Australian cane toad (Rhinella Marina) chemical ecology : the natural degradation and transformation of bufadienolies

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Diplôme : Master en bioingénieur : chimie et bioindustries, à finalité spécialisée
Année académique : 2016-2017
URI/URL : http://hdl.handle.net/2268.2/3090

AUSTRALIAN CANE TOAD (*RHINELLA MARINA*) CHEMICAL ECOLOGY: THE NATURAL DEGRADATION AND TRANSFORMATION OF BUFADIENOLIDES

Michel-Ange Hortegat

Master Thesis presented in order to obtain the Bioengineer Master Diploma Orientation Chemistry and Bio-Industries

Academic Year 2016 - 2017

Supervisors: **Professor François Verheggen**[1] **& Professor Robert Capon**[2]

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ACKNOWLEDGMENTS

What a journey! The last challenge of my study made me pass through a lot of unexpected frustrations (not thank you Australian Immigration!) and push me more than once to the edge. However, if I had to summarise this master thesis, I daresay that it is the people I met on the road that made it 100 % worth it (just kidding, the abstract is on the next page).

I would like to first thank the Professor Capon without whom all this project would have just remained a dream. Thank you to have given me the opportunity to work on a subject that matters to me and I will keep following and supporting the cane toad challenge from my little country. The second person that comes automatically in mind is, of course, the scientist which I have learnt from. My time as a paddawan was enjoyable and I have learnt so much thanks to you. I am really grateful for the time I have spent with you and it is a shame that the distance makes it so complicate now. Good luck on your next adventures Master! Thank you to the all Capon group to have welcomed and counted me as part of the team. Special mention to my bench neighbour, how good is that?!? Thank you to the staff I met at IMB and especially my little Brissy mums, tea times would be missed. I'd like to thank my roomies for the memories; I'll always be a little toady, no worries! How can I say thank you to the one and only Mr Barry? So easy to settle in with you mate! Finally, I cannot speak about Oz without mentioning my second family. Thank you for the support, skype you soon!

This master thesis also marks the fact that I am turning a page on an important chapter of my life. Thank you to the KC for all the memories. The life *en kot* can only be experienced to be fully understood. This little family would definitely be missed. Thank you to the Gembloux family in general for all the funny bloopers, experiences and memories! Keep this ecosystem alive rabbits, it is a precious gem! As much as the student life is about experiencing, it is also about learning. I would never have been able to complete this task without the instructions I received and I would like to acknowledge that. Teaching is a beautiful thing but your job is not always easy so thank you. I close this round with a special person. I will never truly find a way to put in words how important you have become to me. Thank you for your support, especially this last year and your friendship that illuminated my years in Gembloux. We have experienced so much together *maahh tout ça n'est rien à côté de ce que la vie nous réserve encore!*

We then arrive to Brussels. I'd like to first thank my scouts. I find truly amazing that I still have to justify this choice, my commitment to you. You are the next generation that gives sense to what we are doing, to give me hope in a better world. Thank you to give me a sense of purpose. I could not be myself without friendship and I would like to say thank you to all the little stars because even though there are far away, you always shine. Thank you to my family for the support. I can only be proud to be named Hortegat. Finally, yes finally, there is this special person without whom I would be nothing. You have raised me, supported me, had always my back. You are my hero and I hope I would continue to make you proud. Have a good read y'all!

ABSTRACT

The cane toad (*Rhinella marina*) is a performant invasive species that has spread throughout sub-tropical and tropical Australia. Introduced in 1935 in a failed attempt to control agricultural pests, cane toads have since posed a threat to native ecosystems. By a chemical self-defence mechanism, they are poisonous to most Australian anurophagous at all life stages. The cane toad toxins are a complex mixture dominated by bufadienolides, a class of cardiotoxic steroids. The cane toad chemical ecology has become an important part of the fight against this invasive species because it has brought key knowledge that inspired practical control methods. Bufadienolides are principally stored in the form of stable bufotoxins in the adult cane toad parotoid glands. The secretion of parotoid toxins provokes the *ex situ* enzymatic hydrolysis of bufotoxins into more potent bufadienolides called bufagenins. In laboratory conditions, parotoid gland-associated bacteria were found to mediate either the degradation or transformation of bufagenins into oxidised or hydroxylated analogues respectively. However, road-killed cane toads were found to remain toxic to native predators in field studies.

This research project intended to shed some light on the natural evolution of bufadienolides and evaluate the importance of microbiological degradation and transformation within the natural substrate. Firstly, investigations were conducted into the chemistry of parotoid glands of four road-killed cane toads in decomposition. They revealed that bufadienolide profiles first dominated by bufotoxins quickly evolved to the profit of bufagenins in a similar way than for parotoid secretion. The subsequent profiles remained unchanged and no evolution was observed. Only one hydroxylated analogue (11-hydroxymarinobufagenin) was detected but at a constantly low level. Then, parotoid gland-associated bacteria that were previously proved to degrade or transform bufagenins were challenged with marinobufagenin. The results helped describe and differentiate the different microbiological processes: the biodegradation of bufagenins is an oxidation mediated by gram negative bacteria that follows a sequence to produce oxidised scaffolds; the biotransformation of bufagenins is a hydroxylation mediated by gram positive bacteria independently yielding hydroxylated analogues. Finally, strains were isolated from the four road-killed cane toad parotoid glands and challenged with marinobufagenin. Each bufadienolide profile was compared with reference results as to determine if it was originated from microbial degradation or transformation. The comparison confirmed that parotoid glands are a source of degrading and transforming bacteria. Consequently, this research project demonstrated that bufagenins were the agent of the enduring toxicity of road-killed cane toads and remained stable despite the presence of biodegrading and biotransforming bacteria within the parotoid glands. This area of study has still got the potential to bring key knowledge in the general understanding of bufadienolide metamorphosis and inspire new ways to control the Australian cane toad.

Keywords:

Rhinella marina, cane toad, road-killed, bufadienolides, biotransformation, biodegradation

RÉSUMÉ

Le crapaud buffle (*Rhinella marina*) est une espèce invasive qui s'est répandue à travers l'Australie tropical et subtropical. Introduit en 1935 dans une veine tentative pour contrôler des ravageurs agricoles, les crapauds buffles ont représenté une menace pour les écosystèmes natifs. Par un mécanisme chimique d'auto-défense, ils sont toxiques à tout leur stade de vie envers la plupart des anurophages australiens. Les toxines du crapaud buffle sont un mélange complexe dominé par les bufadienolides, une classe de stéroïdes cardiotoxiques. L'écologie chimique du crapaud buffle est devenue un sujet important dans la lutte contre cette espèce invasive car elle a apporté des connaissances clés qui ont inspiré des méthodes de contrôle pratiques. Les bufadienolides sont principalement stockés sous la forme stable de bufotoxines dans les glandes parotoïdes des crapauds adultes. La sécrétion des toxines parotoïdiennes provoque l'hydrolyse enzymatique *ex situ* des bufotoxines en bufadienolides métaboliquement plus actives, les bufagénines. En condition de laboratoire, il a été constaté que des bactéries associées aux glandes parotoïdes catalysaient soit la dégradation soit la transformation des bufagénines en produits d'oxydation ou d'hydroxylation respectivement. Cependant, les études sur le terrain ont montré que les crapauds buffles écrasés sur la route restaient toxiques pour les prédateurs natifs.

Ce projet de recherche visait étudier l'évolution naturelle des bufadienolides et à évaluer l'importance de la dégradation et de la transformation microbiennes au sein du substrat naturel. Tout d'abord, des analyses ont été menées sur la chimie de glandes provenant de quatre crapauds écrasés sur la route et en décomposition. Ils ont révélé que les profils en bufadienolides d'abord dominés par les bufotoxines laissaient rapidement place aux bufagénines de la même façon que pour des sécrétions parotoïdiennes. Les profils obtenus par après restaient inchangés et aucune évolution ne fut plus observée. Un seul produit d'hydroxylation (11-hydroxymarinobufagenine) a été détecté mais toujours à un faible niveau. Dans un deuxième temps, les bactéries associées aux glandes parotoïdes ayant prouvé leur capacité à dégrader ou transformer des bufagénines ont été mises en contact avec de la marinobufagénine. Les résultats ont permis de décrire et de différencier les différentes réactions: la biodégradation des bufagénines est une oxydation contrôlée par l'activité des bactéries Gram négatives qui suit une chaine de réaction pour produire des intermédiaires oxydés; la biotransformation des bufagénines est une hydroxylation contrôlée par l'activité des bactéries Gram positives produisant des produits hydroxylés de manière indépendante. Finalement, des souches ont été isolées depuis les glandes des crapauds buffles écrasés et ont été mises en contact avec de la marinobufagénine. Chaque profil a été comparé aux résultats de référence afin de déterminer s'il provenait d'une dégradation ou d'une transformation microbienne. La comparaison a confirmé que les glandes parotoïdes sont une source de bactéries dégradantes et transformatrices. Par conséquent, ce projet de recherche a démontré que les bufagénines étaient l'agent responsable de la toxicité durable des crapauds buffles morts sur la route et qu'ils sont restés stables malgré la présence dans les glandes parotoïdes de bactéries biodégradantes et biotransformatrices. Ce domaine d'étude a encore le potentiel d'apporter des connaissances clés dans la compréhension générale de la métamorphose des bufadienolides et d'inspirer de nouvelles méthodes de contrôle pour le crapaud buffle Australien.

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ABBREVIATIONS

11hMbg	11α-hydroxymarinobufagenin
12hMbg	12β-hydroxymarinobufagenin
17hMbg	17-hydroxymarinobufagenin
BtH	Bufotoxin hydrolase
BuOH	Butanol
CTRL-	Negative control
CTRL+	Positive control
DAD	Diode array detector
dH_2O	Sterilised dionised water
DNA	Deoxyribose nucleic acid
DP	Potential degradation product
EtoAc	Ethyl Acetate
Gram-	Gram-negative bacteria
Gram+	Gram-positive bacteria
H_2O	Distilled Water
HPLC	High-performance liquid chromatography
m/z	Mass-to-charge
Mbg	Marinobufagenin
MbgPal	Marinobufagenin Palmitate
Mbt	Marinobufotoxin
MeCN	Acetonitrile
MeOH	Methanol
MS	Mass Spectrometer
MWD	Multi Wavelength Detector
Na^+/K^+-ATPase	Sodium-potassium-activated adenosine triphosphatase
NAg	Nutrient Agar
NB	Nutrient Broth
oBlin	$\Delta^{1,4}$-3-oxobufalin
oMbg	3-oxomarinobufagenin
oRbg	$\Delta^{1,4}$-3-oxoresibufogenin
Pregne	Pregnenolone
PrepHPLC	Preparative High-performance liquid chromatography
PS	Parotoid secretion
PT	Stored parotoid toxins
QTOF	Quadrupole time-of-flight mass spectrometer
T	Road-killed cane toad
t	Time point
Tbg	Telocinobufagenin
Tbt	Telocinobufotoxin
Testo	Testosterone
TFA	Trifluoroacetic acid
TP	Potential transformation product
UhMbg	Unknown hydroxymarinobufagenin
UV	Ultraviolet

INTRODUCTION

1.1. INVASIVE SPECIES

1.1.1. A GENERAL CONCEPT

Since the first explorers extended the barriers of our continents, the world economy has progressively grown based on a dynamic of commercial importations and exportations. These movements have led to the displacement of some species from their natural habitat to new environments. Although natural range extensions exist in many species, human activities have recently been associated with many biological invasions carrying plants, animals and microorganisms over greater distances than Mother Nature could do [1,2].

The impacts of biological invasion have attracted the attention of the public over the past half-century. Many examples have been reported of invaders transforming ecosystems and inducing evolutionary pathways by many different types of interaction such as hybridisation, competition, niche displacement, predation, and eventually extinction [1,3–5].

The demand for increased research efforts has grown since certain invaders are considered as pests. The risk assessment associated with invasive species has become a new priority as well [1,6]. Studies have flourished all over the world in recent years bringing with them concepts and definitions. Some ecological reviews have highlighted the confusion that could result from the lack of consensus and the use of simple words such as "exotic" or "naturalised", among others [6,7]. The notion of "invasive species" can, therefore, have divergent interpretations according to the studied organism, the country where the study takes place or the researcher's sensitivity and thus should be defined to avoid any ambiguity.

Based on the definition of the unified framework for biological invasions described by Blackburn *et al.* [4] and the choice to take into account the attributed harmfulness of an invader [7], the words "**invasive species**" will stand in this document for **any non-indigenous species, intentionally or accidentally introduced, that is able to maintain, spread and reproduce to the detriment of any native population and therefore classified as "pest" by the concerned governmental authority**.

1.1.2. AUSTRALIA: A PARTICULARLY AFFECTED COUNTRY

New Zealand, Madagascar or Galapagos are examples of islands that enjoyed millions of years of isolation to develop unique inland ecosystems. Australia is the largest among them and like the others, its insular species count many wonders found nowhere else. Unfortunately, its preserved flora and fauna have suffered since the arrival of the first Europeans back in 1788 [8]. For examples, the country has lost 40% of its original forest area and records 17 extinct mammal species over the past 200 years, which corresponds to 40% of the world's mammalian extinctions over the same period [9].

Deprived of mainland evolutionary pressures for so long, Australia has fulfilled the perfect conditions for the establishment of invasive species. The number of introduced vertebrates on the territory was for example estimated at a minimum of 73 in 2001, along with about 2000 plants [9,10]. The Australian Government takes this issue very seriously because of invasive species' numerous impacts. Besides the threats they pose to biodiversity, they are capable of degrading landscapes and infrastructures. Crops and livestock are often negatively impacted and overall, the economic loss due to the main invasive vertebrates (Table 1) was estimated around AU$ 720 million per annum in 2004 [10]. Australia has therefore put in place an incredible number of resources and measures such as the *Environment Protection and Biodiversity Conservation Act 1999*, *the Threat Abatement Plans*, *the Environmental Biosecurity*, *the National Weeds Strategy*, *the National Landcare Program* or *the Conservation Strategies* to regulate these undesirable populations and to avoid the introduction of new species [8–13].

Table 1 – Main feral vertebrates recognised as invasive species under the Environment Protection and Biodiversity Conservation Act 1999 (adapted from Australian Government Department of the Environment and Energy, 2013)

Species	Origin	Year of Introduction
Feral Cat (*Felis catus*)	Europe	1600s
Feral Goat (*Capra hircus*)	Europe	1788
Feral Horses (*Equus caballus, Equus asinus*)	Europe	1788
Feral Pig (*Sus scrofa*)	Europe	1788
European Rabbit (*Oryctolagus cuniculus*)	Europe	1788
House Mouse (*Mus musculus)*	Europe	1788
Feral Deer (*Axis sp, Dama sp., Cervus sp., Rusa sp.)*	Europe/South Asia	1800s
Water Buffalo (*Bubalus bubalis*)	West and East Asia	1825
Dromedary Camel (*Camelus dromedarius*)	India	1840
Red Fox (*Vulpes vulpes*)	Europe	1855
Cane Toad (*Rhinella marina*)	Latin America	1935

1.2. THE CANE TOAD: FROM A HERO TO A FOE

1.2.1. GENERAL DESCRIPTION

Rhinella marina (Linnaeus 1758), formerly *Bufo marinus*, belongs to the family *Bufonidae* (Table 2), which is distinguished from other anurans by the presence of Bidder's organs located above the testis and the kidneys in males [14]. *R. marina* is the only toad species found in Australia [15].

Figure 1 – Australian *Rhinella marina*, Brisbane 2017

Adult cane toads range in size from 8.5 to 15.0 cm snout-vent length [14] and usually does not exceed 0.7 kg [16], although individuals can be greater than 22.5 cm and weigh over 1.5 kg which put *R. marina* among the largest toads on Earth. Females are generally larger than males. The female skin has a smoother appearance with a lighter yellowish-brown colour although scattered warts and tubercles cover both sexes. The skin is an important organ as it allows water and gas exchange with the external environment of the anurans. The presence of two large self-defence glands onto the back behind the eyes is characteristic as well. They are called parotoid glands and contain the vast majority of cane toad toxins [14].

Cane toads pass through three different life stages like any other anurans: egg, tadpole and postmetamorphic which can eventually be separated into juvenile and adult based on the reproductive capacity. Females lay black eggs in a wide variety of water bodies. The eggs hatch into black tadpoles within 2-4 days. 14-28 days later, those tadpoles metamorphose into juveniles that invest most of their energy in seeking terrestrial refuge and in growth. They reach their maximal size about the time they become adults and can, therefore, spend energy in the search for a partner [17]. At all stages of the cane toad life cycle, the rate of growth and development is temperature dependent [14].

Table 2 – Taxonomic classification of cane toad (adapted from **Australian Government Department of the Environment and Energy, 2017)**

Kingdom	*Animalia*
Phylum	*Chordata*
Sub-Phylum	*Vertebrata*
Class	*Amphibia*
Order	*Anura*
Family	*Bufonidae*
Genus	*Rhinella*
Species	*marina*

R. marina is one of the last animal species to have been voluntarily introduced in Australia. Cane toads are now well established, experiencing an unprecedented expansion over the past 80 years.

1.2.2. A BIOLOGICAL AGENT

In the early 1930s, sugar was one of Australia's major export products, but the economic sector was under threat due to a worldwide sugar depression and the local attack of Frenchi cane beetle (*Lepidiota frenchi*) and Greyback cane beetle (*Dermolepida albohirtum*) on Queensland sugarcane crops. The roots tend to be eaten by the larvae of these beetle species, causing irrecoverable damage [14,17–19]. Pests were a common problem for canegrowers all over the world and their anger put pressure on the competent authorities who were expected to act quickly [17,19,20]. *R. marina* which naturally extends from Central America to tropical South America (Figure 2) was renowned for incorporating beetles in its diet and hold the promise of a biological solution. Specimens from Guyana and French Guiana had already been introduced in four Caribbean islands during the 19th century to protect sugarcane crops [21]. Puerto Rico was the latest country to have used cane toads as biological control agents in the early 20s and held in 1932 the 4th Congress of the International Society of Sugar Cane Technologists. Data from gut contents of Puerto Rican cane toads tend to confirm the benefits of the species. A consensus was found as to test the method on another island – Hawaii – before to generalise the use of cane toads as biological control method [17,19,22]. The introduction operated on the different leading islands were all considered "successful" as cane toads tend to settle in rather easily, the population numbers increasing to an enormous extent in the first years of arrival before to drop and remain at a relatively low level [21].

Figure 2 – Natural distribution of Rhinella marina (taken from Easteal, 1981 adapted from Zug&Zug, 1979)

The first cane toads arrived in 1935 from Hawaii to Australia [17,19,21,23]. Ultimately, this new species was introduced into nine sites along the north Queensland coast in Australia [19,20], as well as about 15 other countries in the Caribbean and Pacific regions [21]. With more recoil, none of the introduced cane toad populations has successfully preyed on the targeted insect pests that they were supposed to control [14].

1.2.3. A PERFORMANT INVASIVE SPECIES

The first toads that trod the Australian soil proved to be completely unsuccessful in controlling cane beetles. Indeed, *R. marina* is a ground-dwelling predator that has little chance to catch the adult beetles that live up in the sugarcane. The beetle larvae would then have been the target if they did not spend their entire lifetime buried in the soil. Despite being supposedly able to eat both, cane toads did not seem to encounter either the grubs or the adult. There is no evidence that risk assessments were conducted prior to cane toad importation and differences between Puerto Rican and Australian beetles seem not to have been considered [20].

The disillusion was even greater when it was realised that cane toads were able to:

- poison and kill native predators;
- poison pets, children and humans;
- prey on native fauna including honey bees (*Apis sp.*);
- compete for food and space with native insectivores;
- host pathogens affecting native frogs and fishes [24].

Cane toads were therefore quickly **considered as pests. In 2005, they were officially considered as threats according to the section 188(4)(a) of *the Environment Protection and Biodiversity Conservation Act 1999*** [11,25].

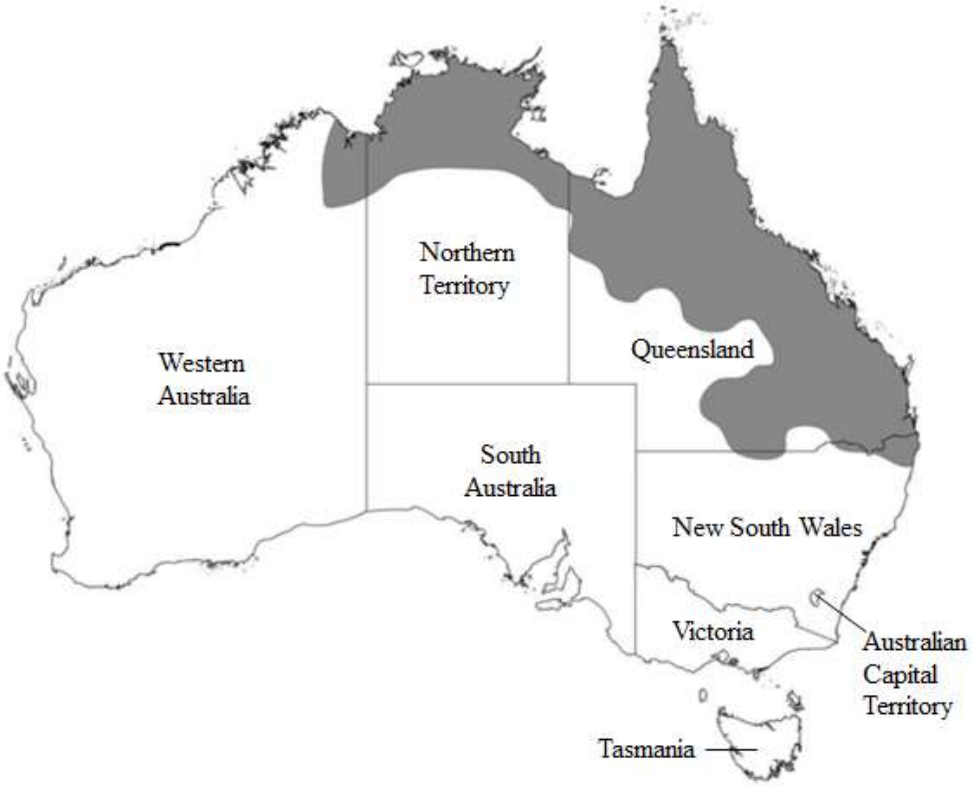

Figure 3 – Approximate mainland distribution of Cane Toads in Australia as of 2016 (adapted from Tingley *et al.*, 2017)

Animals that become established and successful invasive species typically have the following attributes [1,10]:

a) an adaptability to new environments and climatic conditions;
b) a generalised diet;
c) a substantial fecundity rate;
d) few mechanisms of natural control.

Each of these attributes is developed below for *R. marina*.

a) An adaptability to new environments and climatic conditions

Cane toads have proved to be able to prosper in a variety of habitats ranging from sand dunes to rainforest and even mangroves. However, they seem to be more abundant in open clearings which are in contrast to the luxurious tropical forest they are originated from [14]. This incredible achievement demonstrates the flexibility of cane toads which are in addition thought to be able to survive the loss of up to 52.6% of their body water [27] and a salinity level up to 40% sea-water [14]. Although its desiccation tolerance is comparable to other terrestrial anurans [27], Maleek *et al.* report that *R. marina* is more tolerant to saline environments than related species (i.e. *Bufo punctatus*) for all life stages [28].

Tropical and sub-tropical Australia offers a climatic match to cane toads which find in their host country similar conditions that they endure back in Latin America [17,29]. The wet seasons enable them to prosper and the temperatures correspond to their survival range, from 5-40°C [14,27]. Adult cane toads find refuge in holes, crevices and burrows during the day and can remain there for months during the dry season and winter months [14].

