THE DISCOVERY AND CHARACTERISATION OF SPLENDIPHERIN, THE FIRST ANURAN SEX PHEROMONE

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1. INTRODUCTION

Over the course of the last decade, our research group has been studying frog skin secretions with a view to isolation of biologically active compounds. The secretions from over 25 species of Australian frogs have been studied, giving rise to over 200 biologically active compounds. To this end, the secretions from *Litoria splendida* have been studied. *L. splendida,* also known as the magnificent tree frog, was first identified in 1977 (Tyler et al., 1977). This species is immediately characterised by the presence of enlarged parotid glands at the rear of the head and the pale sulphur coloured spots with dark edging, scattered over the entire dorsal surface. *L. splendida* averages 10 cm in length, and has large discs on the end of its slightly webbed fingers, extensively webbed toes and orange or yellow flanks.

Litoria splendida is found in a reasonably small geographical area, in the north-western parts of the Northern Territory, and in the north-eastern parts of Western Australia (Tyler and Davies, 1986; Tyler et al., 1994). Specimens collected for these studies were obtained from the Kimberley region of Western Australia.

During these studies, monthly skin secretions were collected from male and female specimens over a period of three years (Wabnitz et al., 1999). The secretions were collected using the surface electrical stimulation method, in which an electrode of low charge is rubbed gently on the dorsal surface of the animal to produce the alarm response. The secretions are then washed off with deionised water, concentrated, and analysed by high performance liquid chromatography (HPLC). This process can be repeated monthly without causing harm to the animal. No animals were sacrificed for this study.

The chromatograms of these secretions indicated a small component present in the secretions from males only. Comparison of the chromatograms from the three-year period show that this 25 residue peptide (GLVSS IGKAL GGLLA DVVKS KGQPA-

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OH) is produced in the highest levels during the mating season, from January to March each year. During this period the peptide constitutes up to 1% of the total secretion material, dropping to as low as 0.1% from June through to November. It was therefore investigated for a possible role in the breeding cycle of this species.

2. BEHAVIOURAL TESTING

Behavioural tests were conducted in a glass tank containing a 2 cm depth of water totalling 1600 mL. Females of the species exposed to the pheromone at a concentration of approximately 10 pM were attracted to the source with remarkably rapid response times. An increase in alertness and change of posture was noticeable twenty seconds after introduction of the pheromone to the tank (Wabnitz et al., 1999; Wabnitz et al., 2000).

When higher concentrations of peptide were used, the female frogs became agitated, as they were unable to locate the source of the pheromone, and climbed the walls of the tank. Lower concentrations of the peptide went unnoticed.

These tests were repeated with eight different females at different times and in different directions, the pheromone being introduced to random ends of the tank, with a 100% success rate, both with the natural compound and a synthetic version.

Behavioural testing with females from a related species, *Litoria caerulea*, and with males from both species gave negative results. The pheromone was not recognised by any test subjects other than *L. splendida* females. The peptide is therefore a species specific male sex attractant pheromone.

Amphibian pheromones have been previously isolated from newt and salamander species, however, this peptide, which we have named splendipherin, is the first pheromone isolated from any anuran species. The delivery method of those previously isolated amphibian species are very clear. Sodefrin, the ten-residue peptide pheromone of *Cynops pyrrhogaster* and silefrin, the ten residue peptide pheromone of *Cynops ensicauda* are both sent through the water by the male newts by a vigorous shaking movement of the tail (Kikuyama et al., 1995; Yamamoto et al., 2000). The 20 kDa proteinaceous male courtship pheromone of *Plethodon jordani* is applied to the female's skin by direct contact (Rollman et al., 1999).

The pheromone identified from *L. splendida* does not have such an obvious delivery method. Diffusion across the distance of approximately one metre would be expected to take hours (Tanford, 1961). Instead, the female frogs found the source of the pheromone in a matter of minutes (Wabnitz et al., 2000). This infers that the molecule is likely to be moving across the surface of the water with a surfactant-like action.

Indeed, studies on the surface behaviour of the amphibian peptides maculatin and citropin show that these peptides preferentially sit at an air-water interface over bulk solution and tend towards α-helical conformation at the interface (E.E. Ambroggio, personal communication).

3. STRUCTURE DETERMINATION

We have determined the structure of splendipherin using nuclear magnetic resonance (NMR) spectroscopy. NMR is an extremely powerful tool in structure determination,

particularly because the solvent system can be chosen to mimic a specific environment. Because we expect splendipherin to be moving across the surface of the water, the solvent used for the structure determination of splendipherin was a 1:1 mixture of trifluoroethanol and water. This solvent is a good membrane and interface mimic.

The structure of splendipherin has been determined using restrained molecular dynamics and simulated annealing calculations. The resultant structure is currently being employed in molecular modelling calculations using the CHARMM package. The structure and mechanism of movement of splendipherin will be published together when this work is complete.

4. ACKNOWLEDGEMENTS

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