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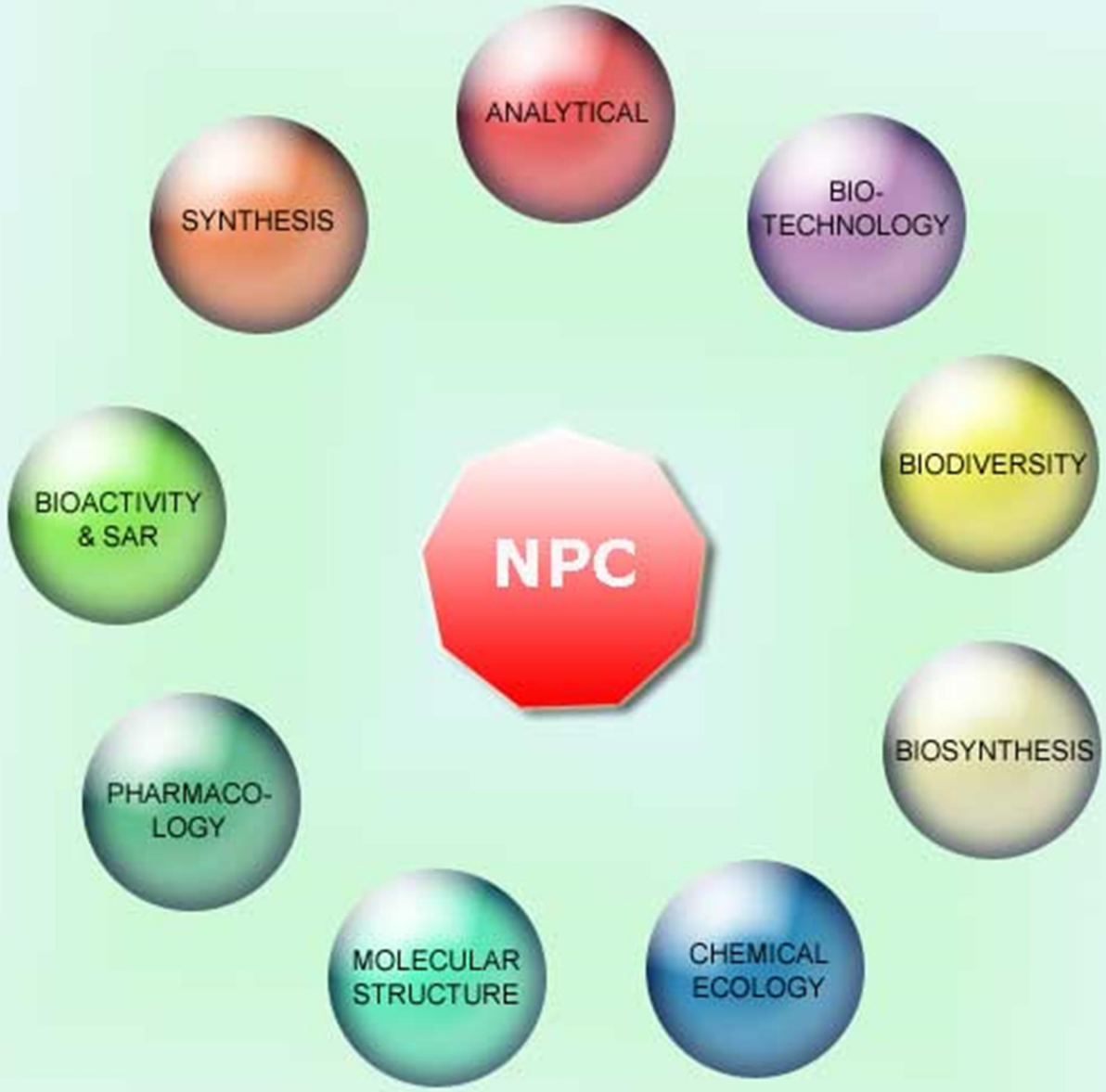

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Establishment of a Phenotypic-based Sand Dollar *Fellaster zelandiae* Embryo Development Assay and its Application in Defining the Structure-Activity Relationship of Discorhabdin Alkaloids

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An embryo development assay using the common New Zealand intertidal species *Fellaster zelandiae* (sand dollar) is presented. The assay was validated by comparing activity profiles of a range of discorhabdin alkaloids, natural products sourced from *Latrunculia* spp. sponges containing a core pyrido[2,3-*h*]pyrrolo[4,3,2-*de*]quinoline tetracyclic skeleton bound to various *spiro*-substituents at the C-6 position. Structural features on the discorhabdin molecule that correlated to the greatest degree of *F. zelandiae* embryo developmental inhibition were the presence of a *spiro*-dienone moiety and a C-2 bromine substituent. Based on the sand dollar embryo development assay results, a mechanism for the activity of the discorhabdin alkaloids is proposed.

Keywords: Sand dollar, Echinoderm embryo development assay, Discorhabdin alkaloids.