Since the time of introduction, cane toad range has ultimately expanded through south and west across tropical and sub-tropical Australia. Cane toads currently cover an area that extends from Queensland to eastern Western Australia passing through Northern Territory and descending as far as north and coastal New South Wales (Figure 3) [30]. Their tremendous rate of dispersion has been facilitated by the use of roads as dispersal corridors [31]. The invasion front can still expand, especially in Western Australia and further down south in Northern Territory [29], and cooler climate will eventually prevent cane toads to invade the entire mainland Australia if no solution is found to stop the conquest.

b) A generalised diet

Cane toad tadpoles eat aquatic plants during the day while juveniles and adults eat ground-dwelling insects and other arthropods during the night. They are particularly voracious and are thought to ingest approximately 200 food items per night [14,25]. They are believed to eat almost any prey of appropriate size and their diet can also include pet food [32], rotting fruits [14] or bird eggs and young offspring [33]. Moreover, cane toads usually eat the gelatinous coating from which they have emerged and often adopt a cannibalistic behaviour towards their conspecific eggs, tadpoles and metamorphs in case of starvation or for genetic competition [32,34–36].

c) A substantial fecundity rate

Cane toads have experienced a demographic explosion. Any time of the year and any particular environment can be suitable for these opportunistic breeders [14,17,25]. While native frogs tend to lay 1000-2000 eggs once a year, cane toads can have one or two clutches per annum of 8000-30 000 eggs [22]. Although tadpole survival is density dependent meaning that all the eggs do not become adults [17], the remaining youngsters only require one year in tropical conditions to reach their maturity [22].

d) Few mechanisms of natural control

Life expectancy of adult cane toads is evaluated at about three years in Australia, three times more than their South American relatives [17]. The first reason for this is that cane toads do not really suffer from predation pressure in Australia. Species of fishes (*e.g. Kuhlia rupestris*), small vertebrates (*e.g. Paraponera clavata*) and reptiles (*e.g. Caiman latirostris, Leptodeira annulata*) are reported to prey upon cane toad eggs, tadpoles and adults in their native distribution range [17]. The anurans toxins affect Australian predators on the contrary of South American species [37]. It can be explained by the absence of a co-evolutionary pathway between *R. marina* and the Australian anurophagous [17]. Although some species of birds (*e.g. Threskiornis molucca, Milvus migrans*), small vertebrates (*e.g. Iridomyrmex purpureus*) and rodents (*e.g. Melomys burtoni, Rattus colletti)* have also been reported to eat cane toads in Australia, they seem to have a very limited impact in regulating the anuran populations [37–39]. Most of the predators tend to avoid the cane toads if they manage to survive their first unfortunate encounter. The second reason is that South American pathogens and parasites were left behind at the time of the introduction and are not able to enter the regulation process. Consequently, when its former and new environments are compared, cane toads seem to have reached an average density about 35 times greater than in South America [17].

e) An extra feature

Cane toads meet all the attributes associated with successful invasive species. What makes these invaders even more peculiar is that part of their population has morphologically evolved to favour sustained long-distance travel [30]. The fastest runners are met at the invasion front and collectively breed, transferring their characteristics to the next generation. The new offspring are ultimately found at the next invasion front and the process repeats years after years, increasing the average speed of each new generation. This evolutionary process is called “spatial sorting” [23,30]. The population bottleneck that introduced cane toads underwent may explain this rapid evolution and the difference with the founding and ancestral populations [21]. Over the 80-year period following their introduction, the cane toad rate of progress has been increasing, from 1-15 km/year in the 1930s to 55-60 km/year currently at the invasion front [16,30]. That record makes *R. marina* the fastest anuran ever been radiotracked [40].

1.2.4. A TOXIC SNACK

As mentioned above, **cane toads are poisonous and toxic to most Australian native predators** [37] **or domestic pets** [41] **that attempt to ingest them**. After the arrival of the invaders on new area, studies reported population declines of Australian anurophagous species including northern quolls (*Dasyurus hallucatus*)[42,43], lizards (*Varanus spp., Carlia gracilis, Tiliqua scincoides intermedia, Chlamydosaurus kingii)* [42,44,45], snakes (*Acanthophis praelongus, Pseudechis australis*) [46,47] and crocodiles (*Crocodylus johnstoni*) [48,49]. Cane toads are toxic during all of their life stages and their eggs and tadpoles represent a threat to native frogs (Hylids, Limnodynastids, Myobatrachids), fishes and turtles (*Chelodina rugosa*) as well [37,50–53].

The protection given by cane toad toxins allows adults to wander in the open and they, therefore, represent an easy prey for an uneducated population of Australian predators [37]. Cane toad toxins are found in skin tissues, bile and plasma but the vast majority are stored in the **parotoid glands.** The sticky white secretion passes through dermal pin hole openings present on the glands and affects the heart and the central nervous system of predators. Cane toad toxins are found to be **rich in bufadienolides**, which are cardiotoxic steroids that act as lethal inhibitors of the sodium-potassium-activated adenosine triphosphatase (Na^+/K^+-ATPase) in the same way as the plant-derived cardenolides such as digitalis. The membrane-incorporated Na^+/K^+-ATPase works like a pump and is essential in the active transport as well as the homoeostasis of sodium and potassium ions across all various vertebrate cells [50,54–56].

Bufadienolides display different profiles in eggs, tadpoles and adults [50] and aim to affect different species as shown above. Although most of the native species are sensitive to bufadienolides, numerous birds, rodents and insects have been reported to tolerate the toad toxins [37–39]. For example, Na^+/K^+-ATPase of insects lacks the binding site for bufadienolides [50] and cane toad cannibalism seem to be possible thanks to a DNA sequence coding for an altered Na^+/K^+-ATPase amino acid profile [19]. Indeed, Na^+/K^+-ATPase exists in multiple isozymes differentiated by variable combinations of subunits isoforms [54]. It is, therefore, simplistic to consider bufadienolides as general toxic agents as it is species (*i.e.* quolls, birds …) and tissue (*i.e.* heart, kidney…) dependent. To date, there are no functional assays that use Na^+/K^+-ATPase from a cane toad predator [57]. Moreover, studies indicate that bufadienolides are presents at traces level in humans and other mammals and are thought to play a role in diverse regulation mechanisms such as renal sodium transport, arterial pressure or cell growth [54,57]. A Chinese traditional medicine (*Ch'an Su*) even uses bufadienolides as active ingredients [58–61]. Toxicity should, therefore, always be considered by which bufadienolides are challenged against which species [62].

1.2.5. Other Ecological Impacts

The toxic ingestion has been highlighted as the major mechanism of cane toad impact but **the aftermath are on multiple scales** and the indirect interactions are more complex to unravel [37]. For example, by hosting indigenous anuran pathogens in one case and parasites on the other, cane toads have been found to respectively increase [63] and decrease [64] native frog infections. Another example is given by their predator-prey relationships where they can be considered as food items for some bird species [39] but predators and nest usurpers for others [33].

Food webs are typically characterised by multiple regulation mechanisms and invasive species such as cane toad can profoundly modify their entire structure, bringing positive outcomes to some species but negative to others [7,65]. Benefits have been reported for species formerly consumed by top predators. It leads to a shift in predator-prey densities by reduction of the top-down regulation [44,48,66]. On the other hand, cane toads have added much more competitions among insectivorous species due to their appetite and densities (see 0). Australian anurans are particularly impacted and competition involves feed, territory and shelter. Moreover, cane toads are found to interfere with native frogs activity levels [67] and sexual communication [68].

1.3. Control Strategy & Methods

1.3.1. The Final Solution: A Failure

The total eradication of the species was the initial goal of the Australian Federal Government and biological controls seemed to be able to bring an enduring broad-scale solution [22,69]. Despite more than AU$ 20 million spent on cane toad research, management and programs development from 1986 to 2009 [69], the cane toad conquest has not stopped nor slowed down as described in 1.2.3. The competent authorities and researchers had to accept that a broad-scale control method would unlikely emerge. As the history of cane toad introduction showed, biological controls can go terribly wrong. Due to many technical hurdles, identified risks and controversy among the general public, most programs were discontinued under recommendations [70]. The considered methods include the use of naturally occurring or engineered viruses, parasitic lungworms, gene-based control strategies and the release of sterile or daughterless males [19,26].

The local communities did not wait for a broad-scale solution and lots of efforts have been displayed in the physical removal of cane toads [71]. However, hand collection cannot alone wipe out the cane toad population for several reasons. First of all, the widespread distribution of cane toads in all sorts of remote environments makes it very difficult for an organised and unified response despite national efforts [19]. Moreover, the cane toad breeding behaviour and high fecundity rate almost ensure that removed toads would always be replaced with new offspring [17]. Subsequently, effective removal would not guarantee a decrease in survival rate as conspecifics are cane toads' main predators [32,34–36]. Finally, the invasion region is colonised by a nomad population and in the hypothetical case where cane toads are wiped out of an area, a new generation would be found at the same place the next night [37].

1.3.2. KNOWING ONE'S ENEMY: CURRENT APPLICATIONS

The focus over the last ten years was to **learn more about cane toad physiology, chemical ecology and mechanisms of impact in order to use that knowledge to ensure the protection of native species and ecosystems** [24]. The shift in strategy as described in the 2011 National Threat Abatement Plan [69] has allowed new control methods to emerge and fulfil different objectives. A better management of the cane toad invasion is thought to come through the combination of different approaches [22,26]. Physical removal is more and more integrated with other approaches maximising field results. A broad range of control methods are now available or in development [26] and do not only focus on reducing toad population size. Some approaches try to monitor toad spread such as the automated cane toad call detection or environmental DNA sampling [26,72]. Those kinds of techniques allow an early detection of incursions when used in sensible cane toad-free environments and maximise the success of rapid eradication. Other methods focus on mitigating toad impacts on ecosystems [26] such as the conditioned taste aversion which uses "toad aversion" baits to teach toad-naïve predators to avoid cane toads [73].

Cane toad weaknesses are found in its biology and a better related knowledge help optimise control methods that target those flaws. The deployment of fences to exclude cane toads from waterbodies comes from the realisation that anurans are subject to hydric stress for example [74,75]. Interestingly, several recent and original methods have sprung from in-depth biology understanding, turning particular aspects of cane toad ecology against themselves [26]. For example, adult traps have been enhanced by the use of olfactory, luminous and acoustic attractants that play an ecological function in cane toad mating or hunting behaviour [76]. Similarly, bufolipins, bufadienolides of the cane toad eggs, were found to specifically attract tadpoles towards conspecific eggs and thus play a fundamental role in the toad cannibalistic behaviour. Therefore, using synthesised bufolipins from adult secretion as baits considerably enhances tadpole traps [34,36]. Other examples are given by the discoveries of tadpole substances [77] and alarm cues [47] that interfere with the development of conspecifics in a context of intraspecific competition. Reducing the size of cane toads could be interesting as some invertebrates such as the Australian meat ants (*Iridomyrmex reburrus*) have been found out to attack and kill small cane toad metamorphs [18].

A detailed account of the control methods that exist or set to appear is held in the recent review of Tingley *et al.* [26]. Although most of the recent approaches hold some promise, there are still barriers that block the way to method success or implementation. A new control method must always prove its effectiveness and its specificity before being accepted. Most of them require substantial investment of resources and time to have a long-term impact. Communities, authorities as well as stakeholders are usually very cautious. Techniques involving the use of genetic tools are yet to win the public opinion for example [26,70]. Without an overall support, none of the control methods could be implemented. A constant and improved knowledge of the cane toad biology as well as a clear communication between involved parties remain the way to drive away those concerns and progressively change the outcome in the cane toad story.

1.4. CHEMISTRY & MICROBIOLOGY OF PAROTOID GLANDS

1.4.1. THE PAROTOID SECRETION

As mentioned earlier, the poisoning of native predators is the cane toad major ecological impact [37]. Studies of cane toad toxins have revealed a complex chemical diversity [36,50,58,60,78,79]. For example, the early Capon group chemical analysis determined that cane toad toxins contain 50 to 100 bufadienolides across all life stages [62].

As described in 1.2.4, **bufadienolides** are present in abundance in the sticky white secretion of cane toads and are responsible for the lethal inhibition of Na^+/K^+-ATPase, thus, have cardiotoxic properties [54,57,59]. They are grouped as cardiac glycosides along with the cardenolides, such as plant derived ouabain and digitalis. Bufadienolides are found in various plants (*e.g.* Crassulaceae, Iridaceae, …) and animals (*e.g.* snakes *Rhabdophis spp.*, fireflies *Photinus spp.*, …) [54,55,57,59,80]. They are characterised by a steroidal structure (Figure 4) with a characteristic junction of a α-pyrone ring at C-17 (D ring). They are, therefore, related to others steroids such as testosterone, progesterone and cholesterol, those two last known to be precursors of cardenolides [54,57,80]. The steroidal rings enable the ultraviolet (UV) detection, characterised for bufadienolides by a maximal intensity at 298 nm thanks to the pyrone ring [80,81].

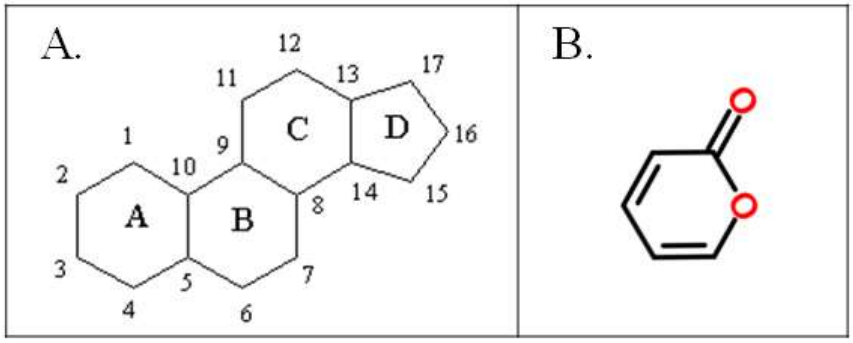

Figure 4 – (A) Steroidal structure and (B) α-pyrone ring

Bufadienolides are heat-stable [82] and hydrophobic [62]. In-depth analysis revealed different C-3 (A ring) conjugated forms among bufadienolides, each differing by the chemical groups added to the main steroidal structure (*i.e.* sulphates, carboxylic acids, amino acids, …) [57,60,81]. The two main bufadienolides sub-classes reported in Australian *R. marina* secretion are **bufagenins and bufotoxins** [58,60,61,78,83]. The first is characterised by the presence of a hydroxyl in C-3 while the second is differentiated by a suberoyl-L-arginyl at the same place. Chemical analysis of parotoid secretion revealed a bufadienolide composition dominated by **marinobufagenin (Mbg)**, followed by less than ten other bufagenins and bufotoxins at moderate or lower levels, the rest being minor bufadienolides at only detectable levels [58,78,83].

Bufagenins are the unconjugated forms of bufadienolides, having a hydroxyl in C-3. They are the major sub-class of bufadienolides in the cane toad secretion [58,60,61,78,83]. Bufagenins can pass through animals' mucous membranes to bind heart and blood vessels Na^+/K^+-ATPase leading to cardiovascular constriction and failure [54,83]. Mbg is the main bufagenins in the parotoid secretion [58,60,61,78,83]. Apart from Mbg, moderate levels of telocinobufogenin (Tbg; also known as telocinobufagin), bufalin and arenobufagenin have been reported as well as low levels of resibufogenin (also known as bufogenin) and trace levels of hellebrigenin (also known as bufotalidin) (Figure 5) [58,78,83].

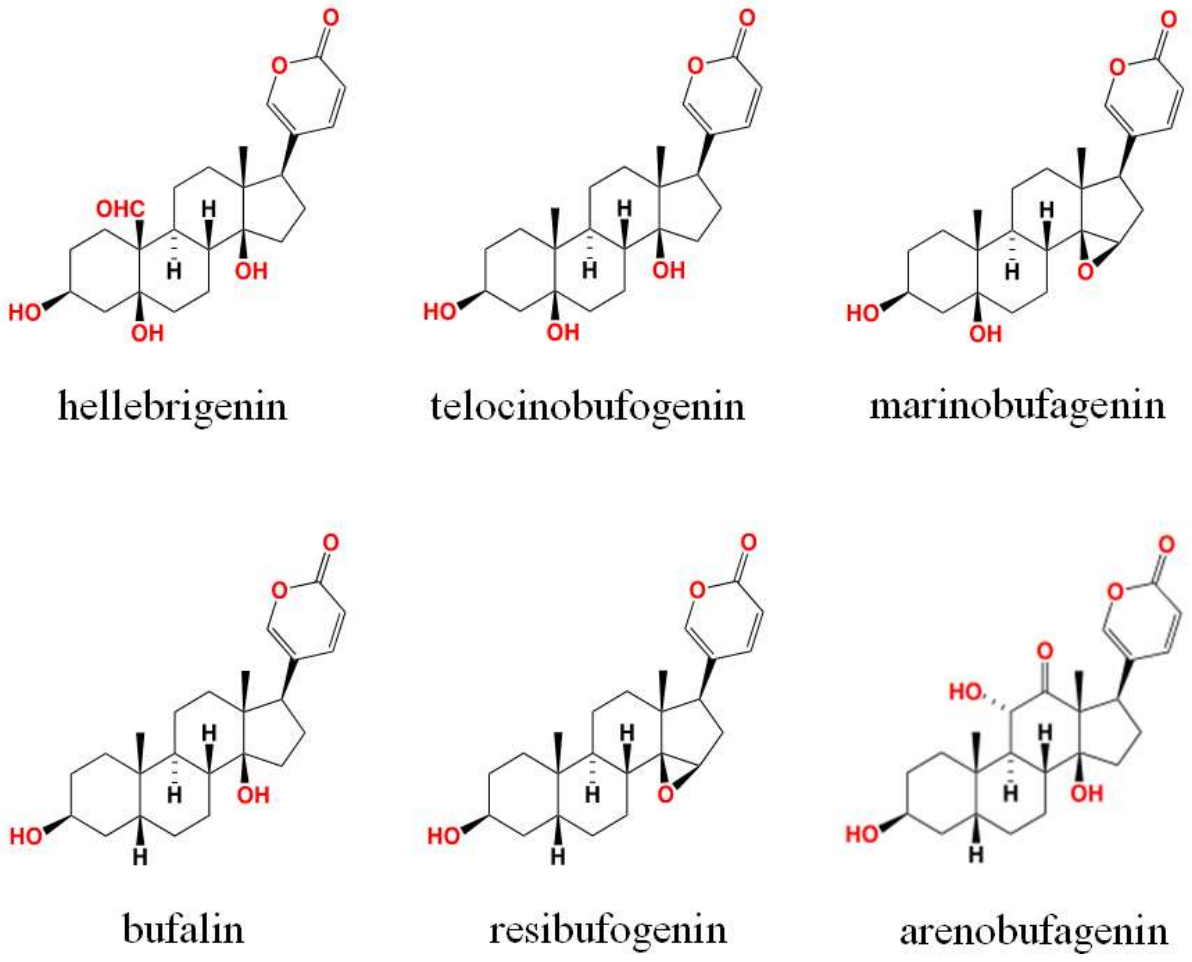

Figure 5 – Bufagenins found in Australian *Rhinella marina* parotoid secretion

Bufotoxins are one of the C-3 conjugated forms of bufadienolides, a suberoyl arginine replacing the hydroxyl present in bufagenins by esterification. They are reported in minor and variable quantities than bufagenins are in cane toad parotoid secretions [58,78,83]. Relative toxicity of bufotoxins is lower than bufagenins [83]. Although bufotoxins are not always reported [60,61], chromatograms have revealed three different forms in *R. marina* secretion: marinobufotoxin (Mbt), telocinobufotoxin (Tbt) and bufalitoxin (Figure 6). Similar C-3 conjugated forms of bufadienolides (arginine-pimeloyl esters) were also identified (Figure 6) [58,78,83].

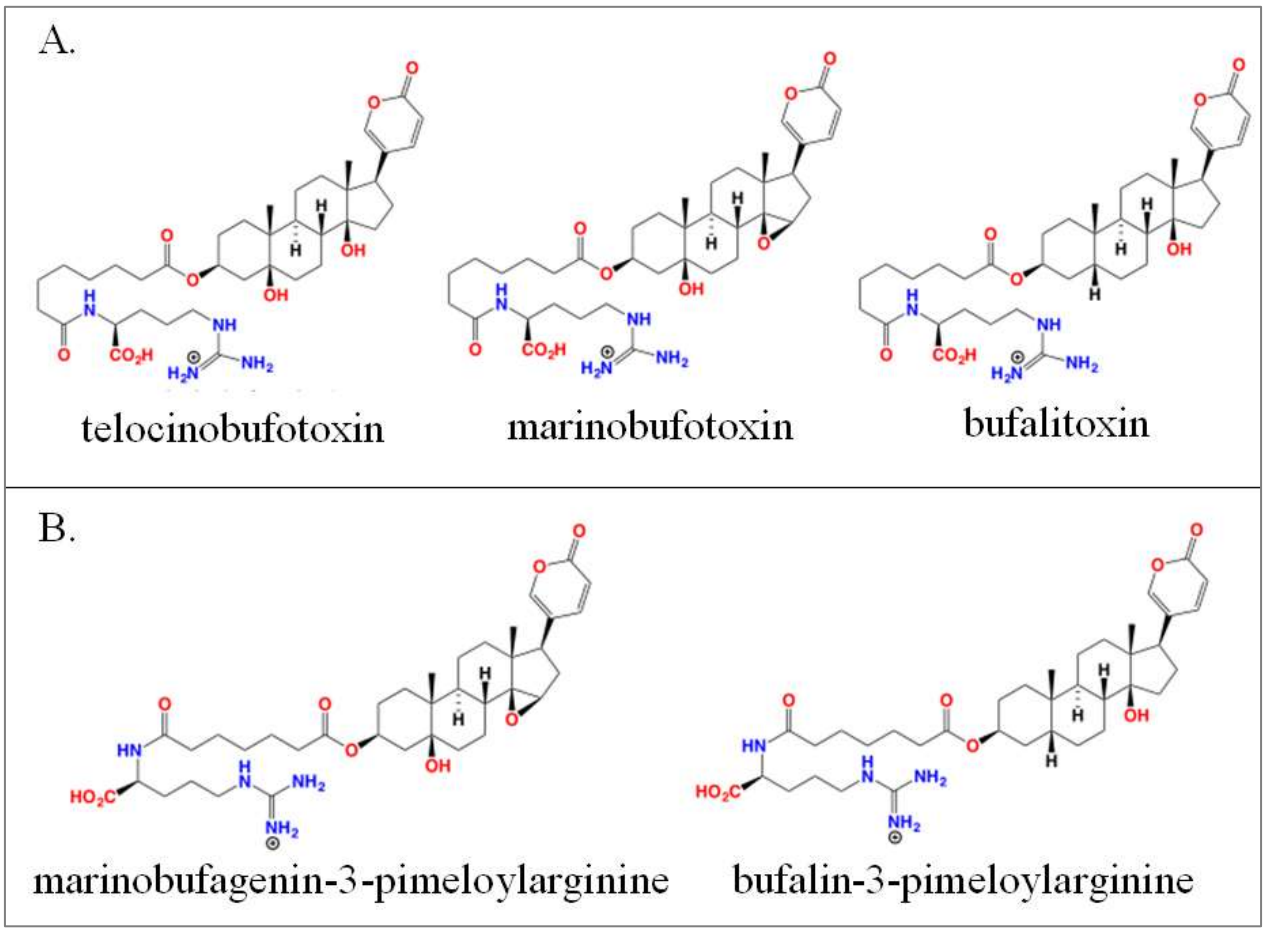

Figure 6 – (A) Bufotoxins and (B) arginine-pimeloyl esters found in Australian *Rhinella marina* parotoid secretion

Free arginyl amides were also reported in parotoid secretion by mass spectrometry [58,60,83]. The presence of suberoyl-L-arginine and pimeloyl-L-arginine (Figure 7), confirmed the theory that bufagenins derive from bufotoxins and is explained here below in 0. It has to be noted that suberoyl-L-arginine is the dominant arginyl amides and that pimeloyl-L-arginine were only present at trace level if detected. Arginyl amides do not exhibit any signs of cytotoxic activity on the opposite of bufadienolides [83]. Derived dicarboxylic and amino acids were logically found in water extracts as well [83].

