubstituted and substituted with methylthio or methylsulphonyl (reviewed in Cooksey, 2001a). Prochromogen hydrolysis by an arylsulphatase enzyme produces cytotoxic bromo-indoleninone monomers and dimers (Benkendorff et al., 2000) and physiologically active choline esters (reviewed in Roseghini et al., 1996). It has recently been shown that 6,6'-dibromoindirubin, a minor pigment in Tyrian purple dye, inhibits cell proliferation by interfering with glycogen synthase kinase-3 (Meijer et al., 2003) and may be useful in the treatment of chronic myelocytic leukemia (Hoessel et al., 1999). Furthermore, research into the cytotoxicity of Tyrian purple precursors has revealed that extracts containing tyrindoleninone display specificity towards rapidly dividing tumor cells (Vine et al., 2007). Although tyrindoleninone holds potential as an anticancer agent, attempts to synthesize this compound have been unsuccessful (Duke, 1974; Vine et al., 2007). Thus histochemical investigation into *in situ* biosynthetic processes may reveal novel approaches for synthetic production of these 6-bromoindole derivatives and ultimately facilitate the isolation of regulatory gene clusters.

Tyrian purple prochromogens are located in the muricid hypobranchial gland (Baker and Sutherland 1968), a conspicuous structure lining the dorsal mantle cavity of most gastropods and various protobranchiate bivalves (Fretter and Graham, 1962). The hypobranchial gland has been shown to function in the transport and eventual expulsion of debris introduced to the mantle cavity by the respiratory stream (Fretter and Graham, 1994). However the bioactivity of compounds secreted by this gland suggests it may also play an important role in molluscan chemical ecology. In addition to the cytotoxins and paralytic choline esters of the Muricidae, a potassium ion channel inhibitor, of striking structural similarity to Tyrian purple precursors, occurs in hypobranchial secretions of *Calliostoma canaliculatum* (Kelley et al., 2003) and functions in defence against sympatric sea stars (Bryan et al., 1997). Furthermore, the hypobranchial gland has been highlighted as a potential defensive structure in the opisthobranch orders Acteonoidea, Cephalaspidea, Sacoglossa and Thecosomata (Wägele et al., 2006).

A wealth of information exists on the biosynthesis of Tyrian purple pigments from prochromogens in the Muricidae (reviewed in Cooksey, 2001a) and although inconclusive, considerable research effort has been directed towards the selective advantage of bioactive Tyrian purple precursors (reviewed in Westley et al., 2006). Of particular interest is the recent isolation of Tyrian purple precursors from muricid egg masses (Benkendorff et al., 2000, 2001, 2004) and observations of dye pigments in the muricid gonoduct (Benkendorff et al., 2004) and non-viable embryos (Spight, 1975; Gallardo, 1979; Pechenik, 1982, 1983; Rawlings, 1996). Although this may indirectly imply a functional role for hypobranchial gland metabolites in reproduction, the biosynthetic origin of these compounds needs to be established

before this proposed role can be confirmed. Consequently, this family represents an ideal model for investigations into the ecological significance of hypobranchial gland natural products in marine molluscs.

Much of the principle research on Tyrian purple genesis has focused on the Australian muricid, *Dicathais orbita* (Gmelin, 1791) (Baker and Sutherland, 1968, Baker, 1974; Baker and Duke, 1976; Benkendorff et al., 2000, 2001, 2004). In contrast to the diversity of prochromogens present in other muricids, *D. orbita* possesses a single dye prochromogen, tyrindoxyl sulphate (Baker and Sutherland, 1968) and therefore, the most simplistic Tyrian purple biosynthetic pathway. Consequently, *D. orbita* is presented as a benchmark species for investigations into the functional advantage of natural products in the Muricidae.

1. 4 Thesis aims, significance, structure and objectives

1. 4. 0 Thesis aims and significance

Through the progressive application of complementary chromatographic, mass spectrometry and histochemistry techniques, this thesis aims to determine the biosynthetic origin, ontogenetic and anatomical distribution of Tyrian purple precursors. However prior to these investigations, the reproductive anatomy of *D. orbita* and the process of larval encapsulation needs to be established to allow functional interpretation of biosynthetic information. In an ecological sense, it is anticipated that this investigation will provide evidence towards a functional role for Tyrian purple precursors in muricid reproduction and insight into the evolutionary significance of the gastropod hypobranchial gland. While in a biomedical sense, it is

hoped that information on the processes regulating precursor biosynthesis will aid future attempts to define the genetic basis of this renowned biosynthetic pathway and ultimately, facilitate the development of Tyrian purple precursors as therapeutic agents.