The echinoderm embryo development assay is a useful tool for obtaining anti-proliferative information in a short time frame and at a relatively low cost. The model widely used for effluent toxicity testing [1a,b] has also found use as an indicator of cytotoxic potency of small molecules. For example, a member of the pyrrolo*ortho*quinone series, zyzzyanone A, was shown to inhibit cellular division of fertilized sea urchin *Strongylocentrotus nudus* eggs at a concentration of 25 µg/mL [1c]. Moreover, the assay system has also been used as a screening platform for tubulin destabilizing activity of small molecules [1d]. In this work, an in-house echinoderm embryo development assay was established using the sand dollar *Fellaster zelandiae* (Gray, 1855). The species was chosen as the test animal for the assay due to several factors: *F. zelandiae* inhabits shallow benthic communities and can be collected in large numbers at low tide, the species is known to have a long spawning season [1b], and adults can be easily maintained in a spawning condition in laboratory aquaria. *F. zelandiae* exhibits deuterostomus embryonic development. The zygote undergoes first cellular division forty minutes post fertilization and by three hours is at the eight cell blastomere stage. After seven hours, the embryos hatch to yield a swimming blastula. Thirty-two hours post fertilization the embryo develops into a fully grown pluteus larva, with well-defined four arms, primitive gut and skeletal elements (Figure 1).

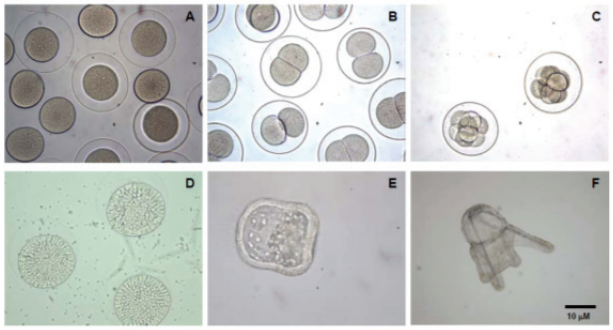

Figure 1: *F. zelandiae* embryo cleavage and development: A) fertilized and unfertilized eggs; B) 2-cell blastomeres; C) 8-cell blastomeres; D) blastulae; E) gastrula; F) pluteus larva.

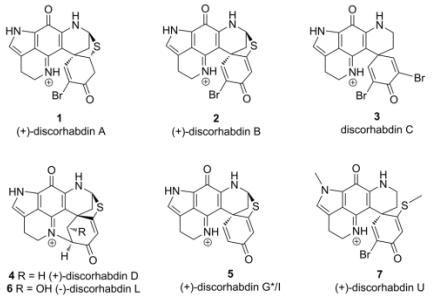

Figure 2: Structures of the discorhabdin alkaloids tested in the embryo development assay.

Our laboratory has an on-going interest in the chemistry and biological activity of the discorhabdin alkaloids sourced from *Latrunculia* spp. sponges. The compounds contain a core pyrido[2,3-*h*]pyrrolo[4,3,2-*de*]quinoline tetracyclic skeleton bound to various *spiro*-substituents at the C-6 position (Figure 2). The class has been reported to exhibit a wide range of anti-proliferative and antibacterial activities [2a-d]. Recently, we have shown that discorhabdin B (**2**) is susceptible to nucleophilic attack by a number of biomimetic thiol and amine nucleophiles and proposed that the observed cytotoxic potency of the alkaloid is due to a combination of electrophilic C-1 and nucleophilic *N*-18 amine functionalities [2e]. Herein, we explore the importance of the substitution on the *spiro*-ring on the discorhabdin skeleton by utilizing a sand dollar embryo development assay to identify the structural characteristics that correlate to the highest anti-developmental effect. Natural products tested in the assay are shown in Figure 2. Preliminary studies using fertilized sand dollar embryos exposed to various concentrations of discorhabdin alkaloids noted that the most dramatic developmental abnormalities were observed within the first seven hours of division, before the onset of differentiation. Figure 3 shows the control embryo seven hours post fertilization at a blastomere stage (A), and three of the abnormalities commonly recorded: inhibition of first cleavage (B), cleavage arrest (C), and irregular differentiation (D). At seven hours post fertilization, the control embryos hatched out of the fertilization membrane whereas the discorhabdin-treated abnormal and undifferentiated embryos were observed to lose structure and rupture into individual cells.

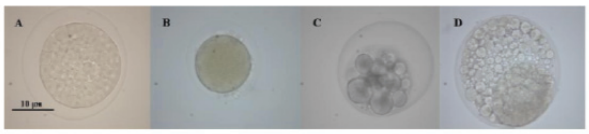

Figure 3: *F. zelandiae* developmental abnormalities recorded at six hours post fertilization A) control (unhatched) blastula, B) zygote, C) 16-cell irregular blastomere, and D) abnormal (unhatched) blastula.

Since this latter effect made identification of single organisms impossible, the discorhabdin treatment assay was terminated one hour pre-hatching, at six hours post fertilization. The readouts were recorded as: inhibition of 1st cleavage, cleavage arrest, irregular blastulae or normal embryo. By noting multiple readouts, rather than a concentration-based IC_{50} value, a morphological profile comparison for each compound at a given concentration was determined. The assay response to compounds **1**-**7** is summarized in Table 1.