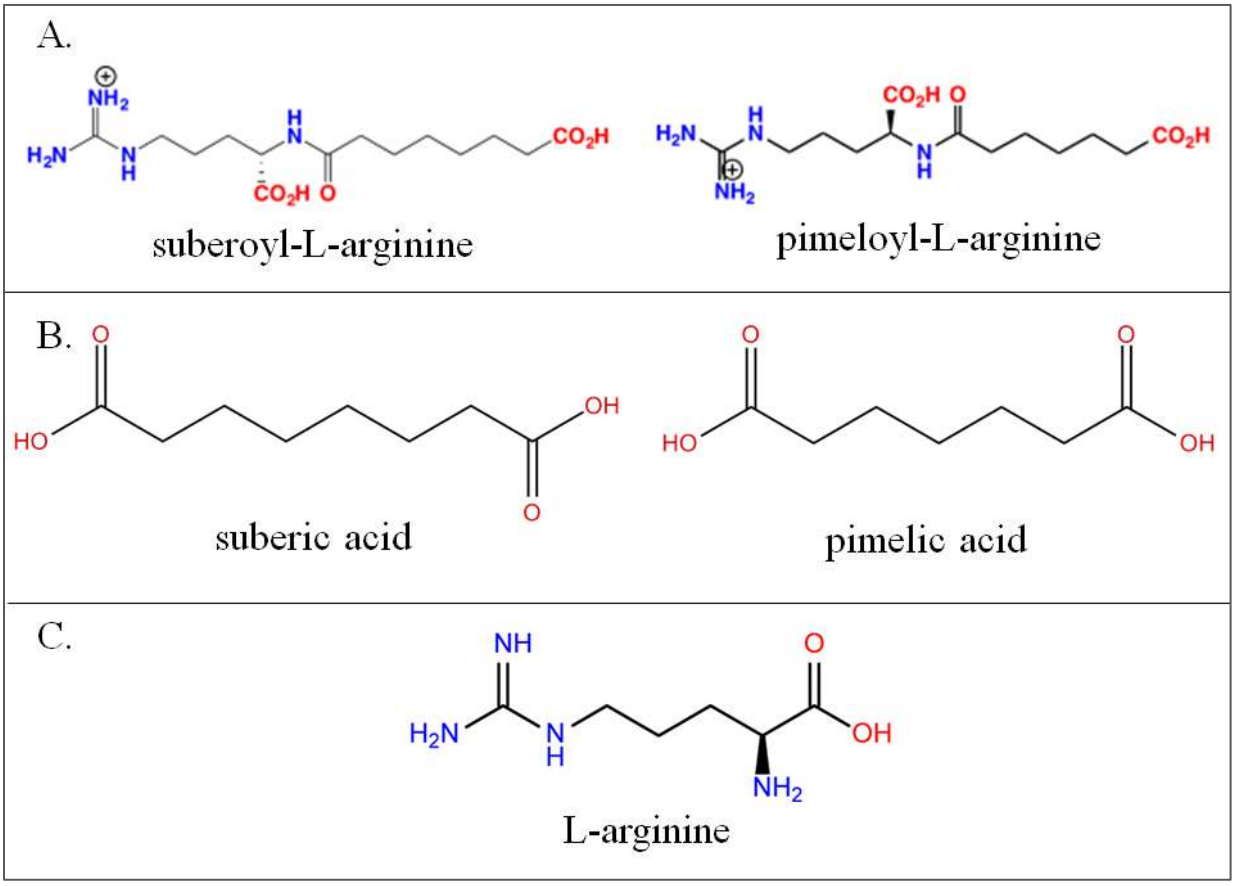

Figure 7 – Examples of (A) Arginyl amides, (B) dicarboxylic acids and (C) amino acid present in Australian *Rhinella marina* parotoid secretion

Other main classes found in the cane toad parotoid secretion include **catecholamines**, such as adrenaline, noradrenaline or dopamine, and **indolealkylamines** such as bufotenine, dehydrobufotenine or serotonin (Figure 8) [57,60,83]. Those biogenic amines are thought to increase the cardiotonic effect of bufadienolides by their activity on the heart and are known to be potent neurotransmitters. Hallucinogenic and other psychotropic effects are assigned to bufotenine and dehydrobufotenine for instance [56,57,84].

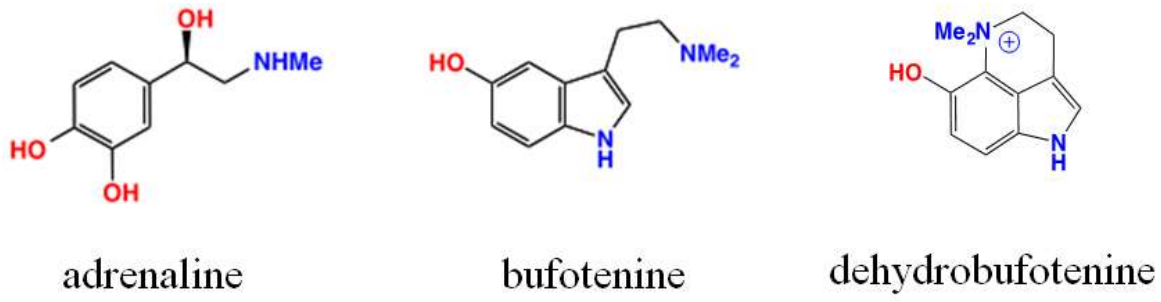

Figure 8 – Examples of biogenic amines found in Australian *Rhinella marina* parotoid secretions

1.4.2. THE STORED PAROTOID TOXINS

As mentioned above, the presence of arginyl amides in the parotoid secretion hints that there is a difference in the chemical composition of cane toad toxins within and out of the parotoid glands. The similar chemical structure suggests that **bufotoxins are precursors of bufagenins and undergo a hydrolysis** which explains the presence of arginyl amides in the parotoid secretion (Figure 9). The work of Dr Kamalakkannan revealed the presence of an **enzyme catalysing the reaction: the bufotoxin hydrolase (BtH)** [83]. This key discovery has cleared up the confusion that dominated cane toad toxins storage and release.

The parotoid glands are composed of about 100 microglands where parotoid toxins are stored mainly as bufotoxins. BtH is stored in other glands localised in the apex of microglands duct and is consequently not in contact with bufotoxins. During a predatory attack, glands compression provokes the release of *in situ* parotoid toxins and the co-secreted enzyme. BtH can, therefore, enter in contact with bufotoxins and mediate the hydrolysis (Figure 10) [83]. The reaction is a rapid process as about 95% of Mbt is converted into Mbg 5 min after secretion [83]. Methanol (MeOH) inactivates BtH and is, therefore, a solvent of choice to isolate bufotoxins. Cane toad microglands and secretion extraction into MeOH have made it clear that parotoid toxin is principally composed of bufotoxins with Mbt dominating and Mbg at low levels [83]. **The various bufotoxin forms come from oxidation-reduction reactions and give the respective bufagenin analogues after hydrolysis**, Mbt and Mbg being respectively the major bufadienolides of parotoid toxins and secretion [83].

Cane toads find several advantages in the use of a pro-toxin mechanism as they stored bufotoxins in parotoid glands, skins and ovaries as well [36,81]. First of all, bufagenins are reported with a greater cytotoxic activity than bufotoxins [57,83]. It is therefore favourable for the toad to store its toxins under less potent chemical forms. Then, the guanidine of the suberoyl arginine present on bufotoxins forms a hydrophilic region that allows the formation of micellar structures [83]. This kind of configuration gives the bufotoxins a physical and chemical stability. For example, apart from BtH and a hog pancreatic lipase [61], no other body fluids (*e.g.* saliva, plasma …) can trigger the hydrolysis of bufotoxins [83]. The accumulation of large amounts of bufadienolides within the parotoid glands (50 - 250 µg of Mbt per microgland) is possible thanks to the physical and chemical stability. Lastly, bufotoxins benefit from a microbiological stability and Dr Kamalakkannan's work suggests that bufotoxins are resistant to fungal and microbial degradation [83]. The microbiological stability of bufagenins contrasts with bufotoxins' one and is developed here below.

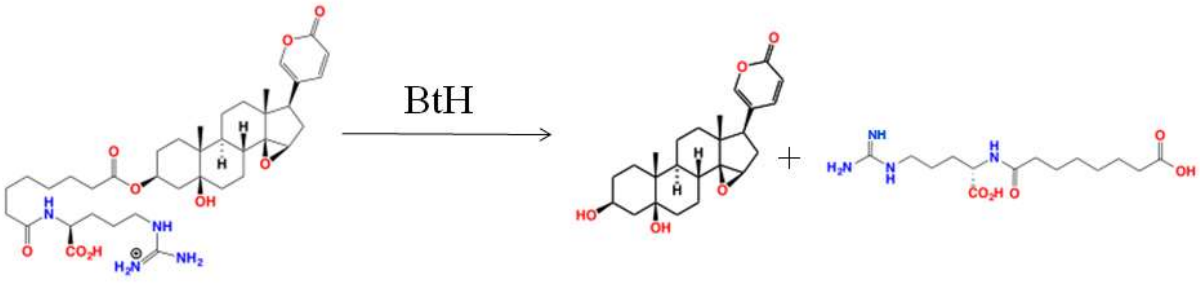

Figure 9 – Hydrolysis of Mbt into Mbg and suberoyl-L-arginine

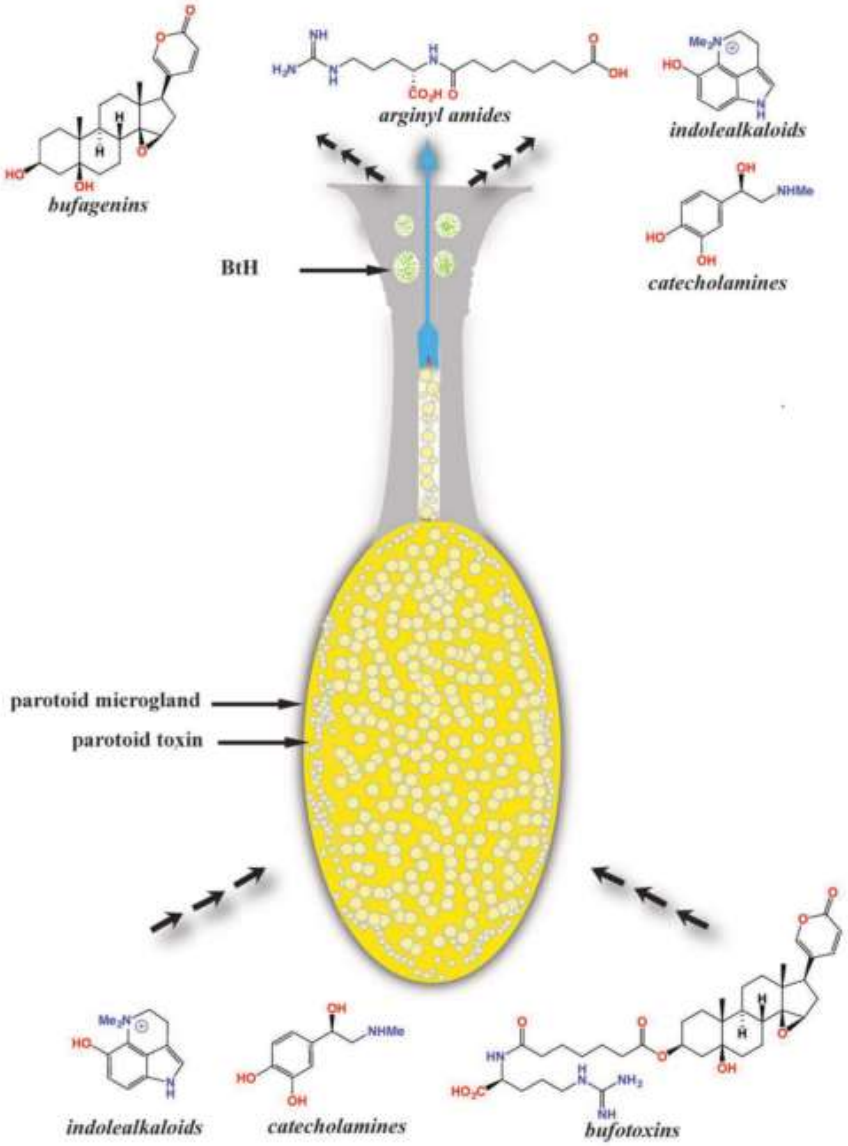

Figure 10 – A diagrammatic representation of storage and release of parotoid toxin from a parotoid microgland (taken from Kamalakkannan, 2014)

1.4.3. THE MICROBIAL TRANSFORMATION

As previously stated, Australian *R. marina* toxins contain a broad range of bufadienolides (50-100). The chemical diversity is greater in cane toad eggs and ovaries [50] with the presence of other bufadienolide sub-classes such as bufolipins [36,83]. This diversity certainly gives an ecological advantage to cane toads that can find among their toxins specific antagonists for a wide array of Na^+/K^+-ATPase isoforms, thereby, improving the lethality of their toxins against different predatory species [50,55,62,78,81,85]. The diversity among bufagenins can be partly explained by oxidation-reduction reactions underwent by bufotoxin precursors, but cannot cover them all.

Deprived of the diacid arginyl amide, bufagenins are more susceptible to microbial attacks [83,86]. For example, hydroxylated products have been isolated when the fungus *Mucor spinosus* is challenged with bufagenins [59,87,88]. In the cane toad context, mono and polyhydroxylated analogues are found in eggs [89] as well as ovaries [81]. Evidence suggests that **biotransformations were mediated by the activity of gram-positive bacteria (Gram+)** [83,86]. Dr Kamalakkannan isolated three monohydroxylated products obtained from the biotransformation of Mbg by *B. cereus* [86]: 11α-hydroxymarinobufagenin (11hMbg), 12β-hydroxymarinobufagenin (12hMbg) and 17-hydroxymarinobufagenin (17hMbg) (Figure 11). By transforming bufagenins, Gram+ associated with cane toads certainly have a diversifying role at an ecological scale, giving a stronger protection to cane toad eggs.

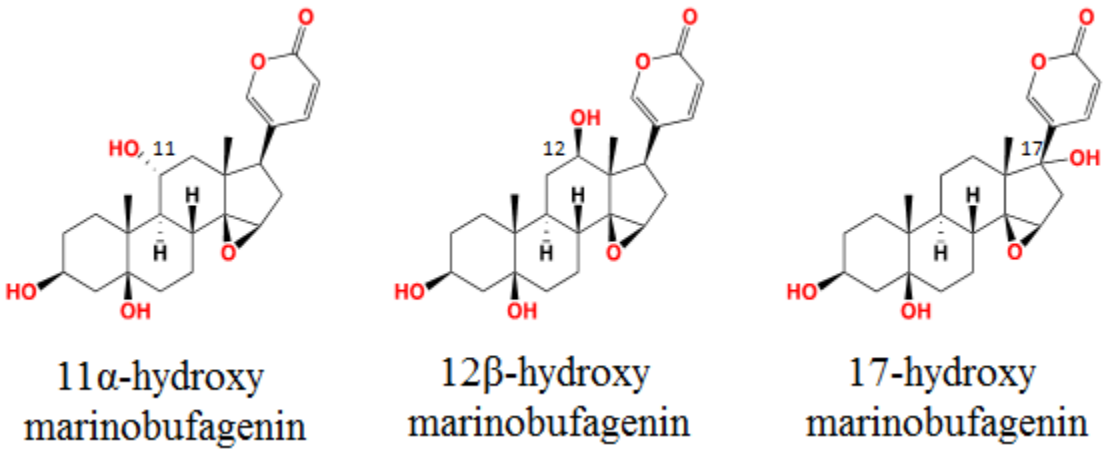

Figure 11 – Hydroxylated products from the microbial transformation of Mbg by *Bacillus cereus*

1.4.4. THE MICROBIAL DEGRADATION

Similarly, some **parotoid gland-associated gram-negative bacteria (Gram-)** such as *Comamonas testosteroni* and *Flavobacterium sp.* were found out to **degrade Mbg** [78,83]. The identified degradation products include 3-epi-marinobufagenin, 3-oxomarinobufagenin (oMbg; also known as 3-dehydromarinobufagin), $\Delta^{1,4}$-3-oxoresibufogenin (oRbg) and $\Delta^{1,4}$-3-oxobufalin (oBlin; also known as helleborogenone) (Figure 12).

The discovery of Gram- ability has brought hopes that they could be used to infect and detoxify cane toads through the *in vivo* degradation of bufadienolides [62]. It seems that they mediate the oxidation of bufagenins, which could be opportunistic or part of a biological function [78,83]. At the difference of the microbial transformation, the bufagenins bio-oxidation follows a chain process and Hayes *et al.* proposed a degradation pathway (Figure 13). The conversion to oBlin requires the reduction of oRbg, not its oxidation. Another Gram-, *Acinetobacter johnsonii*, has been shown to be able to degrade Mbg in a similar way and was isolated from cane toad ovary which reinforces the hypothesis of an ecological relation between bacteria and cane toads [78].

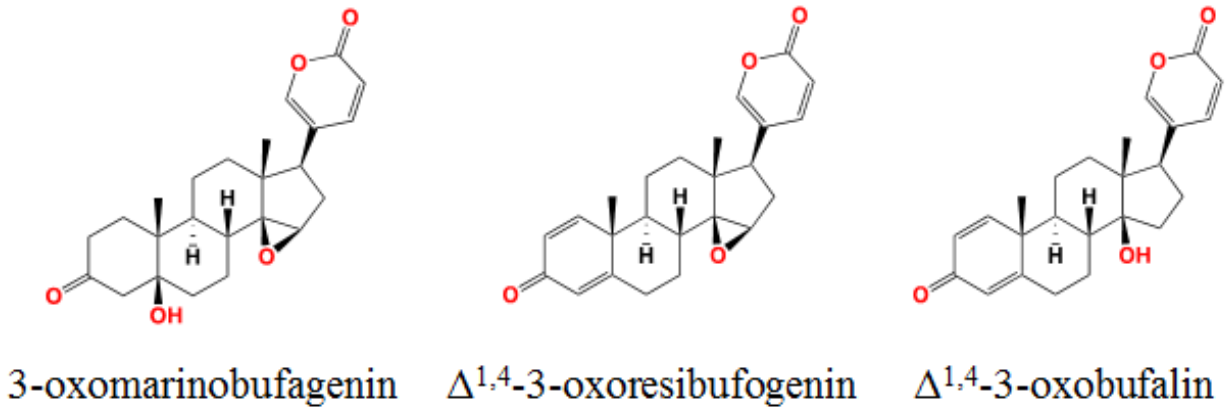

Figure 12 – Oxidised products from the microbial degradation of Mbg by *Comamonas testosteroni*

Little is known about the natural degradation of bufadienolides and the observed biotransformation and biodegradation represent the first accounts of transforming and degrading strains within anuran species [62,78,86]. Bacteria could play a major role in the degradation of bufadienolides and thus loss of cardiotoxic properties. However, Crossland *et al.* revealed that desiccated cane toads found on roads still had the ability to poison and kill native species of frogs *(Cyclorana australis, Litoria rothii)*, fishes *(Mogurnda mogurnda)* and leeches *(Family Erpobdellidae)* [82]. **Those intoxication cases imply that cane toad toxicity does not end with death, even after many months under the Australian tropical sun. Given what is known about parotoid gland-associated bacteria, the cane toad enduring toxicity certainly underlines the remaining gaps in the comprehension of the bufadienolides transformation and degradation.** The recent explorations in the cane toad chemical ecology have unveiled mysteries and lead to the development of several original control methods (*e.g.* tadpole traps).

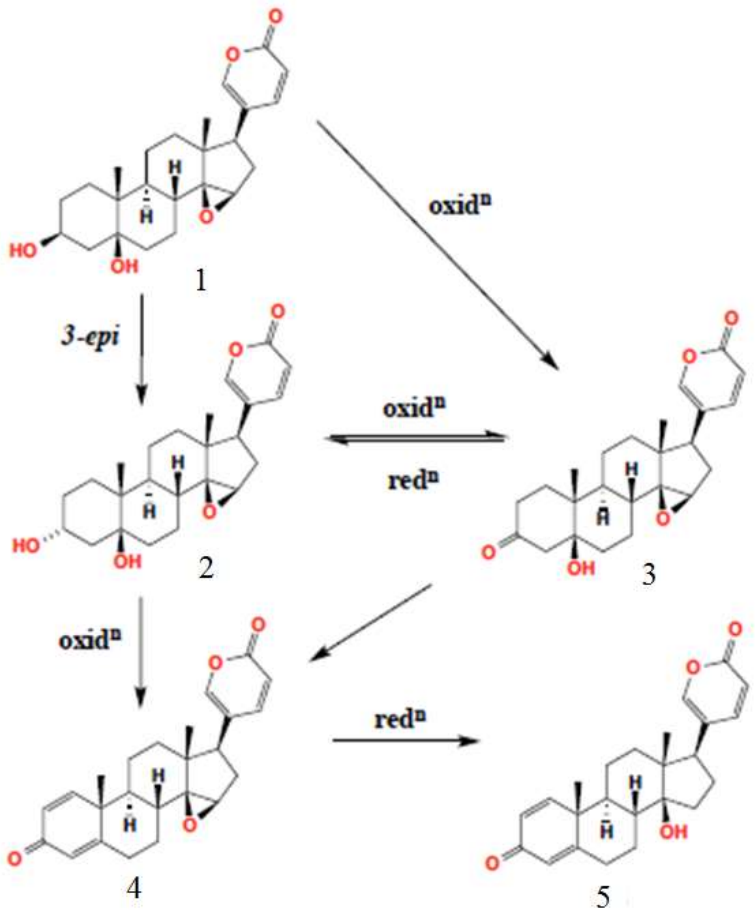

Figure 13 – A possible sequence of degradation of Mbg (1) under the action of *Comamonas testosteroni*, resulting in oxidised products 3-epi-marinobufagin (2), oMbg (3), oRbg (4) and oBlin (5) (adapted from Hayes *et al.*, 2009)

The discovery of BtH has enabled understanding that a compression applied on parotoid glands provokes the secretion of *in situ* bufotoxins which is then converted into bufagenins *ex situ*. If they pass through animals' mucous membrane, bufagenins bind to Na^+/K^+-ATPase. If they do not, the story currently ends here as the way to complete degradation of bufagenins remains unclear. Despite the assumption that bufagenins are less stable than bufotoxins, the remaining toxicity of cane toad carcasses suggests that there are intermediate steps to finalise degradation. The presence of parotoid gland-associated bacteria able to either degrade or transform bufagenins supports that theory as well as a strong relationship between bacteria and cane toads. The bacterial role might be even more important outside the parotoid glands and could help understand what happens to bufagenins once subject to the environment. Bufadienolides natural transformation and degradation is an area of study that certainly needs to be investigated if it is to inspire ways to control Australian cane toads.

