Chapter 7

The ecological significance of Tyrian purple precursors in the Muricidae and future directions in gastropod natural product research

The application of complementary chromatographic, mass spectrometry and histochemistry techniques in this investigation has proven valuable in determining the biosynthetic origin, ontogenetic and anatomical distribution of Tyrian purple precursors. The information afforded by this approach, has provided evidence towards a functional role for Tyrian purple precursors in muricid reproduction and insight into the evolutionary significance of the hypobranchial gland. Furthermore, the localization of biosynthetic regulatory enzymes and tissues will undoubtedly aid future attempts to define the genetic basis of this renowned biosynthetic pathway and the development of muricid secondary metabolites as therapeutic agents.

7. 0. The biosynthetic origin of Tyrian purple precursors

The histochemical approach adopted revealed that the Tyrian purple prochromogen, tyrindoxyl sulphate, is *de novo* biosynthesized through the post-translational bromination of dietary derived tryptophan by bromoperoxidase (Chapter 5). This is the first report of hypobranchial gland natural product biosynthesis from primary metabolites and an example of a novel alternative to radioisotope incorporation in biosynthetic research. Two sites of prochromogen synthesis were identified within the hypobranchial gland of *Dicathais orbita*. Tryptophan is stored within secretory cells of the lateral hypobranchial epithelium and once exocytosed, is united with bromoperoxidase from interspaced supportive cells to form tyrindoxyl sulphate. Prochromogen synthesis also occurs in the subepithelial vascular sinus of the hypobranchial gland for storage and secretion by specialized medial epithelial cells. Tyrindoxyl sulphate hydrolysis and subsequent bioactive precursor synthesis on the medial and lateral epithelial surface is regulated by the liberation of

arylsulphatase from adjacent supportive cells. In addition to the hypobranchial gland, tyrindoxyl sulphate is transported within the vascular sinus to lateral and dorsal capsule gland lobes where bromoperoxidase and arylsulphatase also occur (Chapter 6). Another source of arylsulphatase is the albumen gland, which along with the anteroventral and posterior capsule gland lobes, act as a storage site for tryptophan (Chapter 6).

7. 1 The ecological significance of Tyrian purple precursors in egg masses and early life stages

Biochemical correlations between hypobranchial, capsule and albumen gland secretions and the intracapsular fluid and laminae of capsules identified glandular regions of interest to the origin of egg mass metabolites (Chapter 3) and allowed the process of encapsulation to be deciphered (Chapter 4). Subsequent application of histochemical techniques in combination with liquid chromatography-mass spectrometry revealed the anatomical and early ontogenetic distribution of biosynthetic constituents (Chapter 6). Tyrindoxyl sulphate and the biosynthetic constituents for prochromogen and bioactive precursor synthesis are introduced to intracapsular fluid and capsule laminae by the capsule gland. Tryptophan, bromoperoxidase, tyrindoxyl sulphate and arylsulphatase were also detected in encapsulated larval vitellus, which is elaborated during oogenesis and may also receive secretions from the albumen gland. Due to the absence of a hypobranchial gland in veliger hatchlings, it appears that pelagic larvae rely on vitelline natural products until settlement and metamorphous. Together these findings revealed a

maternal origin for Tyrian purple precursors in egg masses of the Muricidae and provided insight into their ecological significance.

The distribution of Tyrian purple precursors and biosynthetic enzymes within capsules and larvae, coupled with the maternal metabolic expense implicated by these investments, suggests bioactive precursors confer a selective advantage during encapsulated and planktonic development. Prochromogen hydrolysis by arylsulphatase liberates physiologically active choline esters such as murexine (reviewed in Roseghini et al., 1996). Thus, murexine may evoke learned aversion in predators through the paralysis induced by this choline ester (Erspamer and Glässer, 1957). Post ingestion of egg capsules or larvae, arylsulphatase within predator gut lysosomes (Ezeasor and Stokoe, 1980; Arnaud et al., 1984) or autolysis induced by larval stress or death may inadvertently liberate these physiologically active metabolites. To substantiate or disprove this hypothesis, *in situ* demonstration of anti-predatory activity through the presentation of larvae and readily consumed prey laced with crude extract to sympatric predators (e.g. Lucas et al., 1979; Lindquist et al., 1992) is required.