1. 4. 1 Thesis structure

This thesis is presented in manuscript format. Although each chapter is intended for independent publication, the underlying concepts comprise a progressive body of research. To maintain continuity in presentation, all chapters have been formatted in a consistent manner and have adopted the referencing format outlined for the Journal of Chemical Ecology. To avoid repetition, the literature cited in each manuscript has been compiled as a single reference list at the rear of the thesis. Although I am first author on all chapters and personally responsible for the experimental design, conducting the research and preparing manuscripts, the contributions of additional authors on published manuscripts or those currently under review, are outlined in the acknowledgements of each chapter. In such cases, the full reference is provided as a footnote on the first page of the relevant chapter. The following outlines the objective of each chapter with significance to the thesis aim and indicates the publication status.

1. 4. 2 Chapter objectives

Chapter 2: Sex-specific Tyrian purple genesis: precursor and pigment distribution in the reproductive system of the marine mollusc, *Dicathais orbita*. *J. Chem. Ecol*. (2008) 34(1): 44-56.

Objective: Chapter 2 aims to quantify precursor and pigment composition in the female pallial gonoduct, specifically, the capsule, albumen and ingesting glands, as a means for establishing the gross anatomical distribution of Tyrian purple natural products in *D. orbita*.

Chapter 3: Histomorphology of the pallial gonoduct, hypobranchial gland and associated structures in *Dicathais orbita* (Neogastropoda: Muricidae). This chapter will be divided into two manuscripts. The first will be entitled, 'Histomorphology of the hypobranchial gland in *Dicathais orbita* Gmelin 1791 (Neogastropoda: Muricidae): Tyrian purple precursor transfer to the gonoduct?', and is intended for publication in *Zoomorphology*. The second manuscript will adopt a different focus to the thesis aim, entitled 'Histomorphology of the female pallial gonoduct in *Dicathais orbita* (Neogastropoda: Muricidae): Sperm passage, fertilization and sperm storage potential'. This latter manuscript has been accepted for publication in *Invertebrate Biology* (20th February 2009).

Objective: Chapter 3 aims to present the first detailed description of the pallial gonoduct and hypobranchial gland of *D. orbita* with the intention of deciphering an anatomical mechanism for precursor transfer.

Chapter 4: Histochemical correlations between egg capsule laminae and the female gonoduct reveal the process of capsule formation in the Muricidae (Neogastropoda: Mollusca). *Invertebr. Reprod. Dev. Accepted 4th August 2008, in press.*

Objective: Chapter 4 aims to decipher the process of encapsulation to establish the destination of gonoduct secretions in the intracapsular fluid and capsule laminae of *D. orbita* egg masses. This information will be used in Chapter 6 to determine mechanisms for the incorporation of Tyrian purple natural products in various egg mass constituents.

Chapter 5: Evidence for the regulated *de novo* biosynthesis of Tyrian purple in the hypobranchial gland of *Dicathais orbita* (Neogastropoda: Muricidae).

Objective: Through the application of novel histochemical techniques for the localization precursors and enzymes required for Tyrian purple synthesis, this investigation aims to provide insight into the biosynthetic origin and regulatory mechanisms governing bioactive precursor synthesis in the hypobranchial gland.

Chapter 6: The origin of Tyrian purple precursors in egg masses: maternal investment in the chemical defence of encapsulated *Dicathais orbita* larvae (Neogastropoda: Muricidae).

Objective: Through the application of novel and established histochemical techniques in combination with liquid chromatography-mass spectrometry and thin-layer chromatography, this chapter aims to address the origin of bioactive intermediates in the egg masses of *D. orbita*.

Chapter 7: The ecological significance of Tyrian purple precursors in the Muricidae and future directions in mollusc natural product research.

Objective: This chapter provides a summary of the research findings and expands on individual chapter discussions by addressing them in a broad ecological and biomedical perspective. This chapter also highlights future directions for investigations into the functional significance and genetic organization of muricid natural product biosynthesis and the gastropod hypobranchial gland.