OBJECTIVES & EXPERIMENTAL STRATEGIES

Based on the information described above, the goal of this research project was to **investigate the natural degradation and transformation of bufadienolides**. A better understanding of those processes could help answer key questions in the cane toad chemical ecology, enlighten the changes in bufagenins and inspire the development of microbiological control methods.

In the scope of this study, it was decided to investigate the chemistry and microbiology of **road-killed cane toads**. Indeed, working on roadkill offers many advantages since (i) the concept of decomposition is usually associated with the idea of natural degradation [90]; (ii) they are found in abundance on roads, which are used as dispersal corridors that facilitate dispersion [31]; (iii) the compression under a motor vehicle tyre mimics a predatory attack and provokes the release of parotoid BtH [83]; (iv) it alleviates ethical concerns about ways to humanely kill cane toads [71]; (v) it proves to be a convenient and more frequently used route to access microbiome bacteria and natural products [91].

Three distinctive objectives were pursued as to reach the goal of the research project:

1) **Assessing the evolution of bufadienolides levels within cane toad parotoid glands in decomposition;**

2) **Determining the process and specificity of the microbial degradation and transformation;**

3) **Confirming that cane toad parotoid glands contain bacteria that are naturally optimised for the degradation or transformation of bufadienolides.**

Specific experimental strategies were designed as to meet the objectives.

The hypothesis to consider for the first objective was that **bufagenins level would shoot up as a result of the bufotoxins hydrolysis under the action of BtH, then, would progressively decrease through time to the benefit of hydroxylated and oxidised analogues as a result of the bacterial action** (Figure 14). Other degradation products could potentially appear. Parotoid glands from road-killed cane toads should provide appropriate substrates. Several individuals would help generalise the results to the species level. The rest of the experiment would demand to extract the bufadienolides and freeze the transformation/degradation process prior to their analysis through analytical systems such as HPLC, mass spectrometer (LC/MS) and eventually quadrupole time-of-flight mass spectrometer (Q-TOF LC/MS).

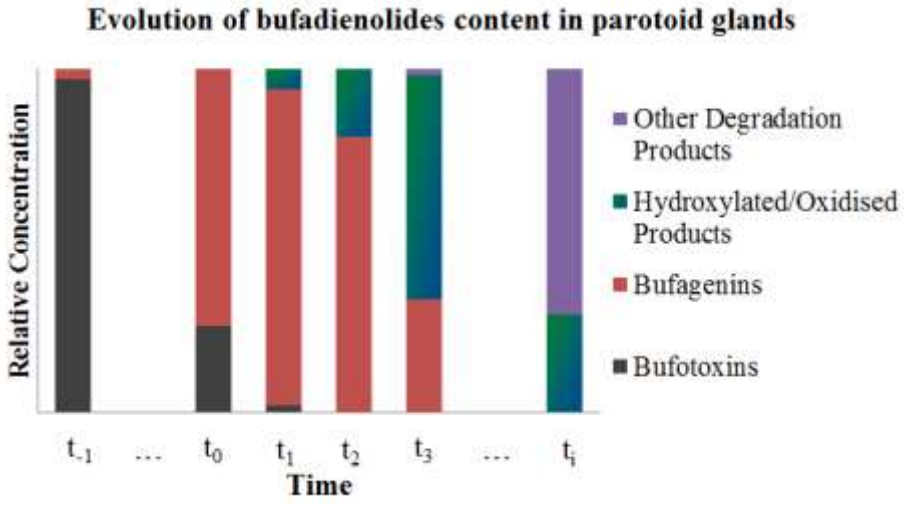

Figure 14 – Hypothesis on the relative evolution of bufadienolides content in road-killed cane toad parotoid glands; t_{-1} represents the situation before death, t_0 - t_i represent different time points after car collision

Considering the second objective, parotoid gland-associated bacteria previously isolated on living cane toads would be cultured and challenged with Mbg as to make sure that they have kept their degrading/transforming ability and to select the most representative Gram- and Gram+ for future positive controls. The remaining bufagenins would be extracted prior to their analysis through HPLC-DAD system. **The evolution of the different profiles would help describe the process of transformation/degradation.** Representative Gram- and Gram+, known to degrade or transform bufagenins, would also be challenged with common and peculiar steroids to assess the specificity of the biodegradation and biotransformation. The rest of the experiment would require the steroids extraction and analysis through analytical systems stated earlier.

For the third objective, parotoid glands in advance phase of decomposition from road-killed cane toads should host active microbiota and provide such strains. Several toad individuals would help assess the results in a species level context. The assumption here being that **roadkill-associated bacteria would be better suited to degrade or transform bufadienolides** than the ones previously identified on living cane toads. Strains from the rotten parotoid glands would be isolated and cultured before being challenged with bufagenins and bufotoxins. The remaining bufadienolides would be extracted prior to their analysis through analytical systems mentioned above. The performance of the strains would need to be compared with positive controls. The identity and type of each isolate would be resolved through microbial genome sequencing. In the eventuality of a natural compound discovery, preparative HPLC instruments would be used to isolate it.

The description of the experiments, results and conclusions drawn to meet the objectives of the research project are reported in the rest of this Master thesis.

MATERIALS & METHODS

3.1. MATERIALS

3.1.1. BIOLOGICAL MATERIALS

a) Road-Killed Cane Toads

Four road-killed cane toads were randomly collected in the area of Pullenvale (Brisbane suburbs, Queensland, Australia) by Pr Capon in the early morning of March 31th. They were directly placed in plastic containers (15×9 cm) and brought to the laboratory the same day (day 0). Motor vehicle collisions are thought to have occurred for all individuals during Wednesday night 29th March. Cane toad weights and sexes were respectively determined by differential weighing and visual observations based on colour skin and pustule density [14].

Each container was kept under hood at 25 °C under laboratory conditions. Two non-standardised holes were drilled on each lid as to enable the presence of air inside containers and let the carcases dry as it happens on roads. After observation of condensation droplets, the surplus of exudates was tipped out of the containers on day 3 and new lids with three openings of 12.5×1 cm replaced the old ones on day 4. Parotoid glands were rehydrated with droplets of distilled water on day 21 and 28 as rain would do on roads.

b) Cane Toad-Associated Bacteria

The bacteria that were proved to have a degrading or transforming activity against Mbg during previous studies [78,83] were retrieved from the Capon group glycerol stock. They were all isolated within cane toad glands or organs and stored at -80 °C in 20 % (w/v) glycerol aqueous solution. Four degrading and three transforming strains (Table 3) were reactivated onto nutrient agar plates (3.2.2) under sterile conditions and left two days at 30 °C in an incubator. They were then sub-cultured onto new nutrient agar plates under sterile conditions and left one day at 30 °C for incubation. A control plate was prepared at each stage. Working in a biosafety cabinet ensured sterile conditions. Fully grown colonies were stored at 4 °C.

Table 3 – Strains retrieved from the Capon group glycerol stock

Strains	Gram	Experimental Code	Origin	Date of Cryopreservation
Acinetobacter sp.	-	Acineto 1	Ovary	08/07/14
Acinetobacter sp.	-	Acineto 2	Ovary	09/07/14
Comamonas testosteroni	-	Coma	Parotoid gland	09/07/14
Flavobacterium sp.	-	Flavo	Parotoid gland	09/07/14
Bacillus cereus	+	G1S14	Parotoid gland	12/04/16
Bacillus cereus	+	G2S14	Parotoid gland	12/04/16
Bacillus sp.	+	G2S7	Parotoid gland	12/04/16

3.1.2. CHEMICAL MATERIALS

The compositions of media used in this research project are described below.

- Solid nutrient agar (NAg) medium: Peptone (Oxoid) 4 g/L, NaCl (Merck) 1.5 g/L and Agar (Labchem) 18 g/L in distilled water.
- Liquid nutrient broth (NB) medium: D-glucose (Univar) 10 g/L, peptone (Oxoid) 2 g/L, yeast extract (Merck) 4 g/L and NaCl (Merck) 1.5 g/L in distilled water.

The different bufadienolides and common steroids used as standards or to challenge bacteria are described in Table 4. Bufagenins and bufotoxins present in the Capon group stock were extracted and isolated on various cane toads between 2012 and 2016 by Dr Kamalakkannan. Mbg was additionally purified and crystallised.

The reagents used to prepare DNA extracts before amplification by polymerase chain reaction (PCR) are described below.

- Reaction mix: EmeraldAmp Max HS PCR Master Mix (2X Premix) from Takara Bio Inc.
- Sterilised deionised water (dH_2O) from Takara Bio Inc.
- Forward primer: 27F (5'-AGAGTTTGATCCTGGCTCAG-3') from Sigma-Aldrich.
- Reverse primer: 1492R (5'-TACGGYTACCTTGTTACGACTT-3') from Sigma-Aldrich.

Table 4 - Bufadienolides and common steroids information

Steroid	Cas Number	Molecular Weight (g/mol)	Supplier	State of the Compound	Purity
11-hydroxy -marinobufagenin (11hMbg)	n/a	416.22	Capon Group	Solution	n/a
12-hydroxy -marinobufagenin (12hMbg)	n/a	416.22	Capon Group	Solution	n/a
Marinobufagenin (Mbg)	470-42-8	400.23	Capon Group	Solution[1]	n/a
Marinobufagenin Palmitate (Mbg Pal)	n/a	638.92	Capon Group	Powder	n/a
Pregnenolone (Pregne)	145-13-1	316.49	Aldrich	Powder	98 %
Testosterone (Testo)	58-22-0	288.42	Sigma	Powder	≥ 98 %
Telocinobufotoxin (Tbt)	n/a	715.43	Capon Group	Solution[1]	n/a

All solvents used in this research project are listed in Table 5.

Table 5 – Solvents information

Solvents	Cas Number	Supplier	Purity
Ethyl Acetate (EtOAC)	141-78-6	Chem-Supply	≥ 99.5 %
Methanol (MeOH)	67-56-1	Honeywell	≥ 99.9 %
Acetonitrile (MeCN)	75-05-8	RCI Labscan	≥ 99.9 %
Trifluoroacetic acid (TFA)	76-05-1	Sigma-Aldrich	≥ 99.9 %
Formic Acid (HCOOH)	64-18-6	Merck	≥ 99.8 %
Butanol (BuOH)	71-36-3	Chem-Supply	≥ 99.5 %
Hexane	110-54-3	Honeywell	≥ 99.9 %

[1] The tare weight was indicated on the stock vial and the solution was dried as to calculate the remaining quantity of Mbg Pal by differential weighing.

The composition of electrophoresis gel and buffer used in this research project are described below:

- Gel: 100 mL agarose 1.5% (w/v) in H_2O, gel stain (Invitrogen, SYBR safe DNA gel stain, 10 000X concentrate in DMSO)
- Buffer: Tris-acetate-EDTA diluted 10-fold from a stock solution 10X.

The loading dye and the DNA ladder are respectively provided by Qiagen (GelPilot, 5X) and Invitrogen (1 Kb Plus DNA ladder, 1 µg/µL).

3.1.3. EQUIPMENT

Information about the general equipment used in this research project can be found in Table 6. When solutions needed to be evaporated, samples were dried in a block heater under nitrogen flow unless specified otherwise.

Table 6 – General Equipment

Apparatus	Brand & Series	Notes
Top Loading Balance	Sartorius – BL1500	Accuracy = 0.1 g
Analytical Balance	Sartorius – CP2245	Accuracy = 0.0001 g
Centrifuge	Thermo Fisher Scientific – Gyrospin	Pulse = 13 500 rpm reached (30 s)
Centrifugal Evaporator	SP Scientific – Genevac EZ-2 Elite	/
Block Heater	Ratek – n.a.	Coupled with nitrogen flow
Rotavapor	Büchi – R-114	/

The instruments described in Table 7 were used to perform high-performance liquid chromatography (HPLC), liquid chromatography coupled with an electrospray ionisation mass spectrometer (MS) or with a quadrupole time-of-flight mass spectrometer (QTOF), and preparative high-performance liquid chromatography (PrepHPLC). The analytical methods used in this research project are described in the section 3.3 Chemical Characterisation. The organic syringe filters that were used to prepare the samples before injection are provided by Kinesis (pore size = 0.45 µm, Ø = 13 mm).

Table 7 - Chromatographic Instruments

Apparatus	Brand & Series	Pump	Detector
HPLC	Agilent – 1100	Quaternary	Diode Array Detector (DAD)
LC/MS	Agilent – 1100	Quaternary	• DAD • MS
LC/QTOF	Agilent – 6545	Binary	• DAD • QTOF
PrepHPLC	Agilent – 1100	Binary	Multiple Wavelength Detector

Instruments used in the scope of nucleic acid applications are listed below:

- Thermal cycler (Applied Biosystems – Veriti);
- Electrophoresis power supply (Thermo Scientific – EC 300XL);
- Imaging system (Bio-Rad – Gel Doc XR);
- NanoDrop Spectrophotometer (Thermo Scientific – NanoDrop 2000).

3.2. EXPERIMENTAL

3.2.1. BUFADIENOLIDES PROFILE OF PAROTOID GLAND

a) Left Parotoid Gland

The experiment was conducted on four road-killed cane toads (3.1). Bufadienolides from left parotoid glands were extracted at seven different time points spread over a month. The experiment was performed on day 0 – 3 – 7 – 10 – 14 – 21 – 28, equivalent to time points 0 to 6 (t_0 - t_6). The developed method was inspired by Dr Kamalakkannan's work [83] and optimised to be finally described in five different steps: **dissection (1)**, **extraction (2)**, **separation (3)**, **preparation (4)** and **analysis (5)**.

1) For each batch, two pieces of parotoid gland no greater than 0.5 cm^3 are dissected with a sterile surgical scalpel (blade n°11). The first gland samples are directly immersed in 2 mL MeOH, while the second ones in 2 mL H_2O. MeOH inactivates the action of BtH and thus prevents the hydrolysis of bufotoxins, freezing the bufadienolides composition at the time of dissection. H_2O is used to control that the hydrolase is still active. Two 4 mL vials are also filled up with MeOH and H_2O to be used as blanks.

2) The parotoid gland samples are disrupted, shaken and left in extraction solvents for at least 30 minutes.
3) The sample solutions are transferred into Eppendorf tubes before centrifugation, leaving the pieces of glands in the vials. 2 pulses are performed to ensure a complete separation. The supernatants are collected in 20 mL vials, previously tare weighed on an analytical balance. 1.5 mL of MeOH is added in every 4 mL vial to resuspend and recover all bufadienolides. The samples are then centrifuged in the same conditions as previously performed and the corresponding supernatants are added to the ones already collected. The samples are left at rest for 1 hour after the second extraction to enhance the separation.
4) All samples are dried overnight at 40°C in a centrifugal rotavapor. The final weight is taken the next day as to get the approximate mass of extracted bufadienolides by differential weighing. All the extracts are redissolved in an adequate volume of MeOH as to obtain a final concentration of 1 mg/mL. The solutions are then filtered through a syringe into 2 mL vials prior to analytical analysis.
5) Samples are injected into HPLC-DAD system. Bufadienolides chromatograms are obtained using the HPLC method (Table 8). If interesting peaks are revealed in a profile, corresponding samples can be injected into MS systems. Mass spectra can be generated by following the MS method (Table 9) and the QTOF method (Table 10) for higher resolution.

It is worth mentioning that this method does not exclusively extract bufadienolides. Other organic compounds found in cane toad toxins can be present in the extracts such as the indolealkylamines or the arginyl amides for example. Contrariwise, tissue residues and other water solubles are left behind.

The separation and evaporation were performed differently for the two first batches. At t_0, syringe filters (organic: Kinesis, pore size = 0.45 µm, Ø = 13 mm, PTFE; aqueous: Grace, pore size = 0.45 µm, Ø = 13 mm, Nylon) were used to filter the crude extracts and the samples were dried in a block heater under nitrogen flow. The syringe filters tended to clog and the samples took much space in the block heater which led to find another solution. At t_1, the separation was performed by filtration of the crude extracts on filter paper (Whatman, pore size = 11 µm, Ø = 42.5 mm, cellulose) placed on suction Hirsch funnel under vacuum. The contact surface was expanded, increasing the risk of sample loss. Besides, Mbg peaks were detected in blank samples despite washing steps between each samples filtration. Centrifugation was therefore considered as an alternative and superior separation technique.

b) Right Parotoid Gland

The right parotoid glands from the road-killed cane toads were preserved and left intact from their entry into the laboratory until their dissection. Indeed, the regular sections made in the left parotoid glands nurtured the concern that they could sensitively disturb the *in situ* microbiota growth and activity. If such was the case, bufadienolides profiles from the right parotoid glands should be similar or in the same trend to what was observed at t_6. The parotoid glands were rehydrated on day 21 and 28 as described in 3.1. The experiment started on day 34 where the glands were used in parallel to culture bacteria (3.2.5 Isolation Of Optimised Degrading/Transforming). Bufadienolides were extracted on day 35 (t_7).

The developed method took the same five steps as for the left parotoid glands – **dissection (1)**, **extraction (2)**, **separation (3)**, **preparation (4)** and **analysis (5)** - with slight changes due to the condition and size of the crude samples. Indeed after 35 days, cutting through the cardboard-like right glands represented a challenge without mentioning the overall state of the roadkill. The decomposition had its effect over time, most of the tissue samples being degraded, but surprisingly, did not seem to affect the parotoid gland tissues much. Moreover, interrupting the hydrolysis was not a necessity at that stage because BtH was assumed to either be degraded or have completed its task already (confirmed by the results: 4.2.1). Finally, the size of the parotoid samples was considerably greater than the small dissected pieces described above and called for an adapted method, including a dilution step before the HPLC analysis.

1) The whole parotoid glands are dissected with a sterile surgical scalpel (blade n°11) and place into 20 mL vial filled up with 5 mL H_2O. They are left overnight to soften the tissues and facilitate the bufadienolides release. One blank sample is also prepared.

2) The parotoid glands are chopped into pieces and shaken as to facilitate the release of cane toad toxins. The samples are dried at 40°C then redissolved in 10 mL MeOH to extract the bufadienolides and organic compounds. They are left in MeOH for at least 30 mins.

3) An aliquot from each vial is withdrawn and transferred into Eppendorf tubes before centrifugation. 2 pulses are performed to ensure a complete separation.

4) For each supernatant, 100 mL is transferred in a syringe before filtration. 100 mL of MeOH is added to dilute the sample. The final solution is filtered into a 2 mL vial prior to analytical analysis.

5) Samples are injected into HPLC-DAD system. Bufadienolides chromatograms are obtained using the HPLC method (Table 8). If interesting peaks are revealed in a profile, corresponding samples can be injected into MS systems. Mass spectra can be generated by following the MS method (Table 9) and the QTOF method (Table 10) for higher resolution.

3.2.2. BIODEGRADATION/BIOTRANSFORMATION OF MARINOBUFAGENIN

The bufagenins degradation/transformation profiles of bacteria should be confirmed by challenging Mbg to bacterial cultures. The developed method was inspired by Dr Kamalakkannan's work [83] and optimised to be finally described in four different steps: **challenge (1), extraction/separation (2), preparation (3)** and **analysis (4)**. The seven bacteria tested in this experiment (Table 3) were retrieved from the Capon group glycerol stock, as described in 3.1. Four extractions were performed on day 3 – 4 – 5 – 7 after Mbg challenge as to assess the evolution of degradation/transformation profiles. A negative control (CTRL-) was obtained by adding Mbg to NB. Working in a biosafety cabinet ensured sterile conditions.

1) One single colony per sub-culture is picked and cultured in culture tubes containing 5 mL NB under sterile condition. The inoculation is not performed on one culture tube for CTRL- purpose. The cultures incubate overnight at 27 °C in aerobic condition and under agitation at 200 rpm. Each culture is then challenged with 50 µL Mbg 10 mg/mL in acetone under sterile condition. The incubation lasts 3 days at 27 °C in aerobic condition and under agitation at 200 rpm.

2) For each batch, 500 µL of each culture is pipetted out into 500 µL of EtOAc. A manual shake is given to each vial as to enhance the extraction and allow the separation between EtOAc and H_2O phases. The light phase is collected after at least 30 min of rest, leaving water solubles and cells in the heaviest solvent. 500 µL of EtOAc are added to the original vials and the extraction/separation is repeated as to collect more bufagenins. The samples are left at rest for 1 hour after the second extraction to enhance the separation.

3) The collected phases are dried at 40 °C. The samples are then redissolved in 100 µL MeOH and filtered into 2 mL vials prior to analytical analysis.

4) Samples are injected into HPLC-DAD system. Bufadienolides chromatograms are obtained using the HPLC method (Table 8). If interesting peaks are revealed in a profile, corresponding samples can be injected into MS systems. Mass spectra can be generated by following the MS method (Table 9) and the QTOF method (Table 10) for higher resolution.

The results helped select the best biodegrading and biotransforming strains among the glycerol stock bacteria that serve as Gram- and Gram+ references in other experiments.

3.2.3. BIODEGRADATION/BIOTRANSFORMATION OF STEROIDS

As Mbg is a steroid, the method employed in this experiment was the same as described in 3.2.2, bacteria being challenged with related steroids instead of Mbg. The Gram- and Gram+ references selected through the method described in 3.2.2 were used to perform biodegradation and biotransformation respectively. Three steroids were tested – **MbgPal, Pregne and Testo** – and Mbg was used as positive control (CTRL+). EtOAc was selected as the extraction solvent for all steroids after preliminary tests with BuOH and hexane. Working in a biosafety cabinet ensured sterile conditions.

Bufolipins, sub-class bufadienolides found in cane toad eggs and ovaries, were supposed to be the first tested steroids. If oxidised or hydroxylated products from the experiments were the same as in cane toad eggs, it would give evidence of a potential biological role of bacteria towards cane toads. Due to the difficulty to isolate and build up pure compounds, the Capon group stock needed to be managed carefully. The method required too much bufolipins with no clue about the outcome of the experiment. Marinobufagenin palmitate (Mbg Pal; also known as marinobufagenin-3-palmitoyl ester) is a bufagenin molecule structurally similar to bufolipins (Table 16). Because availability was not a problem, it was decided to use it instead. If the Gram- and Gram+ references could degrade or transform Mbg Pal, bufolipins could be employed in an arranged method described further down (3.2.4 Biodegradation/Biotransformation Of Bufolipins).

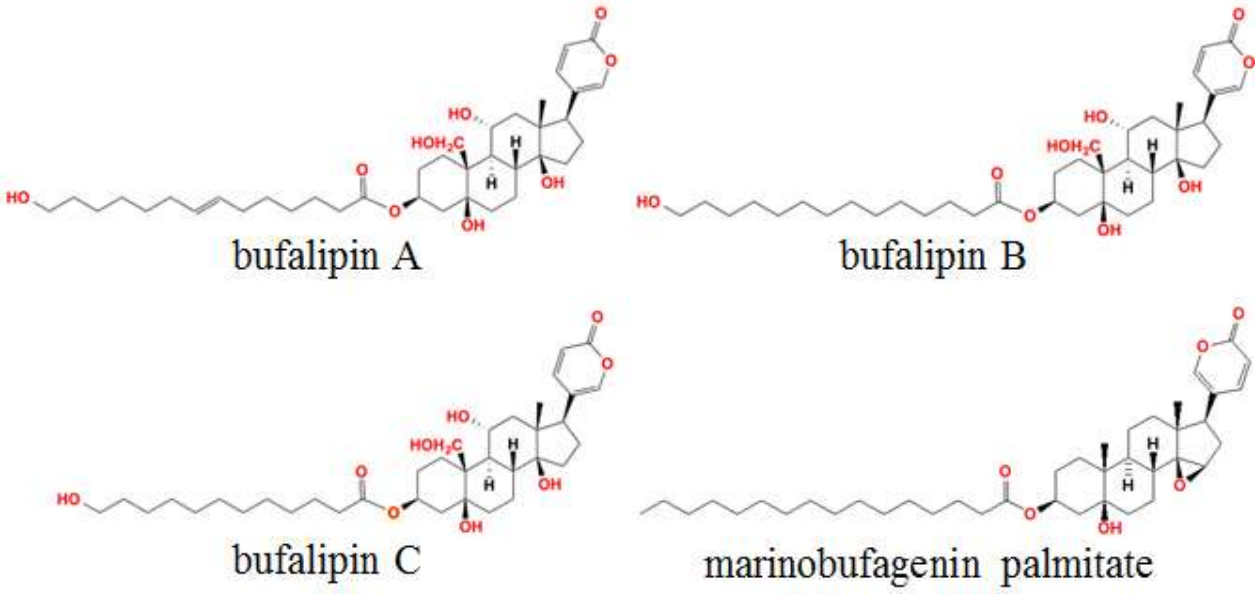

Figure 15 – Bufolipins & MbgPal

Progesterone, a common steroid, is a known precursor of cardenolides and was intended to be the second tested steroids. Its location in the laboratory being unknown, it was decided to use another precursor with a similar structure instead. The replacement was justified as pregnenolone (Pregne) not only offers another suitable precursor but also has a hydroxyl group linked in C-3 like in Mbg (Figure 16). Testosterone (Testo) was the last intended and used steroid for this experiment.