In addition to choline esters, prochromogen hydrolysis also promotes the oxidative genesis of antimicrobial bromoindoles (Benkendorff et al., 2000). Although the heterogeneous activity of these metabolites implies a multifaceted functional role, their simultaneous synthesis suggests they may be intrinsically linked. The functional significance of liberating choline ester salts in synchrony with antimicrobial indoles may reflect their ability to reversibly increase cell permeability. Choline fatty acid esters salts are administered in conjunction with antibiotics, antiviral and anticancer agents to enhance absorption (Alexander and Fix, 1989). Thus, it is possible that

choline esters of sulphate, such as murexine, facilitate the absorption of antimicrobial indoles by pathogens and biofilm-forming microorganisms, thereby enhancing their effect. The division of precursors and catalytic enzymes between capsule laminae appears to facilitate natural product synthesis as the physical structure degrades. In light of this, and the limited fouling of muricid egg capsules (Lim et al., 2007), it appears that antimicrobial indoles provide protection against bacterial colonization throughout encapsulated development. Similarly, the incorporation of precursors and biosynthetic enzymes in larval vitellus may prevent infection by pathogens inhaled into the mantle cavity during pelagic development.

Future investigation into this proposed synergistic effect would not only clarify the ecological significance of their simultaneous synthesis, but provide an endogenous mechanism of increasing muricid natural product bioavailability. Tyrindoleninone is a cytotoxic Tyrian purple precursor with promise as a novel anticancer agent (Vine et al., 2007). As effective delivery is often a challenging aspect of drug development and the absorptive effect of choline esters is pronounced within the gastrointestinal tract (Alexander and Fix, 1989), investigation into the therapeutic benefits of administering tyrindoleninone in conjunction with murexine is clearly warranted.

Whether intended for defence against predators, pathogens or both, the findings of this investigation strongly suggest Tyrian purple precursors are incorporated into muricid egg masses as a maternal investment in larval defence. Furthermore, the transport and storage of indole precursors and biosynthetic enzymes to specific hypobranchial secretory cells for regulated release, implies this gland functions in natural product biosynthesis. As the gonoduct of all caenogastropods

evolved from an ancestral right hypobranchial gland (Kay et al., 1998) it is possible that a capacity for hypobranchial gland natural product synthesis has been retained in the reproductive glands of other families. In addition to the Muricidae, egg masses of the Conidae, Buccinidae, Cassidae and Cypraeidae (Santhana Ramasamy and Murugan, 2005), Littorinidae, Ranellidae, Naticidae and Mitridae (Benkendorff et al., 2001) also display antimicrobial activity. Furthermore, hypobranchial gland secretions of Volutidae (Weaver and Du Port, 1970) and Olividae (Marcus and Marcus, 1959) neogastropods are purple, with the latter also possessing chemically defended egg masses (Santhana Ramasamy and Murugan, 2005). Thus, it would be of taxonomic significance to determine if biochemical correlations between hypobranchial gland, gonoduct and masses are generic to caenogastropods.

7. 2 The ecological significance of Tyrian purple precursors in the adult hypobranchial gland

Maternal investment in larval defence is not the principal ecological role of bioactive Tyrian purple precursors in the Muricidae for several reasons; 1) the primary site of prochromogen storage is with specialized epithelial cells of the hypobranchial gland, 2) hypobranchial gland secretory products are liberated onto the epithelial surface lining the dorsal mantle cavity, 3) bioactive precursors biosynthesis on the epithelial surface is regulated, and 4) males also invest considerable metabolic energy into the *de novo* biosynthesis of Tyrian purple precursors (Westley and Benkendorff, 2008).