Structural features that correlated to the greatest degree of developmental inhibition were the presence of a *spiro*-dienone moiety and a C-2 bromine substituent. At a concentration of 10 µg/mL discorhabdins B (**2**), C (**3**), and U (**7**) showed complete cleavage inhibition, while discorhabdins D (**4**) and L (**6**) were least active. At 2.5 µg/mL compounds **4** and **6** did not cause any developmental abnormalities, whereas **2** at the same concentration completely inhibited embryo third cleavage. These findings were consistent with the results of the P388 anti-proliferative assay, where the *N*-18-C-2 ring closure (as present in **4** and **6**) was shown to drastically reduce the cytotoxic potency of the discorhabdin alkaloids [2b,d]. In contrast to the P388 assay, where **1**, **2**, **3**, and **7** were essentially equipotent (Table 1), the sand dollar development inhibition model was more sensitive to changes in alkaloid structure. Thus compounds **2**, **3**, and **7** with a C-2 bromo substituent and a di-enone moiety were found to be more active at inhibiting embryo development, while the de-bromo analogue **5** and the enone **1** were less active, allowing the embryo to progress to a further developmental stage at all test concentrations. The results of the sand dollar embryo assay clearly demonstrated the importance of the electrophilic C-1 carbon and a good leaving group at C-2 for potent biological activity, consistent with our previous report on the electrophilic reactivity of discorhabdin B (**2**) [2e]. Thus the enhanced P388 activity and anti-developmental effect observed for the Michael acceptor discorhabdin molecules may be due to the ability of the compounds to react with intracellular nucleophilic species such as glutathione or other nucleophilic groups of proteins. Although less active, alkaloids **4** and **6**, which lack electrophilic C-1 and a C-2 leaving group, still exhibited anti-developmental activity in the embryo assay, suggesting the presence of other alternative mechanisms of bioactivity for the discorhabdin alkaloids. The opacity of the embryos, combined with our recently reported bioactive, fluorescently-labeled discorhabdin analogs [2h], will facilitate localization and target pull-down studies, the results of which will be reported in due course.

Experimental

F. zelandiae* assay*: Adult *F. zelandiae* were collected at low tide on Cheltenham beach, Auckland, and kept in a seawater aquarium in the laboratory. Spawning was induced by an intro-coelomic injection of 0.5 M KCl (1.0 mL), and the gametes collected by inversing the sand dollars over 20 mL glass vials overfilled with filtered seawater. Fertilization was initiated by the addition of a single drop of the dense sperm solution and the success measured by the appearance of a fertilization membrane enveloping the zygote 3 min following the addition of sperm. The embryos were added to the test solutions no later than 10 min following fertilization. Test solutions were prepared by the addition of filtered seawater (9.8 mL), a solution of 200-300 fertilized eggs (0.2 mL) and the treatment compound dissolved in deionized water (5 µL), all in a 30 mL test tube. The assay was kept at 20 ± 3°C, at the environmental light regime for 6 h. The assay was terminated by the addition of borax buffered 50% formalin solution (0.5 mL) and the embryos then examined by light microscopy.

The isolation of discorhabdins A, B, C, D, G*/I, L and semi-synthesis of discorhabdin U have been described elsewhere [2d]. Cytotoxicity against the P388 D1 murine leukemia cell line was measured using a standard protocol [3].

Acknowledgments - This work was supported by the University of Auckland. We thank Dr V. Webb (NIWA) for supplying the sponge material and Mrs G. Ellis (University of Canterbury) for P388 assay results.

Table 1: *F. zelandiae* assay response and P388 *in vitro* antitumor activity of compounds **1**-**7**.

Sand dollar							**P388**
Concentration (µg/mL)	**10**	**5**	**2.5**	**1**	**0.5**	**Control** [a]	**IC_{50} (µg/mL)**
1	cleavage arrest	cleavage arrest	irregular blastulae	irregular blastulae	normal embryo	normal embryo	0.11[b]
2	inhibition of 1st cleavage	cleavage arrest	cleavage arrest	cleavage arrest	irregular blastulae	normal embryo	0.08
3	inhibition of 1st cleavage	cleavage arrest	cleavage arrest	irregular blastulae	irregular blastulae	normal embryo	0.11
4	irregular blastulae	irregular blastulae	normal embryo	normal embryo	normal embryo	normal embryo	14.9[c]
5	cleavage arrest	irregular blastulae	irregular blastulae	irregular blastulae	normal embryo	normal embryo	0.65
6	irregular blastulae	irregular blastulae	normal embryo	normal embryo	normal embryo	normal embryo	1.1
7	inhibition of 1st cleavage	cleavage arrest	cleavage arrest	irregular blastulae	irregular blastulae	normal embryo	0.16

[a] Deionized water without test compound was added. [b] value taken from Perry *et al*. [2f]; [c] value taken from Perry *et al*. [2g].

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