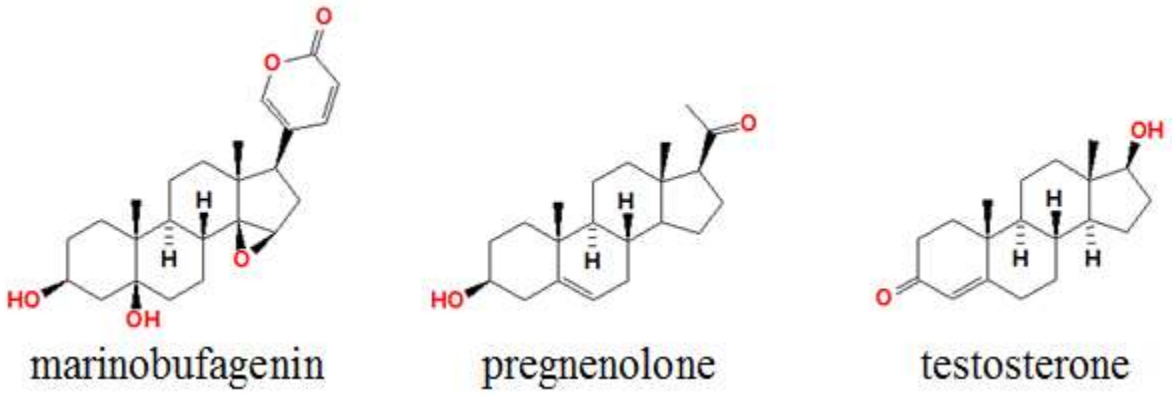

Figure 16 – Structures of Mbg and common steroids

3.2.4. BIODEGRADATION/BIOTRANSFORMATION OF BUFOLIPINS

If MbgPal showed signs of biodegradation or biotransformation, the method described below was designed to test one of the bufolipins on Gram- and Gram+ references while reducing the use of pure compounds. It follows four different steps: **challenge (1)**, **extraction/separation (2)**, **preparation (3)** and **analysis (4)**.

1) One single colony per sub-culture is picked and cultured in a well filled up with 1.5 mL NB under sterile condition, for the samples and CTRL+. The inoculation is not performed on one NB medium as to serve as CTRL-. The cultures incubate overnight at 27 °C in aerobic condition and under agitation at 150 rpm. Each culture is then challenged with 3 µL bufolipin A/B/C 15 mg/mL in acetone under sterile condition. The incubation lasts 3 days at 27 °C in aerobic condition and under agitation at 150 rpm.

2) For each batch, 1.5 mL of each culture is pipetted out into 4 mL vial filled up with 1.5 mL of EtOAc. A manual shake is given to each vial as to enhance the extraction and allow the separation between EtOAc and H_2O phases. The light phase which contains the bufagenins is collected after at least 30 min of rest, leaving water solubles and cells in the heaviest solvent. 1.5 mL of EtOAc are added to the original vials and the extraction/separation is repeated as to collect more bufagenins. The samples are left at rest for 1 hour after the second extraction to enhance the separation.

3) The collected phases are dried at 40 °C. The samples are then redissolved in 100 µL MeOH and filtered into 2 mL vials prior to analytical analysis.

4) Samples are injected into HPLC-DAD system. Bufadienolides chromatograms are obtained using the HPLC method (Table 8). If interesting peaks are revealed in a profile, corresponding samples can be injected into MS systems. Mass spectra can be generated by following the MS method (Table 9) and the QTOF method (Table 10) for higher resolution.

The method would have been performed on a 24 deep-well block but the experiment never started as the scarcity of the bufolipins and the results were not favourable.

3.2.5. ISOLATION OF OPTIMISED DEGRADING/TRANSFORMING STRAINS

As stated earlier, the right parotoid glands from the road-killed cane toads were preserved and left intact from their entry into the laboratory (day 0) until their dissection as to favour and host an active microbiota. The parotoid glands were rehydrated on day 21 and 28. The experiment started on day 34 and the parotoid glands were used afterwards to extract the bufadienolides as described in 3.1. The developed method was inspired by Dr Kamalakkannan's work [83] and optimised to be finally described in six different steps: **microbial sampling (1), isolation (2), challenge (3), extraction/separation (4), preparation (5)** and **analysis (6)**. The Gram- and Gram+ references selected through the method described in 3.2.2 were used as CTRL+. Bacteria were challenged in deep-wells. Working in a biosafety cabinet ensured sterile conditions.

1) The whole parotoid glands are dissected with a sterile surgical scalpel (blade n°11), set out as a grid and spread out onto NAg plates under sterile conditions. A control plate is also prepared. The culture plates are incubated at 30 °C and left in the incubator until complete bacterial growth.

2) Once mature heterogenic colonies are observed, they need to be discriminated and marked for each plate. Every selected colony is then spiked and streaked onto a NAg plate under sterile conditions. The plates are incubated at 30 °C and left in the incubator until complete bacterial growth. The operation is repeated until the isolation of individual clonal population. The culture plates are compared and the repeats are discarded by visual assessment. The remaining strains are sub-cultured onto NAg plate under sterile conditions and incubated for 2 days at 30 °C. A control plate is prepared at each stage.

3) One single colony per sub-culture is picked and culture in a well filled up with 1.5 mL NB under sterile condition, for the samples and CTRL+. The inoculation is not performed on one NB medium as to serve as CTRL-. The cultures incubate overnight at 27 °C in aerobic condition and under agitation at 150 rpm. Each culture is then challenged with 3 µL Mbg 15 mg/mL in acetone under sterile condition. The incubation lasts 5 days at 27 °C in aerobic condition and under agitation at 150 rpm.

4) 1.5 mL of each culture is pipetted out into 1.5 mL of EtOAc. A manual shake is given to each vial as to enhance the extraction and allow the separation between EtOAc and H_2O phases. The light phase is collected after at least 30 min of rest, leaving water solubles and cells in the heaviest solvent. 1.5 mL of EtOAc are added to the original vials and the extraction/separation is repeated as to collect more bufagenins. The samples are left at rest for 1 hour after the second extraction to enhance the separation.

5) The collected phases are dried at 40 °C. The samples are then redissolved in 100 µL MeOH and filtered into 2 mL vials prior to analytical analysis.

6) Samples are injected into HPLC-DAD system. Bufadienolides chromatograms are obtained using the HPLC method (Table 8). If interesting peaks are revealed in a profile, corresponding samples can be injected into MS systems. Mass spectra can be generated by following the MS method (Table 9) and the QTOF method (Table 10) for higher resolution.

Due to the loss of activity of the Gram- reference (A.4), samples were also **prepared for a co-injection (5bis),** respectively between the Gram-/Gram+ reference and the best degrading/transforming strains from road-killed cane toad parotoid gland. The co-injection enables to confirm that the peaks produced by roadkill-associated strains correspond to the ones previously observed on proven degrading/transforming bacteria from the glycerol stock.

5bis) The samples are dried at 40 °C. They are re-dissolved in an adequate volume of MeOH as to reach the original concentration. 15 µL of each sample are withdrawn and mixed in a new common 2 mL vial prior to analytical analysis.

3.2.6. Evaluation Of Biodegradation/Biotransformation Capacity

In order to get a sense of replicability and evaluate the capacity of the road-killed cane toad-associated bacteria, the applied method was the same as in 3.2.2 but included replicates (n=3) and CTRL+. The biodegradation/biotransformation testing of Mbg was performed on the best degrading and transforming strains selected through the method described in 3.2.5. The Gram- and Gram+ references selected through the method described in 0 were used as CTRL+. Five extractions were performed on the hour 0 – 12 – 24 – 48 – 96, the hour 0 corresponding to day 3 after Mbg challenge. Working in a biosafety cabinet ensured sterile conditions.

3.2.7. Biodegradation/Biotransformation Of Telocinobufotoxin

Bufotoxins are the second sub-class bufadienolides found in cane toad parotoid secretion. No bacteria have been reported to be able to degrade bufotoxins and Dr Kamalakkannan's work suggests that bufotoxins are resistant to microbial degradation/transformation [83]. Cane toad parotoid gland-associated strains should, therefore, be challenged with bufotoxins in order to support or refute the precedent conclusion.

Due to its nature, pure Mbt is difficult to isolate and accumulate. The Capon group stock needed to be managed carefully and Tbt was therefore chosen instead. However, Tbt was also available in a small amount. Consequently, the applied method was the one designed to save bufolipins stock (3.2.4), bufolipins being replaced by Tbt. Furthermore, only relevant isolated strains demonstrating degradation or transformation capacity through the method described in 3.2.5 were challenged. The Gram- and Gram+ references selected through the method described in 3.2.2 were used as CTRL+. Bacteria were challenged in deep-wells block. Working in a biosafety cabinet ensured sterile conditions.

3.2.8. PURIFICATION OF BIODEGRADATION/BIOTRANSFORMATION PRODUCTS

In order to isolate pure degradation and transformation products, a scale-up method was designed. Only relevant isolated strains demonstrating degradation or transformation capacity were challenged as to preserve the Mbg stock. The developed method follows four steps: **challenge (1)**, **extraction/separation (2)**, **preparation (3)** and **isolation (4).** The cultures were performed in 250 mL flasks where the caps were loosely screwed to ensure the presence of air. Working in a biosafety cabinet ensured sterile conditions.

1) One single colony per sub-culture is picked and cultured in 100 mL NB under sterile condition. One culture flask does not undergo the inoculation and is used as blank sample. The cultures incubate overnight at ambient temperature in aerobic condition and under agitation at 200 rpm. Each culture is then challenged with 1 mL Mbg 15 mg/mL in acetone under sterile condition. The incubation lasts 7 days at ambient temperature in aerobic condition and under agitation at 200 rpm.

2) For each batch, the culture medium is poured into a separating funnel previously filled up with 100 mL EtOAc. A manual shake is given to each vial as to enhance the extraction and separation between EtOAc and H_2O phases. After at least 30 min of rest, the light phase which contains the bufagenins is collected in a round-bottomed flask. The separating funnel is then filled up again with 100 mL EtOAc and the heaviest phase is poured over as to perform a second extraction. The separating funnel is left at least 1 hour before collecting the EtOAc phase.

3) The samples are dried in a rotavapor at 40 °C. They are then dissolved in MeOH and filtered into 4 mL vials, previously tare weighed. When the samples are dried, the vials are weighed then filled up with MeOH as to get a final concentration of 50 mg/mL.

4) The samples are injected into a PrepHPLC system. Separation and isolation are obtained using the PrepHPLC method (Table 11). Similar fractions are regrouped to acquire degrading/transforming product solutions. In the case of doubt, aliquots can be withdrawn and injected in a LC/MS system to link masses to the fractions.

3.2.9. TAXONOMIC DETERMINATION OF MICROORGANISMS

A genus and species names were assigned to each strain isolated from road-killed cane toad parotoid gland and demonstrating a degradation or transformation activity through the method described in 3.2.5. The taxonomy of left aside isolated strains (3.2.5) was also determined if they were found to be visually similar to a degrading/transforming bacterium and present on a different toad. The identity of mysterious microorganisms can be revealed through 16sRNA gene sequencing as long as the nucleic acid material is in sufficient quantity.

The identification was performed by following several steps: **preparation (1), extraction/amplification (2), differentiation (3), extraction (4), preparation (5), amplification (6), confirmation (7) and sequencing (8)**. DNA extracts were obtained via PCR for Gram+ and via the extraction kit DNeasy blood&tissue kit from Qiagen for Gram-. Details about the 16sRNA gene sequencing and the taxonomic characterisation of the parotoid gland-associated bacteria can be found in 0.

1) One single colony per sub-culture is picked to inoculate 22 µL of DNA free dH_2O in a PCR tube. 25 µL of reaction mix is added, as well as 1.5 µL of forward and reverse primers.

2) The tubes are injected into a thermal cycler. The PCR amplification conditions are as follows: initial denaturation at 95ºC for 2 min, followed by 40 cycles of 95°C for 20 s + 56 °C for 20 s + 72 °C for 30 s, a final extension at 72 °C for 5 min, followed by a hold at 4 ºC. PCR is sufficiently traumatic to extract Gram+ DNA.

3) An electrophoresis is run to differentiate extracted DNA samples (Gram+) from non-extracted DNA samples (Gram-). Gel and buffer are conditioned by applying a potential difference of 70 V for 30 min. 2 µL of loading dye is added to 10 µL of DNA samples and 3 µL of reference ladder. Wells are loaded with 10 µL of dyed samples. A 90 V potential difference is applied for 90 min. The electrophoresis gel is revealed through an imaging system under UV light. Gram+ DNA samples are stored at -30 °C.

4) Gram- DNA is extracted by following the instructions of an appropriate DNA extraction kit. The necessary liquid cultures are obtained by sub-culture single colony into culture tubes containing 5 mL NB under sterile condition. The cultures incubate overnight at 27 °C in aerobic condition and under agitation at 200 rpm.

5) 1 µL of Gram- DNA extract was poured into 21 µL of DNA free dH_2O in a PCR tube. 25 µL of reaction mix is added, as well as 1.5 µL of forward and reverse primers.

6) The tubes are injected into a thermal cycler and the same method is repeated. The PCR amplification conditions are as follows: initial denaturation at 95ºC for 2 min, followed by 40 cycles of 95°C for 20 s + 56 °C for 20 s + 72 °C for 30 s, a final extension at 72 °C for 5 min, followed by a hold at 4 ºC.

7) The electrophoresis run is repeated to confirm the extraction of Gram- DNA. Gel and buffer are conditioned by applying a potential difference of 70 V for 30 min. 2 µL of loading dye is added to 10 µL of DNA samples and 3 µL of reference ladder. A 90 V potential difference is applied for 90 min. The electrophoresis gel is revealed through an imaging system under UV light.

8) Concentrations are determined through a nanodrop spectrometer before sequencing. Gram- and Gram+ DNA samples are sent for 16S rRNA gene sequencing.

3.3. CHEMICAL CHARACTERISATION

3.3.1. HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY ANALYSIS

HPLC analysis enables to identify the different compounds present in the samples as well as their relative concentration. All the samples were injected into an analytical HPLC-DAD instrument (Table 7). The method used was a fast version of the one optimised for bufadienolides analysis by Dr Kamalakkannan, who determined that the use of TFA as solvent modifier and of a reversed phase column C_{18} provide the best resolution [83]. The linear gradient elution goes from 90% H_2O/MeCN to 100% MeCN over 10 min, hold at 100% MeCN over 5 min, with constant 0.01 % TFA. Preliminary tests helped determine the optimal UV wavelength for Pregne and Testo among 210, 230, 254 and 298 nm. The data analysis was performed with the software Agilent ChemStation. Details on the HPLC method applied in this research project are found in Table 8.

Table 8 – The HPLC method

Parameters	**Characteristics and Settings**
Column	Agilent Zorbax-C_{18} (4.6 x 150 mm, 5 µm)
Elution	Gradient[2]
Mobile phases	• H_2O – Solvent A • MeCN – Solvent B • 0.1% (v/v) TFA/MeCN – Solvent C
Mobile phase flow rate	1 mL/min
Injection volume	10.0 µL
Column temperature	Ambient
Optimal Wavelength	• Bufadienolides: 298 nm • Pregne: 210 nm • Testo: 254 nm

[2] Gradient elution program: step 1 - 10 min from 10 % C to 90 % B & 10 % C; step 2 - 5 min at 90 % B & 10 % C; step 3 – 1 min from 90 % B & 10 % C to 10 % C; step 4 – 5 min at 10 % C.

3.3.2. MASS SPECTROMETRY ANALYSIS

The use of a LC/MS enables to link a mass to a corresponding peak and bring supporting data for the identification of compounds diluted in a solution. By ionising analytes, MS can sort out the ions based on their mass-to-charge ratio (m/z). Representative samples were injected into the analytical LC-MS to attribute molecular mass to known as well as unknown peaks. The method used was a fast version of the one optimised for bufadienolides analysis by Dr Kamalakkannan [83] and is similar to the one described above. TFA is replaced with HCOOH due to the sensitivity of the MS detector. The linear gradient elution goes from 90% H_2O/MeCN to 100% MeCN over 10 min, hold at 100% MeCN over 5 min, with constant 0.05 % HCOOH. The data analysis was performed with the software Agilent ChemStation. Details on the MS method applied in this research project are found in Table 9.

Table 9 – The MS method

Parameters	**Characteristics and Settings**
Column	Agilent Zorbax-C_{18} (4.6 x 150 mm, 5 µm)
Elution	Gradient[3]
Mobile phases	• H_2O – Solvent A • MeCN – Solvent B • 0.5% (v/v) HCOOH/MeCN – Solvent D
Mobile phase flow rate	1 mL/min
Injection volume	10.0 µL
Column temperature	Ambient
Electrospray ionisation mode	• Positive $(M+H)^+$ • Negative $(M-H)^-$

[3] Gradient elution program: step 1 - 10 min from 10 % D to 90 % B & 10 % D; step 2 - 5 min at 90 % B & 10 % D; step 3 – 1 min from 90 % B & 10 % D to 10 % D; step 4 – 5 min at 10 % D.

The use of a QTOF LC/MS enables obtaining the same information given by LC/MS but at a higher resolution. The technique is more rapid, selective, sensitive and accurate which is why it is increasingly used in the cane toad context [56,58,92]. As consequences, the instruments require particular attention. Solutions need to be diluted before analysis for instance as not to damage the system. Only samples with HPLC chromatograms revealing unknown peaks were prepared for Q-TOF analysis. They were diluted ten-fold. The linear gradient elution goes from 90% H_2O/MeCN to 100% MeCN over 4.5 min, hold at 100% MeCN over 1 min, with constant 0.01 % HCOOH/MeCN modifier. Details on the QTOF method applied in this research project are found in Table 10.

Table 10 – The QTOF method

Parameters	**Characteristics and Settings**
Instrument	Agilent series 6545
Column	Agilent Zorbax-C_{18} (50 x 2.1 mm, 1.8 µm)
Elution	Gradient[4]
Mobile phases	• H_2O – Solvent A • MeCN – Solvent B • 0.01 % (v/v) HCOOH/MeCN – Solvent E
Mobile phase flow rate	0.5 mL/min
Injection volume	1.0 µL
Column temperature	Ambient
Electrospray ionisation mode	Positive $(M+H)^+$

[4] Gradient elution program: step 1 – 4.5 min from 10 % E to 90% B & 10 % E; step 2 – 1 min at 90 % B & 10 % E; step 3 – 0.1 min from 90 % B & 10 % E to 10 % E; step 4 – 2.5 min at 10 % E.

3.3.3. PREPARATIVE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

PrepHPLC is not strictly speaking an analytical method as its main function is to isolate and purify compounds from a complex mixture rather than to identify and quantity an analyte. This is why samples in a PrepHPLC system go from the detector to a fraction collector instead of the waste. More samples can be loaded on a PrepHPLC than a semi-preparative HPLC which saves a considerable amount of time. The method used was developed by Dr Kamalakkannan [83]. The linear gradient elution goes from 10 % to 80 % H_2O/MeCN over 15 min, then from 80 % H_2O/MeCN to 100 % MeCN over 5 min, and hold at 100 % MeCN over 5 min, with constant 0.01 % TFA. The collection time frame is set in concordance with the degradation/transformation profile at small scale. The data analysis was performed with the software Agilent ChemStation. Details on the PrepHPLC method applied in this research project are found in Table 11.

Table 11 - The PrepHPLC method

Parameters	**Characteristics and Settings**
Column	Waters SymmetryPrep-C_{18} (19 x 150 mm, 7 µm)
Elution	Gradient[5]
Mobile phases	• 0.01% (v/v) TFA/H_2O – Solvent F • 0.01% (v/v) TFA/MeCN – Solvent G
Mobile phase flow rate	15 mL/min
Injection volume	N.A.[6]
Column temperature	Ambient
Collection	• Column by column • 40 fractions

3.4. TAXONOMIC CHARACTERISATION

The taxonomic characterisation of road-killed cane toad parotoid gland-associated strains was made through DNA sequencing. By ordering the nucleotides, sequencing programs can recreate specific DNA sequences as to assigned genus and species names to a DNA sample. After the 16sRNA genes were amplified by PCR and nucleic acid concentrations determined by spectrometry (3.2.9), the samples were sent to the Australian Genome Research Facility at the University of Queensland for DNA sequencing.

[5] Gradient elution program: step 1 - 15 min from 10 % to 80 % G; step 2 - 5 min from 80 % to 100 % G; step 3 – 5 min at 100 % G; step 4 – 1 min from 100 % to 10 % G; step 5 – 4 min at 10 % G.

[6] As the objective is to purify and isolate all the compounds, the injection volume varies with the sample.

RESULTS & SPECIFIC DISCUSSIONS

4.1. PRELIMINARY RESULTS

Road-killed cane toads were required in this research project. Four individuals were randomly collected, brought into the laboratory on day 0 and described as explained in 3.1.1. Information about cane toad weight and sex can be found in Table 12. Odors indicated that tissue decomposition had started. T1 appeared to be the largest of the roadkill and managed to keep its initial shape despite the motor vehicle collision. Contrariwise, T2 was damaged after the crash, with visible gut content out. Some cane toad secretions could be observed on the surface of T2 and T4's parotoid glands. T3 was the smallest individual.

Table 12 – Road-killed cane toad weight and sex

Road-Killed Cane Toad Code	Weight[7] (g)	Sex
T1	142.6	female
T2	92.1	female
T3	60.6	female
T4	98.2	female

Figure 17 – Road-killed cane toads at t_0; from left to right, T1 – T2 – T3 – T4

Despite females being generally larger than males, the four road-killed cane toads were far from the largest anurans previously described [14,16,27]. T3's weight even suggested that it had recently reached the adult stage.

[7] Wet weight

4.2. Experimental Results

4.2.1. Bufadienolides Profile Of Parotoid Gland

a) Left Parotoid Gland

Bufadienolides within dissected pieces of left parotoid glands from four different road-killed cane toads were extracted and analysed through analytical system at seven different time points over a month as explained in 3.2.1. Analysis performed at the initial time point revealed very complex profiles. Based on previous studies [78,83,86], bufadienolides were expected to be detected between 5 and 12 minutes of elution. Five bufotoxins, three other arginine esters and four bufagenins were detected in the initial analysis as revealed by the QTOF analysis of T3 (Table 13). Biogenic amines were also detected in the four first minutes of analysis. The chromatogram of T1 parotoid toxins shows the different peak regions (Figure 18). The great variety of bufadienolides in different proportions gave unique looks to each toad chromatogram. Death did not occur at the same time nor did have the same impact on the roadkill and could explain the heterogeneity displayed.