It has been proposed that physiologically-active choline esters are utilized during prey capture (Keyl et al., 1957; Whittaker, 1960; Roseghini et al., 1970; Huang and Mir, 1971; Ottaviani, 1978; Bolognani-Frantin and Ottaviani, 1981; Roller et al., 1984; Srilankshmi, 1991; Roseghini et al., 1996). However, the lack of an effective delivery mechanism and failure to demonstrate ecologically relevant activity in prey renders this hypothesis unlikely (Roller et al. 1995). One proven function is in the removal of debris introduced to the mantle cavity in the respiratory stream. It has been shown that particles trapped on the ctenidium are swept by ciliary action onto the hypobranchial gland where they are cemented together to facilitate expulsion in the exhalent stream (Fretter and Graham, 1994). An extension of this function is the binding of respiratory borne pathogens in epithelial secretions (Westley et al., 2006), which may induce antimicrobial indole biosynthesis and the liberation of choline esters to increase indole absorption and maintain a sterile environment within the mantle cavity. Production of choline esters may also trigger continued prochromogen exocytosis, as the nicotinic action of these compounds is known to stimulate mollusc nerve cells (Avoli et al., 1978; 1979), secretory activity and muscular contraction (Roesghini et al., 1996). Thus, it is possible that choline esters not only facilitate antimicrobial indole absorption, but create a positive feed-back loop, promoting epithelial secretion for sustained biosynthesis. To better understand the evolutionary significance of this gland, it would be useful to demonstrate the binding and antimicrobial capacity of hypobranchial secretions, through fluorescent viability staining of epithelial microbial communities. In addition, assessment of marine pathogen viability after incubation within

antimicrobial precursors in the presence and absence of choline ester salts would provide further insight into the significance of their simultaneous synthesis.

The choline esters murexine, dihydromurexine and senecioylcholine are also common to hypobranchial secretions of neogastropods from the Nassariidae, Coralliophillidae, Olividae and Fasciolariidae, while acryloylcholine occurs in the Buccinidae (see Roseghini et al., 1996). As the functional advantage conferred by choline esters is likely to provide insight into the evolutionary significance of this gland in neogastropods, investigation into the concurrence of antimicrobial compounds in these families is clearly warranted. Although the presence of choline esters or antimicrobial compounds in hypobranchial secretions of the Trochoidea is unknown, a 6-bromo-2-mercaptotryptamine ion channel inhibitor has been isolated from this taxonomically distant vestigastropod family (Kelley et al., 2003). Furthermore, biosynthesis of 6-position brominated tryptophan metabolites also occurs in the Conidae (Jimenez et al., 1997). The structural similarity of these compounds to Tyrian purple precursors may indicate that the synthesis of bioactive 6-brominated indole-thiol metabolites is conserved throughout the Gastropoda. Although, the biochemical and bioactivity profiles of hypobranchial gland secretions from other representative gastropod families are not currently available, it is likely that the hypobranchial gland represents a virtually untapped source of natural products with biomedical and biotechnological potential.

7. 3 The genetic basis of Tyrian purple biosynthesis and the biomedical potential of muricid natural products

The histochemical approach adopted has provided valuable information on the processes regulating Tyrian purple biosynthesis, which is of significance to identifying the genetic basis of this biosynthetic pathway and hence the sustainable development and production of muricid natural products. In cases where biotic or abiotic factors appear to influence the products of secondary metabolism, it becomes desirable to comprehend how the biosynthetic pathway is regulated at the molecular level (Garson, 2001). The findings of this investigation indicate that both sex and reproductive status affect natural product biosynthesis in *D. orbita*. Investigation into the sex-specific genesis of Tyrian purple (Chapter 2) revealed that male hypobranchial gland extracts yield high concentrations of bromoindirubins, which have been shown to inhibit cell proliferation (Meijer et al., 2003; Magiatis and Skaltsounis, 2006). In contrast, female extracts contain the intermediate precursors, tyrindoleninone and tyriverdin, with reported anticancer and bacteriostatic activity, respectively (Benkendorff et al., 2000; Vine et al., 2007). Consequently, this information is not only valuable for the optimized extraction of novel bioactive compounds from the Muricidae, but subsequent genetic investigations, as biosynthetic genes may be expressed differently depending on sex. Increases in bromoperoxidase staining in egg-laying females and the demand for tyrindoxyl sulphate incorporation into capsules suggests that prochromogen biosynthesis may be heightened during capsule elaboration. Consequently, the up-regulation of genes encoding for bromoperoxidase, and possibly other enzymes thought to be involved in

prochromogen synthesis (e.g. tryptophanase and oxidase), may be correlated with the expression of genes involved in capsule laminae deposition.