The resolution of the peaks was not always optimal and a couple of overlaps suggest that some components were hidden (Figure 18). For example, Mbt (**1**) and Tbt (**2**) shared a common peak which was the dominating figure for T1 and T3 amongst the major detected bufadienolides. In T2 and T4, the level of Mbg (**2**) was the most important followed by the one of Tbg (**3**). The first step in the evolution of bufadienolides content is the hydrolysis of bufotoxins into bufagenins. According to the hypothesis (Figure 14), T1 and T3 were behind in the conversion process when compared to T2 and T4 as their major bufadienolides were Mbt and Tbt. The motor vehicle collision had provoked the release of the sticky white secretion onto T2 and T4 surface (Figure 17). Parotoid glands lose about 50 % of their content when compressed [83]. T2 and T4 chromatograms were, therefore, dominated by Mbg/Tbg rather than Mbt/Tbt because they had half the quantity of bufotoxins of T1 and T3 when death occurred and the conversion started.

Table 13 – QTOF results: major bufadienolides detected in T3 on day 0

#	RT[8] (min)	Compound	Calculated mass (m/z) $(M+H)^+$	Measured mass (m/z) $(M+H)^+$	ΔmDa	Molecular Formula
/	6.14	marinobufagenin-3-adipoylarginine	684.3734	684.3748	-1.38	$C_{36}H_{52}N_4O_9$
/	6.23	hellebritoxin	726.4217	726.4217	0.02	$C_{38}H_{56}N_4O_{10}$
/	6.32	marinobufagenin-3-pimeloylarginine	698.3891	698.3894	-0.34	$C_{37}H_{54}N_4O_9$
2	6.54	Tbt	714.4204	714.4217	-1.29	$C_{38}H_{58}N_4O_9$
1	6.61	Mbt	712.4047	712.4060	-1.31	$C_{38}H_{56}N_4O_9$
4	7.11	Tbg	402.2406	402.2405	0.15	$C_{24}H_{34}O_5$
/	7.36	bufalin-3-pimeloylarginine	684.4098	684.4107	-0.93	$C_{37}H_{56}N_4O_8$
/	7.68	bufalitoxin	698.4255	698.4263	-0.86	$C_{38}H_{58}N_4O_8$
3	7.87	Mbg	400.2250	400.2238	1.19	$C_{24}H_{32}O_5$
/	7.96	resibufotoxin	696.4098	696.4112	-1.38	$C_{38}H_{56}N_4O_8$
5	8.31	bufalin	386.2457	386.2456	0.15	$C_{24}H_{34}O_4$
7	9.31	resibufagenin	384.2301	384.2314	-1.29	$C_{24}H_{32}O_4$

[8] Retention time corresponding to the HPLC chromatograms

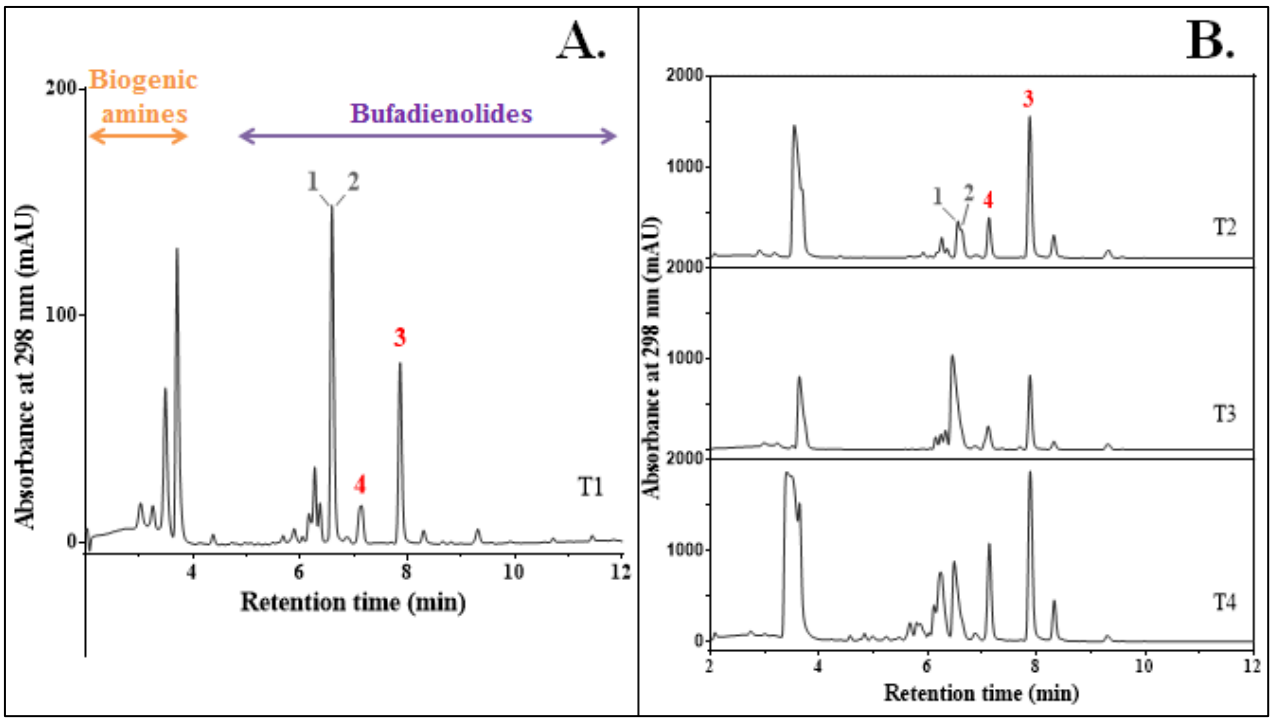

Figure 18 – (A) Chromatogram and (B) stacked chromatograms of parotoid toxins on day 0

On day 3, Mbt had considerably decreased and Mbg became the dominating figure in all toxin profiles. All proportions held, the chromatograms obtained from day 3 to day 28 were all similar for all toads and only slight variations could be noticed (Appendix A.1 and A.2). The chemical profiles did not fluctuate and the same compounds were found on day 3 as well as on day 28. Three different sub-classes of bufadienolides and one of biogenic amines were present. Bufagenins constitute the main sub-class, in proportion and number, and were represented by Mbg (**3**), Tbg (**4**), bufalin (**5**), and at a lower level by hellebrigenin (**6**) and resibufogenin (**7**) (Figure 5); dehydrobufotenin (**8**) (Figure 8) was the only constituent of indolealkylamine and at lower levels, Mbt (**1**) (Figure 6) and 11hMbg (**12**) (Figure 11) were respectively the representatives for bufotoxins and hydroxylated bufagenins (Table 14; Figure 19).

Hydroxylated analogue emerged on day 3. 11hMbg was a sign that bufadienolides were evolving and that a transformation of bufagenins took place. A co-injection in the LC-MS with standards from the Capon Group stock confirmed the presence of 11hMbg (**9**) as shown in Figure 20. The apparition of a hydroxylated analogue was expected according to the hypothesis (Figure 14). However, the 11hMbg did not soar and stayed at a constantly low level, hardly detectable. MS spectra could not confirm the presence of 12hMbg too. Those pieces of evidence suggested that the transformation of Mbg had rapidly started to stop as suddenly. A possibility is that the 11hMbg level reached a maximum and kept a constant level through a dynamic oxidoreduction reaction between 11hMbg and Mbg. Nevertheless, the non-presence of oxidised products does not support that theory. Another explanation lays in the concern that the multiple dissections could have disturbed and ultimately prevent the bacterial development. Further experiments should confirm it.

Table 14 – Main biogenic amine & bufadienolides found in parotoid toxins since day 3

#	RT (min)	Compound	Molar mass (g/mol)	Molecular Formula
8	3.74	Dehydrobufotenine	202.11	$C_{12}H_{14}N_2O$
12	5.78	11hMbg	416.22	$C_{24}H_{32}O_6$
6	6.24	Hellebrigenin	416.22	$C_{24}H_{32}O_6$
1	6.61	Mbt	712.41	$C_{38}H_{56}N_4O_9$
4	7.11	Tbg	402.24	$C_{24}H_{34}O_5$
3	7.87	Mbg	400.23	$C_{24}H_{32}O_5$
5	8.31	Bufalin	386.25	$C_{24}H_{34}O_4$
7	9.31	Resibufogenin	384.23	$C_{24}H_{32}O_4$

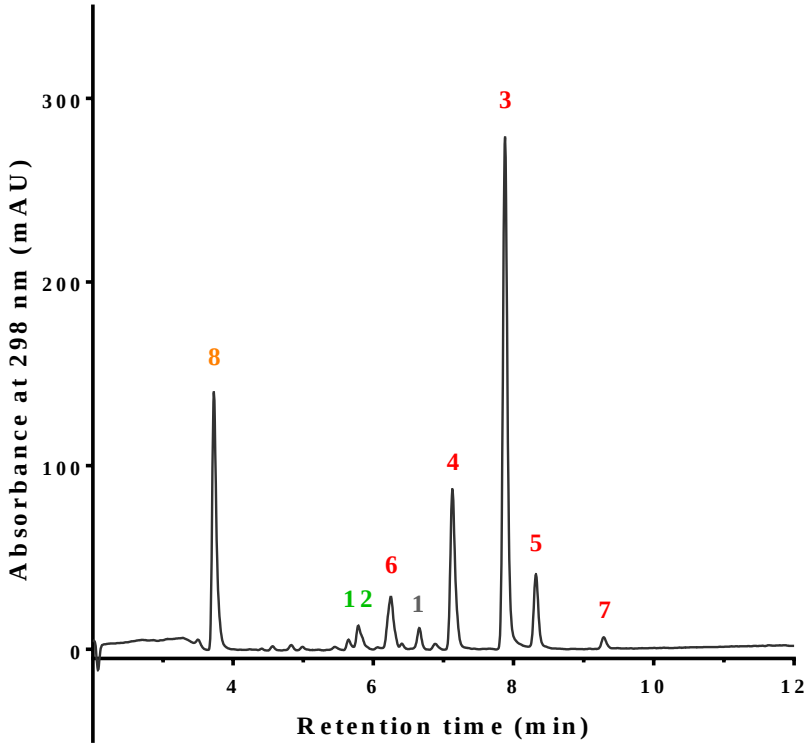

Figure 19– Chromatogram of T4 parotoid toxins on day 14

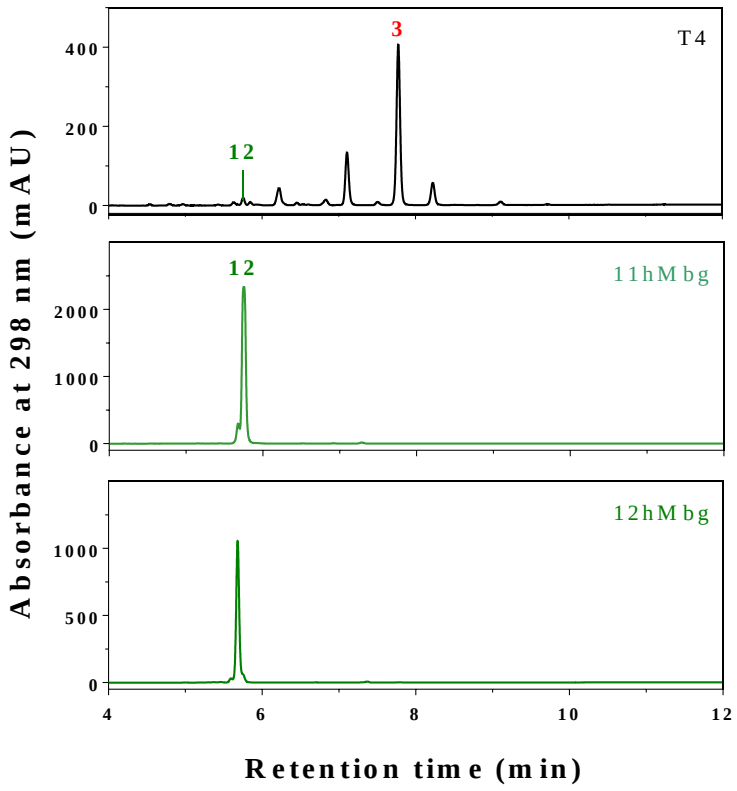

Figure 20 – Co-injection of T4 on day 4 and standards of 11hMbg and 12hMbg in LC-MS

The evolution of bufadienolide relative concentrations confirmed the constancy in the chemical composition of parotoid glands since day 3 (Figure 21). As arginine esters are similar in structure and function to bufotoxins, they were both grouped for the sake of clarity. Only integration values of detected bufadienolides described above were considered in the confection of this graph. It also has to be noted that some components were certainly under and overrated on day 0 due to bad resolution of some peaks. It surely does not influence the general trends. At day 0, bufagenins were present in minority in T1 and T3 while they were already the main sub-class of bufadienolides in T2 and T4. This difference is thought to be the result of T2/T4 secretions as about 50 % of the parotoid content had been lost after compression. The relative Mbt concentrations for the different cane toads supported that explanation (T1 = 62.64 %, T2 = 30.03 %, T3 = 64.14 %, T4 = 45.37 %). Then, bufagenins rocketed after day 0 and remained the major sub-class from day 3 to 28 for all road-killed cane toads. The analysis of the parotoid glands revealed the presence of 11hMbg at a low concentration since day 3. T4 was the road-killed cane toad with the highest proportion of it. Bacteria might have mediated 11hMbg production in the early stage of the decomposition but were quickly limited. It has to be noted that no oxidised analogue or product of degradation appeared during the course of the experiment.

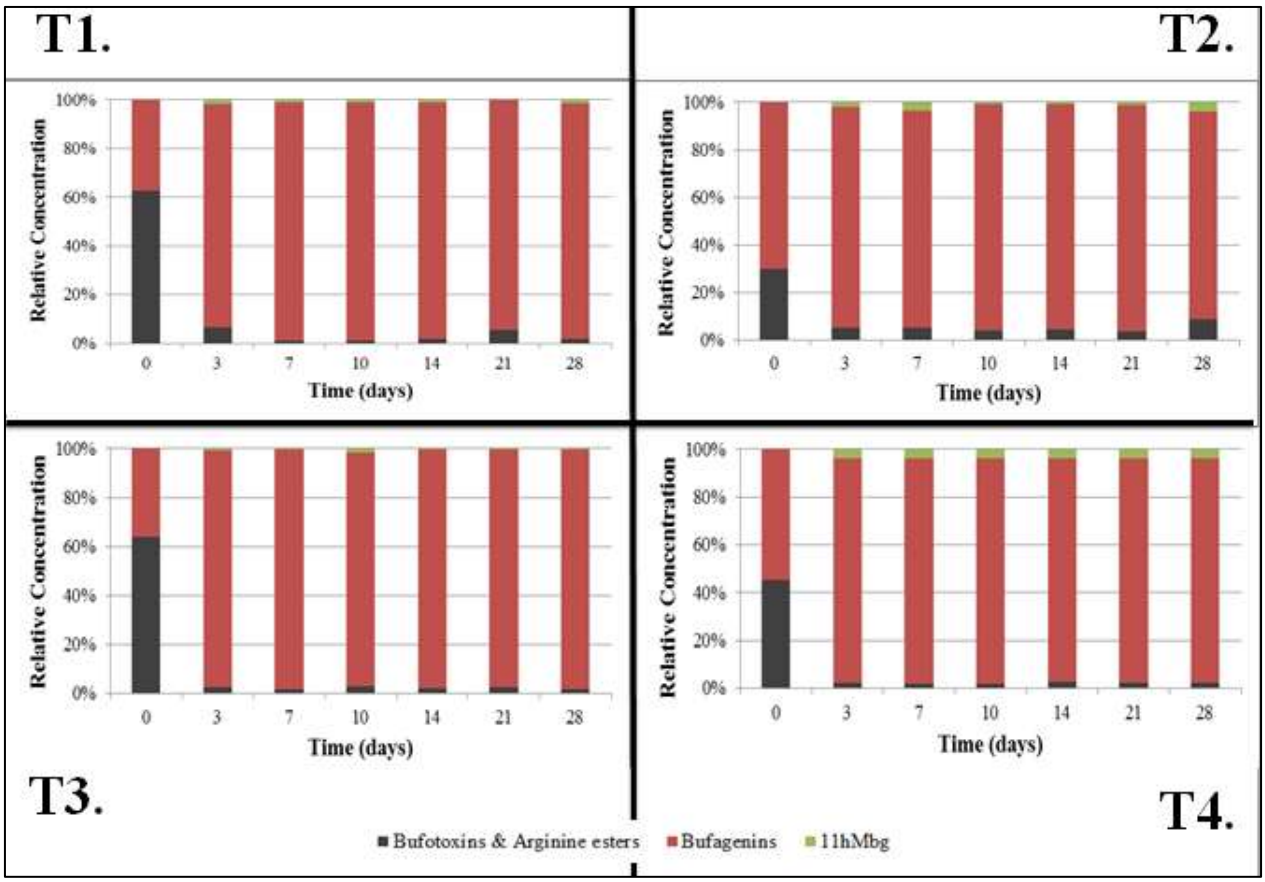

Figure 21 – Evolution of bufadienolides content in four road-killed cane toad parotoid glands

Analysis of parotoid glands extracted in H_2O confirmed that MeOH effectively inactivates BtH. Indeed, chromatograms of MeOH and H_2O extracts were different on day 0 while they were similar at the other time points, the hydrolysis of bufotoxins having already occurred. Interestingly, the secretions present onto T2 and T4 parotoid glands were also analysed and revealed profiles that matched the ones of the last batches. This highlighted the fact that bufotoxins left in the glands underwent the same hydrolysis process as within the parotoid secretion. Corresponding chromatograms are represented for T2 in Figure 22.

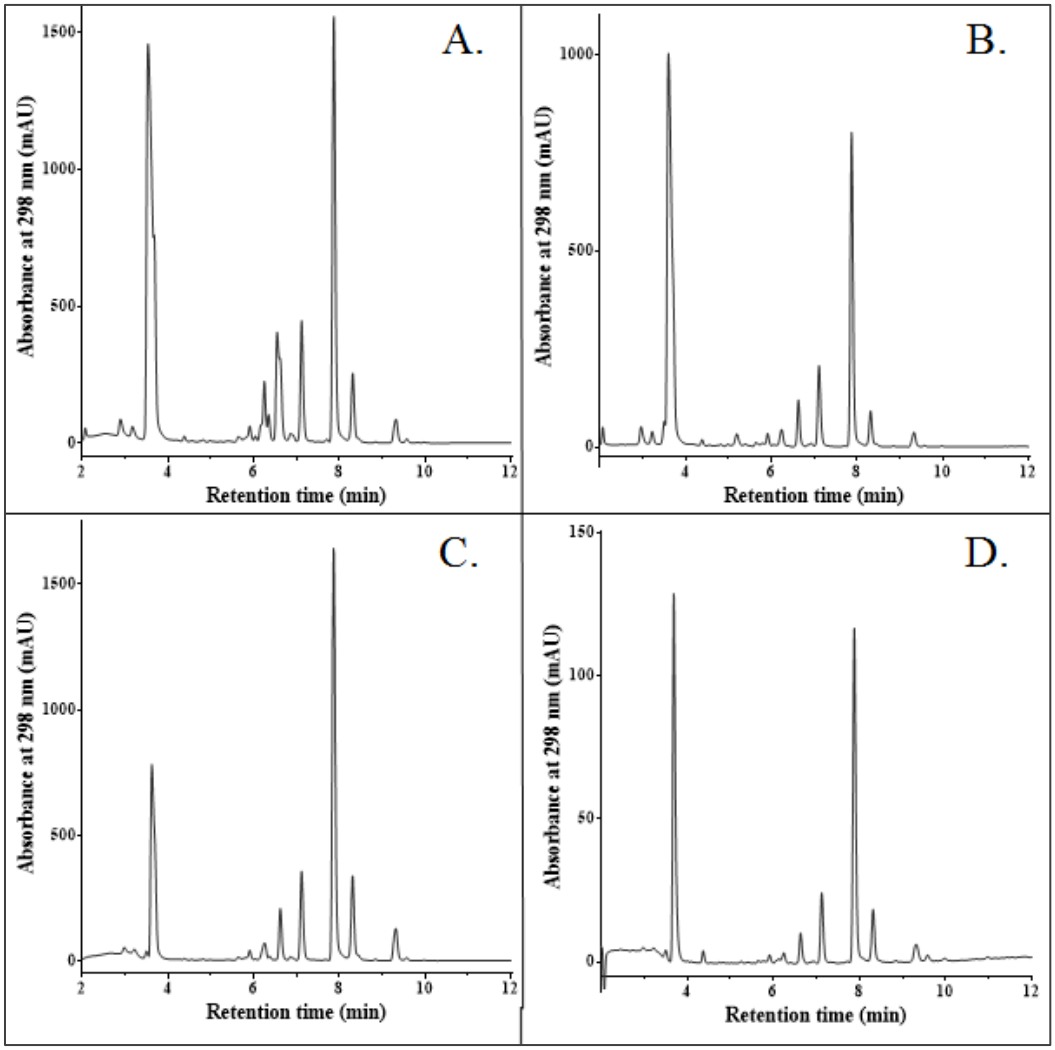

Figure 22 – Chromatograms of T2 parotoid (A) toxins extracted in MeOH on day 0; (B) toxins extracted in H_2O on day 0; (C) secretion extracted in MeOH on day 0; (D) toxins extracted in MeOH on day 28

In the first batch, a great variety and quantity of bufotoxins and arginine-pimeloyl esters were found. Bufagenins could only become the major sub-class of bufadienolides after the hydrolysis of *in situ* bufotoxins. The cell walls of the microglands respectively enclosing the BtH and bufotoxins had to be disturbed to allow the hydrolytic interaction. The motor vehicle collision and the natural decomposition of dead tissue were assumed to bring that outcome. Although the hydrolysis of bufotoxins by the catalysing BtH is a rapid process [83], the uniformity of the parotoid toxin profiles came rather quickly considering that they were quite unique on day 0. The observed profiles dominated by Mbg demonstrated similarities with the analysis of parotoid secretion described in the literature [58,78,83] and for T2/T4. The dissection operated on day 0 may have therefore accelerated the process by disturbing the microglands cell walls and subsequently, brought the confined BtH to the *in situ* bufotoxins. The frequent removal of parotoid gland's pieces could have also prevented the development of a microbial population either able to degrade or transform bufagenins. It was, therefore, necessary to leave intact a gland and analyse it as to assess the impact of the method on the results.

b) Right Parotoid Gland

The results were in the continuity of the ones obtained over a month on the left parotoid glands. Chromatograms of the right parotoid gland extracts revealed a similar composition for all toads (A.3) corresponding to the observations made for the left parotoid gland. The analysis of the bufadienolide contents also matched the results previously obtained (Figure 23). More 11hMbg and remaining Mbt were present in T2/T4 than T1/T3. The secretion of toxins might have had an influence. It is especially accurate in the case of Mbt if the productions of BtH and bufotoxins are proportional. Giving the fact that part of the hydrolase was lost during the secretion, the remaining BtH was certainly used out before entirely hydrolysing all bufotoxins.

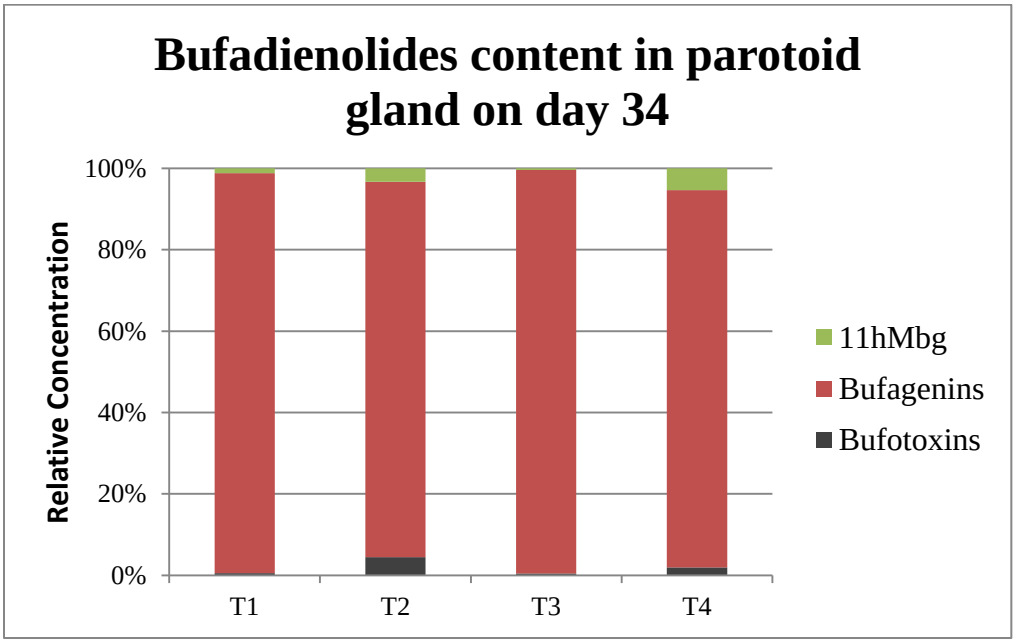

Figure 23 – Bufadienolides content in parotoid gland on day 34

The results suggest that bufagenins within road-killed cane toad parotoid glands remained stable over time and were not degrading. Mbg stayed at a constant level and was still the major components of road-killed cane toad parotoid toxins after more than a month since death occurred. They can, therefore, explain the enduring toxicity attributed to road-killed cane toads [82]. No oxidised analogues appeared during that period meaning that the degrading bacteria either were not grown or active. Hydroxylated analogues were represented by 11hMbg right after the bufotoxins hydrolysis. Its level was hardly detectable and remained low throughout the study suggesting that the transforming bacteria were active only for a limited period of time.

4.2.2. BIODEGRADATION/BIOTRANSFORMATION OF MARINOBUFAGENIN

Parotoid gland-associated bacteria that were proven to degrade or transform bufagenins in previous studies are kept in the Capon group glycerol stock. They were cultured and challenged with Mbg to control that they kept their degrading/transforming ability, to help describe the biodegrading and biotransforming process as well as to select a representative Gram- and Gram+. Four extractions of the culture media were performed in a week and analysed through analytical systems as described in 3.2.2.

The evolution of the Mbg level for each parotoid gland-associated bacteria is represented in Figure 24. All the bacteria underwent a reduction of their Mbg level except Acineto2 which kept a Mbg level constant around 78 %. Acineto2, thus, lost its degrading activity during storage. In the case of Gram- (blue), the Mbg levels decreased at a similar rate and seemed to reach a minimum on day 5 as the Mbg concentrations remained constant until day 7. Flavo hit the minimal value (27.1 %) and was selected as the Gram- reference. In the case of Gram+ (green), the Mbg levels constantly decreased but at different rates. The values on day 7 did not look like minima and it seemed that the Mbg levels could have declined more. G2S14 had the quickest rate of transformation and hit the minimal value (37.3 %). However, the result on day 3 (55.2 %) was confusing because it was not in the trend of the three other time points. Despite being certainly an odd point, G2S14 was left aside and G2S7 was selected as Gram+ reference for its constancy.

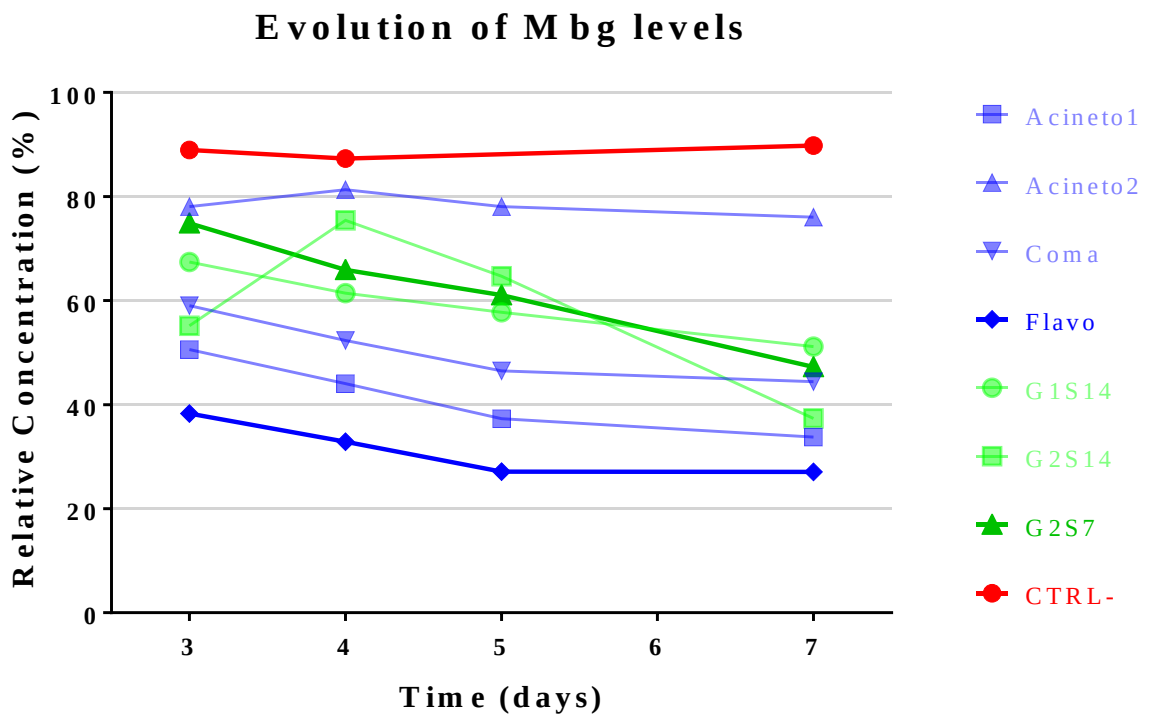

Figure 24 – Evolution of Mbg levels through time

a) Biodegradation

Flavo displayed a profile similar to Acineto1 and Coma. The main oxidised analogues produced were oBlin (**11**), oMbg (**9**) and oRbg (**10**) (Table 15; Figure 12). A typical chromatogram is given in Figure 25. Those results correspond to what was previously obtained with *Comamonas testosteroni* by *Hayes et al.* [78].

Table 15 – Oxidised analogues resulting from the bacterial degradation of Mbg by *Flavobacterium sp.*

#	RT (min)	Compound	Molar mass (g/mol)	Molecular Formula
9	7.4	oMbg	398.21	$C_{24}H_{30}O_5$
10	8.9	oRbg	378.18	$C_{24}H_{26}O_4$
11	9.3	oBlin	380.20	$C_{24}H_{28}O_4$

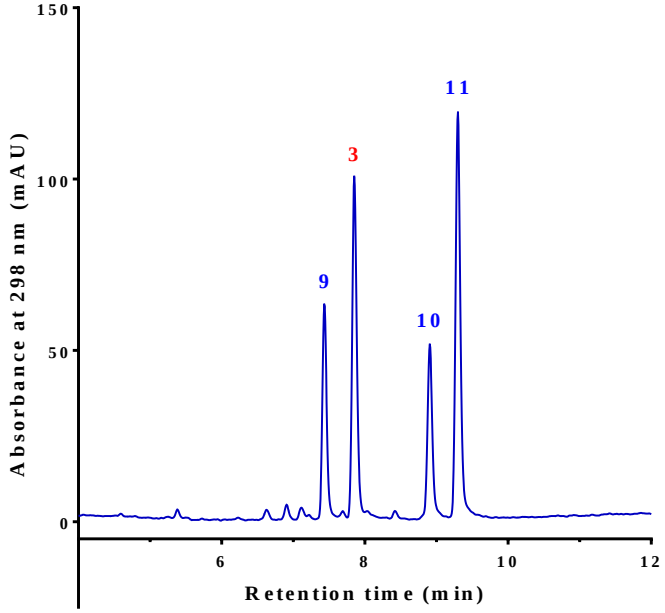

Figure 25 – Chromatogram of Mbg degrading profile by Flavobacterium sp. on day 7

The evolution of the formation of oxidised analogues when Flavo was challenged with Mbg is given in Figure 26. On day 3, the three main degradation products were already formed: oMbg was the major one followed by oBlin then oRbg. oMbg level stayed constant until day 4 where it decreased. On the contrary, oRbg and oBlin constantly increased, even when Mbg seemed to mark a plateau. oBlin finished as the main oxidised products, above the Mbg level. It seems that the microbial degradation follows a sequence where Mbg must be first oxidised into oMbg before degrading into oRbg and oBlin. The final product, oBlin, cannot be obtained without passing by intermediates first, oMbg and oRbg. This joins the possible sequence of biodegradation suggested by Hayes *et al.* (Figure 13).

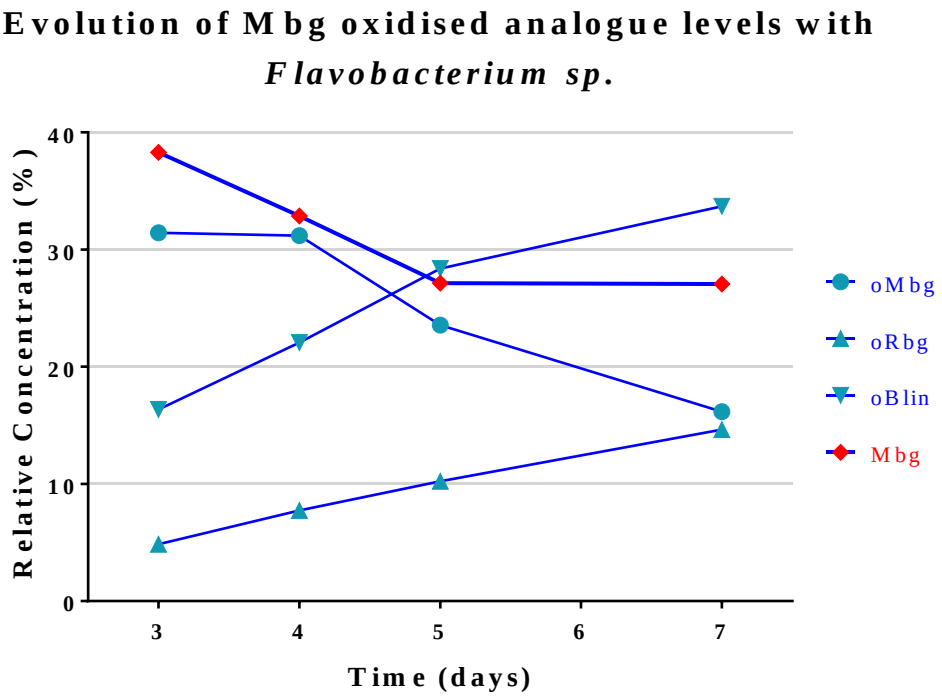

Figure 26 – Evolution of Mbg oxidised analogue levels with Flavobacterium sp.

b) Biotransformation

G2S7 displayed a profile similar to G1S14 and G2S14. The main hydroxylated analogues produced were 11hMbg and 17hMbg (Table 16; Figure 11). A typical chromatogram is given in Figure 27. The small peak present between 11hMbg (**12**) and 17hMbg (**13**) was associated with an unknown hydroxymarinobufagenin but at a level too low to be characterised while the peak in 7.1 min of retention time (*****) was an artefact also present in the control sample. A significant fronting can be observed for 11hMbg as well as a tailing for 17hMbg. When compared with the results of Dr Kamalakkannan [86], the profiles looked similar although a third hydroxylated analogue was described, 12hMbg, and appeared around 5.7 min. Consequently, 12hMbg was precisely hidden in the fronting of 11hMbg. By the same reasoning, 17hMbg tailing could overlay another unknown hydroxylated analogue.

Table 16 – Hydroxylated analogues resulting from the bacterial transformation of Mbg by *Bacillus sp.*

#	RT (min)	Compound	Molar mass (g/mol)	Molecular Formula
12	5.8	11hMbg	416.22	$C_{24}H_{32}O_6$
13	6.2	17hMbg	416.22	$C_{24}H_{32}O_6$

* Artefact also present in the CTRL-

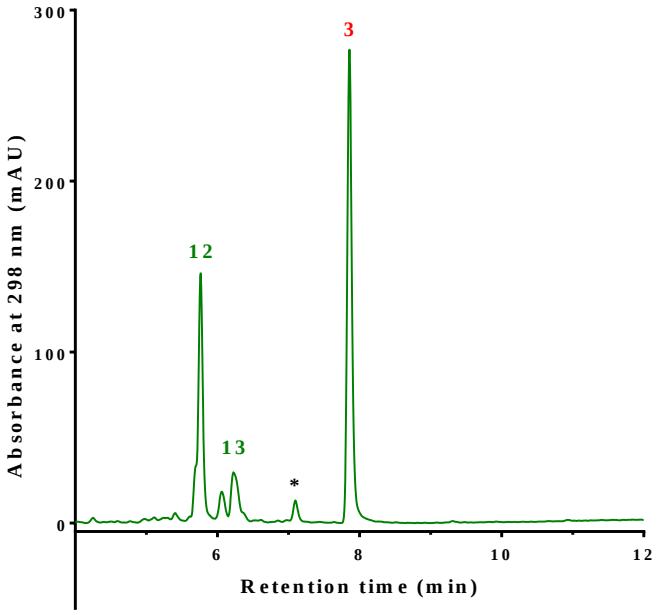

Figure 27 – Chromatogram of Mbg transforming profile by Bacillus sp. on day 7

The evolution of the formation of hydroxylated analogues when G2S7 was challenged with Mbg is given in Figure 28. On day 3, the two main transformation products were formed: 11hMbg was the major one followed by 17hMbg. They marked a steady upward trend over the five days of measurement but 11hMbg had the fastest rate of emergence and finished over 25 % on day 7 while 17hMbg did not reach 10 %. The formation of hydroxylated analogues seems to happen simultaneously. No intermediates are needed and the different transformation products independently appear.

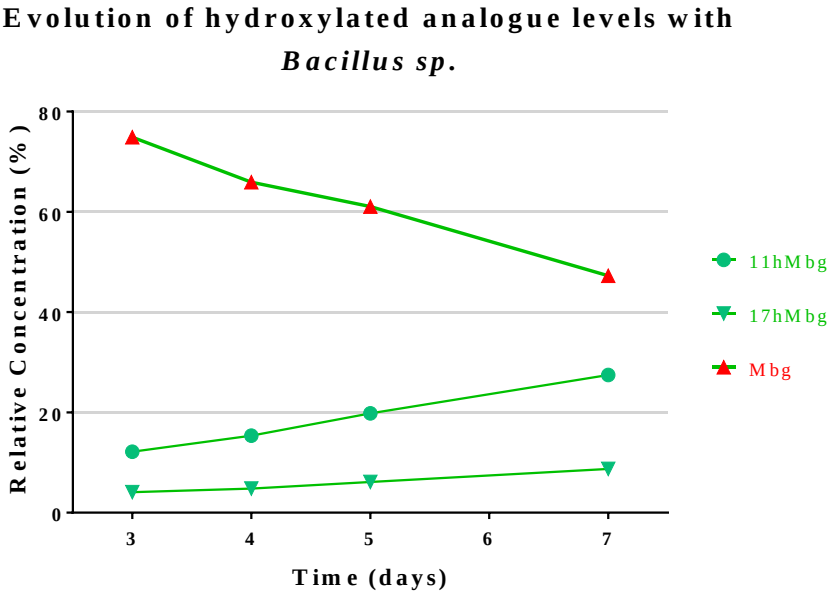

Figure 28 – Evolution of Mbg hydroxylated analogue levels with *Bacillus sp.*

4.2.3. BIODEGRADATION/BIOTRANSFORMATION OF STEROIDS

As degrading and transforming references, Flavo and G2S7 were challenged with common and peculiar steroids: MbgPal, Pregne and Testo. Four extractions of the culture media were performed in a week and analysed through analytical systems as described in 3.2.3. Pregne and Testo as precursors of bufadienolides were structurally relevant. MbgPal was close related to bufolipins which were ecologically relevant and served as a preliminary test compound. Unfortunately, the results showed that no MbgPal was present in the stock vial and could not be exploited (A.3). Moreover, Flavo did not grow in the culture tube challenged with Testo and Mbg (CTRL-) and did not provide any results.

a) Biodegradation

In the case of Pregne, the maximum absorbance was detected at an optimal 210 nm. Most of the media compounds were also detected at that UV wavelength and created complex profiles. The zone of interest was between 5.7 and 12 min of retention time where the peaks of Pregne and a potential degradation product (DP) appeared (Figure 29). The detected levels were low throughout the experiment and the noise level was critical. The QTOF results were inconclusive and could not confirm the nature of the DP. Any conclusion must be cautiously taken given the results but if biodegradation occurred, it was definitely different from what is described above as only one major compound was produced.

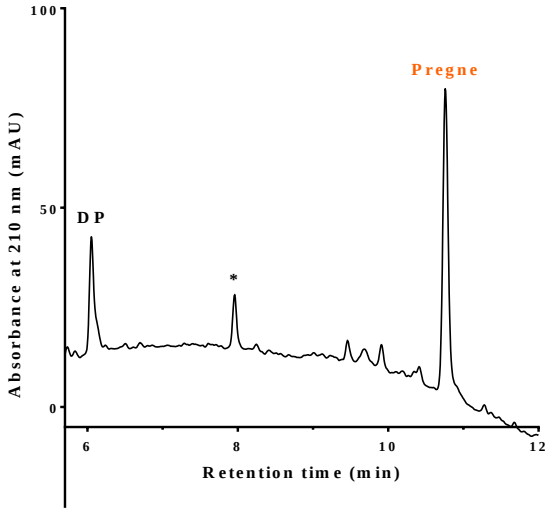

Figure 29 – Chromatogram of Pregne and DP resulting from the activity of *Flavobacterium sp*. on day 7

b) Biotransformation

In the case of Pregne, the zone of interest was between 4.7 and 12 min of retention time where the peaks of Pregne and potential transformation products (TP) appeared (Figure 30). The TP between 4.7 and 5.7 min of retention time overlayed peaks originated from the media. Despite a weaker noise, the QTOF results were inconclusive and could not confirm the nature of the TP. Any conclusion must be cautiously taken given the results but if biotransformation occurred, it was definitely different from what is described above as more major compounds were produced demonstrating the activity of G2S7 on Pregne.

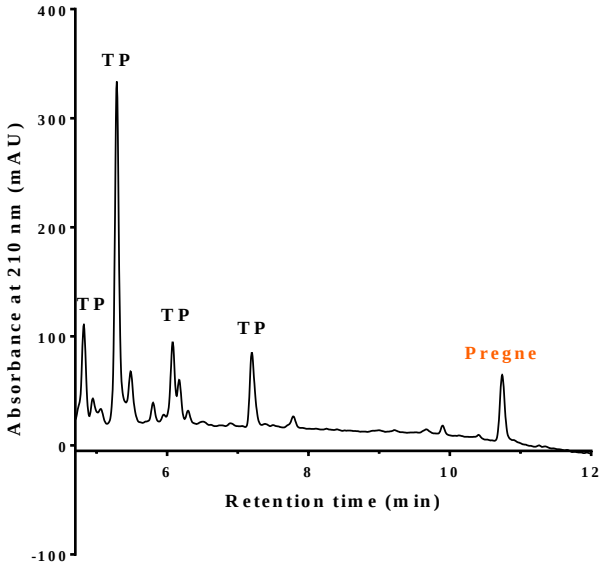

Figure 30 – Chromatogram of Pregne and TP resulting from the activity of *Bacillus sp.* on day 7

In the case of Testo, the maximum absorbance was detected at an optimal 254 nm. The zone of interest was between 4.7 and 5.7 min of retention time where the peaks of Testo and several TP appeared (Figure 31). The observed profile was more complex than the one of Pregne. Despite a noise level more acceptable than for Pregne, the QTOF results were inconclusive and could not confirm the nature of the TP. Any conclusion must be cautiously taken given the results but if biotransformation occurred, it was definitely different from what is described above as more major compounds were produced demonstrating the activity of G2S7 on Testo.

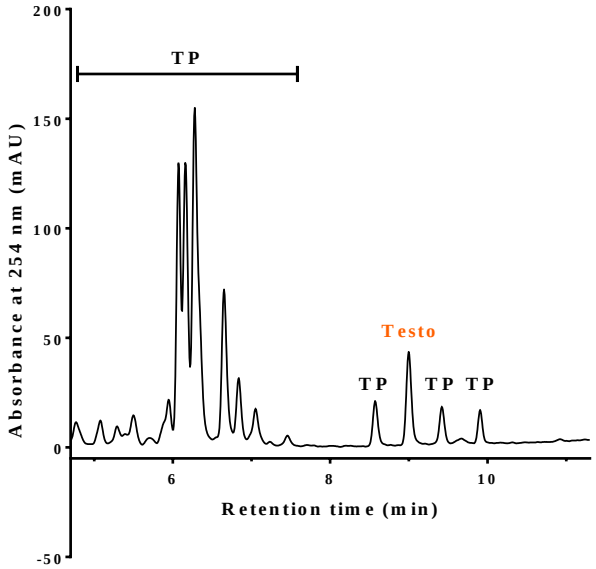

Figure 31 – Chromatogram of Testo and TP resulting from the activity of *Bacillus sp.* on day 7

4.2.4. BIODEGRADATION/BIOTRANSFORMATION OF BUFOLIPINS

In the absence of MbgPal results, it was finally decided to preserve the bufolipin stock and not to use any in this experiment as explained in 3.2.4.

4.2.5. Isolation Of Optimised Degrading/Transforming Strains

Parotoid gland-associated bacteria from four different road-killed cane toads were isolated, cultured and challenged with Mbg. The degrading/transforming products were then extracted on day 5 and analysed through analytical systems as described in 3.2.5. The first step of the experiment gave four master plates representative of the microbial population concealed inside the parotoid glands. They looked all different. For each plate, heterogenic colonies were marked and sub-cultured as to be isolated: 13 strains from T1, 3 from T2, 8 from T3 and 11 from T4. The isolates were then visually compared as to work on different strains only and preserve the Mbg stock. This step reduced the number of plates from 35 to 17. After analysis, 10 strains out of 17 showed activity when challenged with Mbg: 4 with a degrading profile (chromatograms similar to Figure 25), 5 with a transforming profile (chromatograms similar to Figure 27), 1 with an unexpected profile. At least one degrading and one transforming strain could be linked to each roadkill confirming their presence within parotoid glands.

a) Biodegradation

The presence of oxidised analogues demonstrates the degradation of Mbg mediated by the activity of Gram-. In this experiment, 4 strains out of 17 met those criteria and were, therefore, assumed to be Gram-: T1S4, T1S5, T2S2 and T3S3. It has to be noted that the chromatograms of CTRL+ and CTRL- perfectly matched meaning that Flavo lost its degradation activity on Mbg (A.4). The media could be at fault as bacteria could find all the nutrients that they needed in the NAg and NB. Multiply the sub-cultures could, therefore, promote the use of the nutrients found in the media to the detriment of the Mbg metabolic pathway. The plate containing the isolated colony where this strain of Flavo was originated from was not used in further experiments.