To facilitate the total synthesis of muricid natural products for biomedical purposes, suppressive subtractive hybridization (SSH) of mantle and hypobranchial cDNA is currently being applied to sequence the enzymes, bromoperoxidase and arylsulphatase (Laffy et al., in progress). As this technique has revealed over 400 up-regulated genes uniquely expressed in the hypobranchial gland (Laffy et al., submitted), the process of identifying genes specific to Tyrian purple biosynthesis is tedious. The histochemical approach adopted in this investigation has enabled the identification of specific cells involved in fundamental biosynthetic processes, which could be used to increase enzyme sequencing efficiency. Conversion of tryptophan to indole occurs within or on the surface of Type 1 hypobranchial cells (Chapter 5). As tryptophanase is required for the production of indole from tryptophan by gut bacteria in humans (see Allegeri et al., 2006), this enzyme may be present within these cells or adjacent supportive cells. Supportive cells also contain arylsulphatase, of which there may be two types, one involved in the provision of sulphate for prochromogen synthesis and the other in the hydrolysis of prochromogens (Chapter 3). The former of these is most likely interspaced between sulphated mucopolysaccharide secretory cells (Type VII), while in the latter is present in all lateral supportive cells (Chapter 5). Although bromoperoxidase also occurs in these cells, the proposed source of this enzyme is symbiotic bacteria within rectal gland epithelial cells. Due to the known location of these key biosynthetic enzymes, laser capture microdissection (LCM) could be employed to select and isolate these cells for subsequent DNA purification

and amplification. SSH of cDNA could then be applied to facilitate the identification of sequences encoding for biosynthetic enzymes.

The sequences of these biosynthetic enzymes could also be useful in the development of primers for identifying gene clusters, and microarray probes. The formation of genes clusters encoding for enzymatically functional proteins are favored under the pressure of natural selection (Maplestone et al., 1992). As the *de novo* biosynthesis of muricid natural products is a highly evolved process involving a suite of enzymes, one would expect that the genes encoding for this biosynthetic pathway would be present on a chromosome as a cluster devoid of unrelated genetic material (Maplestone et al., 1992). Thus, a process called "primer walking" on genomic DNA (Heiner et al., 1998) could be employed to link the sequences of known biosynthetic enzymes within a gene cluster. This could subsequently be used to identify additional resistance and regulatory genes, such as those encoding for enzymes facilitating methane thiol incorporation into the prochromogen indole ring. This would ultimately aid the isolation and expression of biosynthetic genes for the sustainable development and production of muricid natural products with biomedical potential.

Key biosynthetic enzymes sequences could also be used in the development of microarray probes for biosynthetic genetic analyses. Although commonly focused on gene expression, microarrays have recently been applied for the identification of various marine bacteria (Peplies et al., 2003, 2004), phytoplankton (Metfies and Medlin, 2004; Metfies et al., 2005; Godhe et al., 2007) and fish (Kochzius et al. 2008). In such investigations, 16S rDNA or rRNA oligonucleotides have been used as highly selective microarray probes for species differentiation. Similarly,

complementary open reading frames or entire enzyme sequences could be used as probes to identify the biosynthetic characteristics of other gastropods and marine invertebrates. As bromine (Shaw et al., 1974) and thiol (Munday, 1985, 1989) enhance compound bioactivity, probes based on bromoperoxidase sequences and enzymes involved in methane thiol incorporation, may be useful in identifying species capable of synthesizing natural products with biomedical potential.

Although confirmation by scanning or trans-electronmicroscopy is required, bromoperoxidase localization within symbiotic bacteria could be of great significance to the synthetic production of cytotoxic indoles as anticancer pharmaceuticals. As chemical bromination of indoles typically produces 5-Br and 7-Br compounds (Verhecken, 1989), previous attempts at the total synthesis of 6-bromoindole-thiol have been unsuccessful and economically unviable (Duke, 1974; Vine et al., 2007). Thus, the identification of an endogenous bacterial bromoperoxidase would not only facilitate regiospecific incorporation of bromine into the 6-position of the indole ring, but provide a more economically viable enzyme source. These implications clearly substantiate the benefits of employing a histochemical approach to natural product research.

7. 4 Conclusion

The findings of this investigation clearly demonstrate the importance of understanding the ecological significance of secondary metabolites to progression in natural products research. As our knowledge of marine chemical ecology grows, more rigorous examination of correlations among phylogeny, complementary biosynthetic pathways, defence mechanisms and the evolution of reproductive and

life-history characteristics will be possible. This will not only facilitate the identification of biomedically important lineages, but aid the environmentally sound development and production of marine natural products.