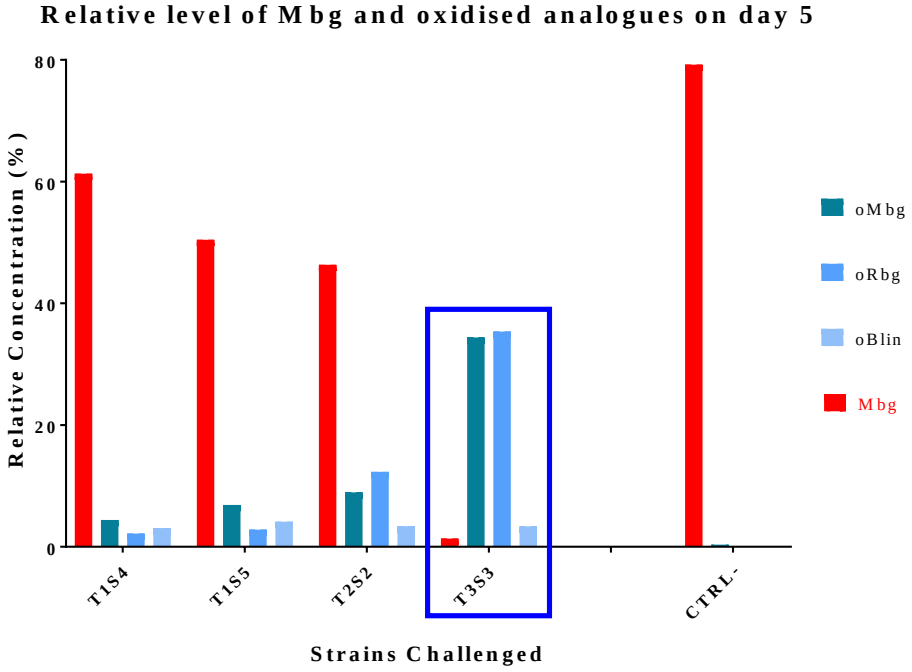

Figure 32 – Relative level of Mbg and oxidised analogues on day 5

In the absence of CTRL+, the relative level of Mbg and oxidised analogues were compared with CTRL- and are presented in Figure 32. The relative levels of Mbg fell and the three oxidised analogues presented earlier – oMbg, oRbg and oBlin – appeared in all strains. This was confirmed by a co-injection analysis (Figure 33) and QTOF results (A.4). T3S3 stood out as it had almost entirely degraded Mbg and possessed significant levels of oMbg and oRbg. Interestingly, its profile was slightly different from what was observed earlier as its major and minor oxidised analogues were respectively oRbg and oBlin on day 5 while it was the contrary for Flavo and the other Gram- from the Capon group glycerol stock (Figure 26). This was also observed in T2S2 which could mean that those strains belonged to a gender not described yet. Taxonomic results should confirm it. Three strains originated from T4 were discarded because they visually appeared similar to T3S3. It was, therefore, assumed that T3S3 was found in 2 road-killed cane toads out of 4 which made this strain ecologically relevant. Taxonomic results should confirm it. For all the reasons here described, T3S3 was selected for further experimentations.

As stated earlier, a co-injection experiment was conducted on samples of T3S3 (deep-well, day 5) and Flavo (culture tube, day 7; 4.2.2) on 15th June 2017 as described in 3.2.5 and shown in Figure 33. The chromatograms confirmed that oRbg (**10**) and oBlin (**11**) were present in both samples but oMbg peak (**9**) was missing from the Flavo profile. The oBlin peak had considerably increased too which hinted that the degradation sequence was continuing within the Flavo sample despite the vial being sealed with paraffin film and kept from the light at ambient temperature after HPLC injection on 15th May 2017. No bacteria could be active in the samples because all the extracts were dissolved in MeOH and filtered before injection. This suggests that the oxidation of Mbg into oMbg must be catalysed by Gram- while the subsequent oxidoreduction reactions into oRbg and then oBlin do not need to be mediated by the activity of bacteria.

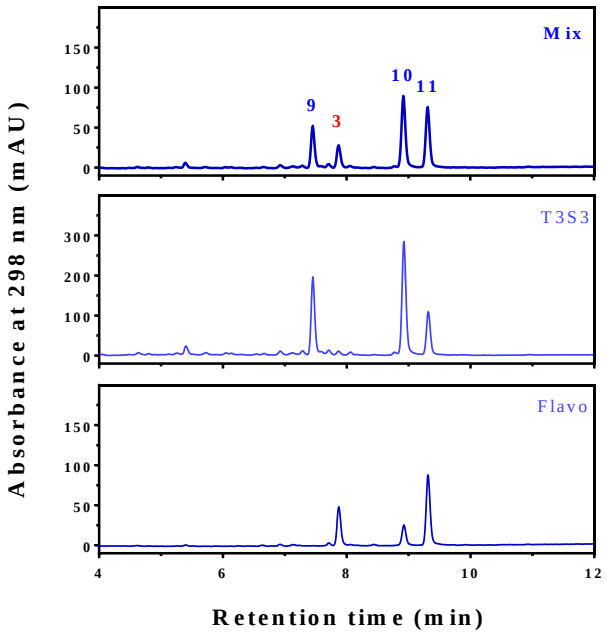

Figure 33 – Co-injection between T3S3 (deep-well, day 5) and *Flavobacterium sp.* (culture tube, day 7)

b) Biotransformation

The presence of hydroxylated analogues demonstrates the transformation of Mbg mediated by the activity of Gram+. In this experiment, 5 strains out of 17 met those criteria and were, therefore, assumed to be Gram+: T1S1, T1S13, T2S3, T3S6 and T3S7. G2S7 was used as CTRL+ and provided a profile similar to what was previously observed except for the proportions. The level of Mbg went down noticeably in this experiment after 5 days of challenge (16.1 %; Figure 34) than in the previous one after 7 days of challenge (47.3 %; Figure 28). The opposite was observed when the formations of 11hMbg and 17hMbg were compared. This suggests that the experiment in deep-well is more suited to transforming Gram+ than in culture tubes.

The relative level of Mbg and hydroxylated analogues were compared with CTRL+ and CTRL- and are presented in Figure 34. The relative levels of Mbg fell and the two hydroxylated analogues presented earlier – 11hMbg and 17hMbg – appeared in all strains. This was confirmed by a co-injection analysis (Figure 35) and QTOF results (A.4). T1S13, T2S3 and T3S7 were brought out. Mbg level of T3S3 reached a lower point than the CTRL+ while it levelled out for the two other strains. 11hMbg was the major bufadienolides in T2S3 and T3S7 even though it did not overtake the level exhibited by the CTRL+. By the same reasoning used for T3S3, T1S13 was assumed to be found in 3 road-killed cane toads out of 4, 2 out of 4 for T3S7 and 1 out of 4 for T2S3 which made those two first strains ecologically relevant. Taxonomic results should confirm it. More 11hMbg and 17hMbg were produced with T2S3 than with T3S7. However, in the case of T3S7, Mbg marked a greater decreased which implied that it was biotransformed into other forms of hydroxymarinobufagenin (see below). For all the reasons here described, T3S7 was selected for further experimentations.

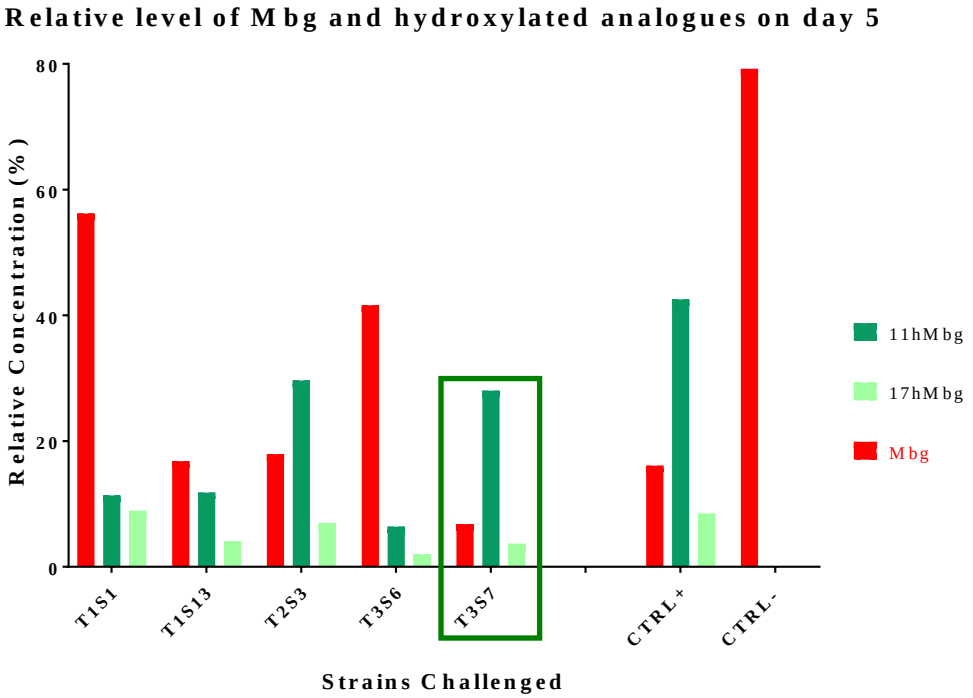

Figure 34 – Relative level of Mbg and hydroxylated analogues on day 5

As stated earlier, a co-injection experiment was also conducted on samples of T3S7 and G2S7 (deep-well, day 5) as described in 3.2.5 and shown in Figure 35. The chromatograms confirmed that 11hMbg (**12**) and 17hMbg (**13**) were present in both samples. They also underlined the presence at low levels of other peaks associated with hydroxylated analogues in T3S7. The tailing observed for 17hMbg in G2S7 is replaced with a resolute peak in T3S7 and supports the idea that the fronting of 11hMbg hides the missing 12hMbg as suggested in 4.2.2.

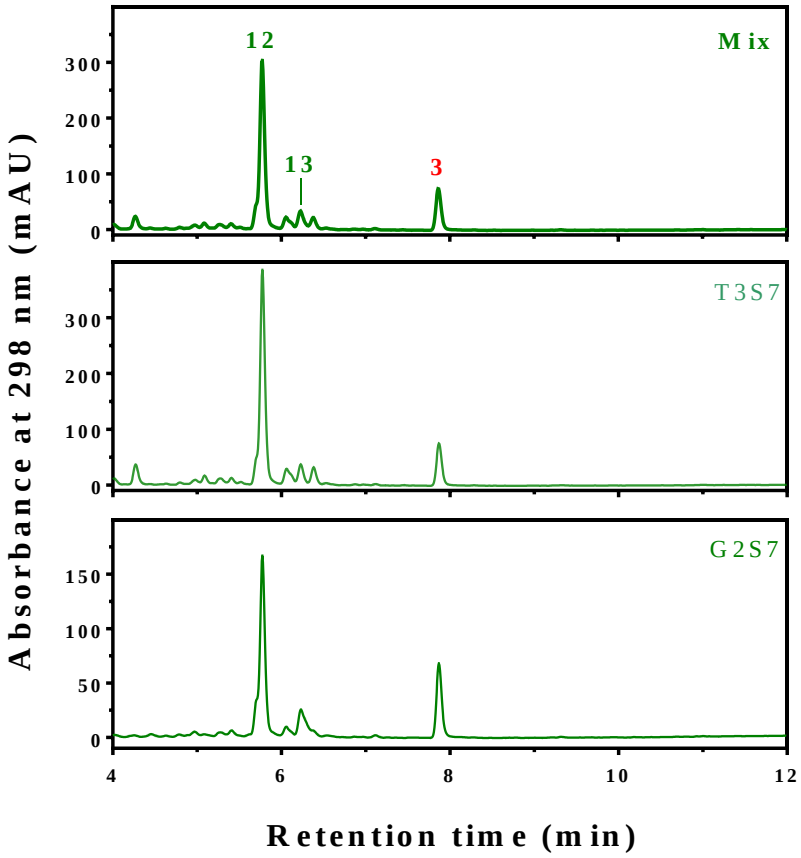

Figure 35 – Co-injection between T3S7 and *Bacillus sp.* (deep-well, day 5)

c) Unexpected

The chromatogram of T1S12 did not match any of the profile previously described as shown in Figure 36. An undescribed peak at a level never equalled in this study appeared around 6.0 min of retention time. However, the Mbg peak (**3**) had an absorbance similar to the CTRL- which suggested that no transformation occurred over the time of the experiment. The QTOF results revealed the presence of aspergillic acid (**14**) (Figure 37), a naturally occurring hydroxamic acid produced by certain strains of the fungus *Aspergillus flavus* and known for its antibiotic and antifungal properties [93,94]. T1S12, therefore, produced the aspergillic acid independently or in response to the presence of Mbg. The taxonomic results should confirm T1S12 fungal nature.

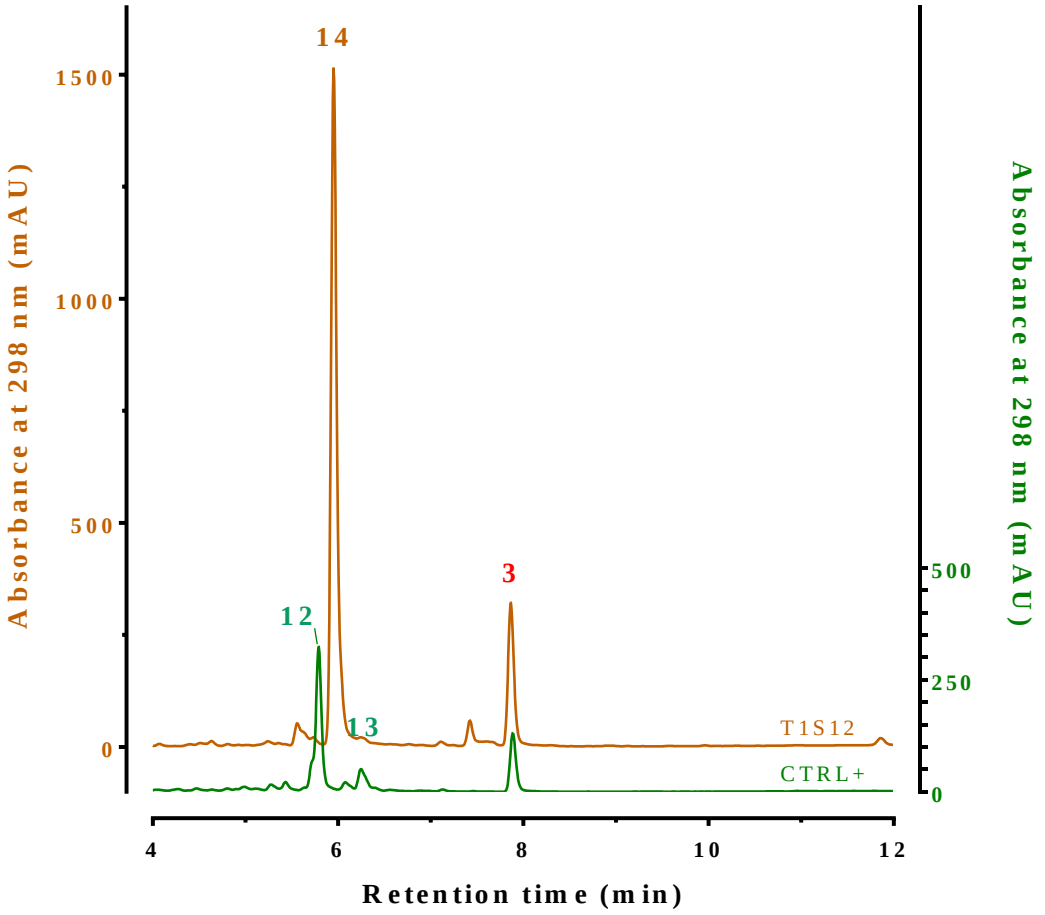

Figure 36 – Stacked chromatograms of T1S12 and CTRL+

Table 17 – QTOF results: Aspergillic acid from T1S12

#	RT (min)	Compound	Calculated mass (m/z) $(M+H)^+$	Measured mass (m/z) $(M+H)^+$	ΔmDa	Molecular Formula
14	6.0	Aspergillic acid	224.1524	224.1527	-0.3	$C_{12}H_{20}N_2O_2$

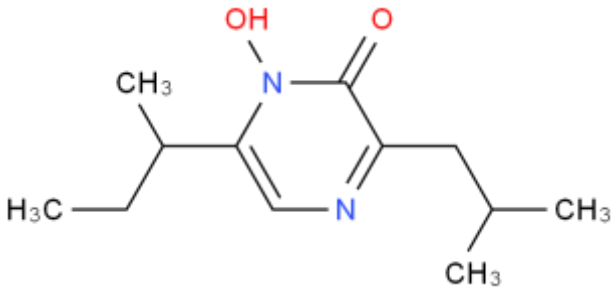

Figure 37 - Aspergillic acid

4.2.6. Evaluation Of Biodegradation/Biotransformation Capacity

In this experiment, the selected strains isolated from road-killed cane toad parotoid glands were cultured and challenged with Mbg in three replicates as to assess their capacity. Five extractions of the culture media were performed in a week and analysed through analytical systems as described in 3.2.6. The results described in 4.2.5 pointed out the loss of activity of *Flavobacterium sp.* In order to avoid such scenario, the Gram- and Gram+ reference were retrieved from the Capon group glycerol stock and reactivated on solid media before to be sub-culture for this experiment purpose. Despite this effort, the Flavo and CTRL- chromatograms matched again meaning that Flavo results could not serve as control and base of comparison.

a) Biodegradation

The selected and tested parotoid gland-associated strain assumed to be Gram- through its degrading profile was T3S3 (4.2.5). The reduction of Mbg and formation of oxidised analogue is given in Figure 38. Means (n = 3) and standard deviations are represented on the graph. T3S3 still degraded Mbg but not in the same proportions as in the deep-well. The bufadienolide profile and evolution were more linear and constant than what had been previously observed in culture tubes with Flavo. Despite some fluctuations, the Mbg level decreased following a steady downward trend while the oxidised analogue slowly increased. The profile evolution suggested that the biodegradation was still in the first step of the process where the oMbg level was increasing. In the case of Flavo, the Mbg level had reached a plateau on day 5 (Figure 26) which had been a sign of the shift in the phases. It did not occur in this experiment which consequently meant that T3S3 degraded Mbg at a slower rate than Flavo earlier.

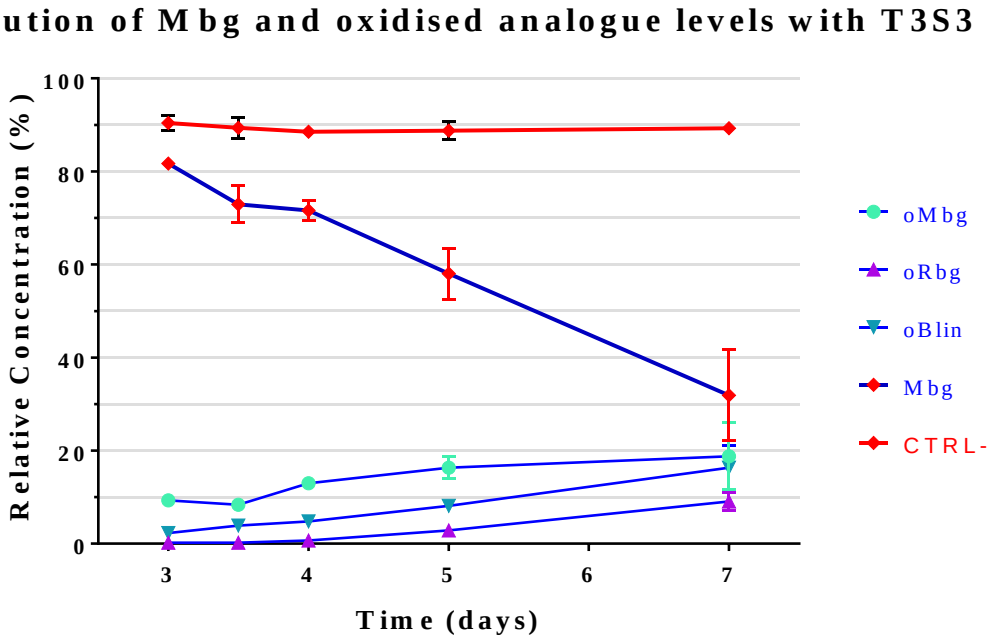

Figure 38 - Evolution of Mbg and oxidised analogue levels with T3S3

The oxidised analogue evolution supported this hypothesis. As shown in Figure 39, the major and minor compounds were respectively oMbg and oRbg from day 3 to day 7. Despite the high variability of the means at the last time point, independent results of the three replicates confirmed it (see chromatogram in Figure 40 for example). Flavo profile had previously revealed that a second phase of the degradation process was characterised by the regression of oMbg as it is an intermediate in the formation of oRbg and oBlin (Figure 13). It did not occur in this experiment. However, the rate of progression of the oMbg level lessened while it grew in parallel for the two other products. This hinted that T3S3 was still in the first phase of the degradation process.

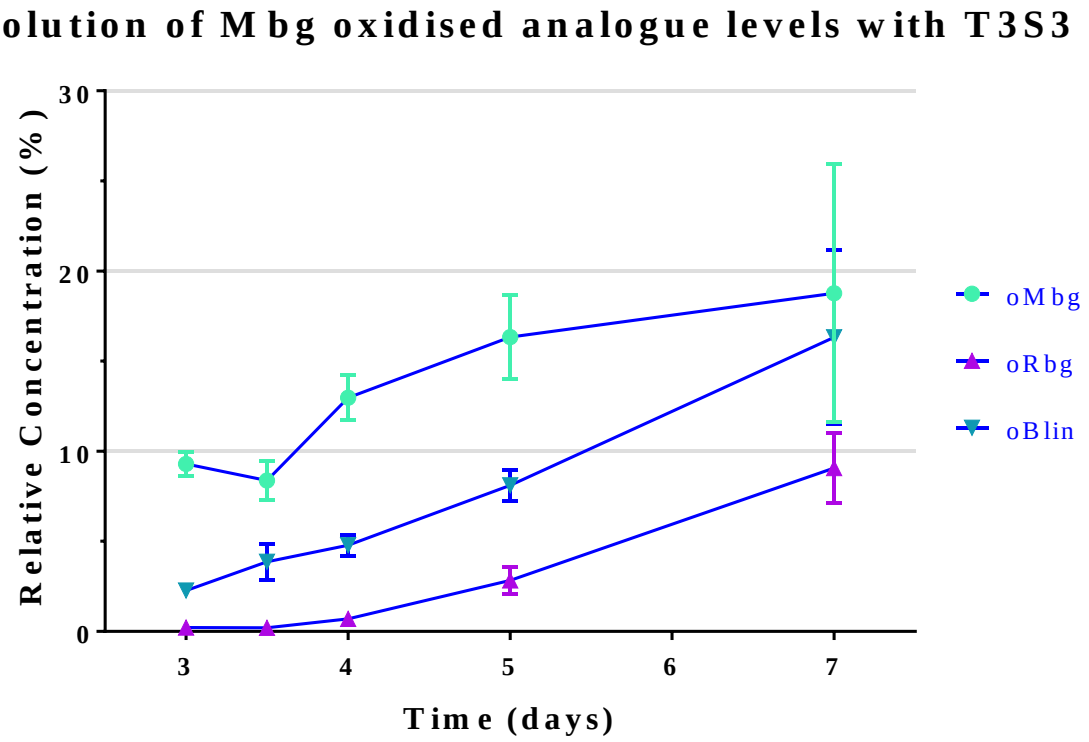

Figure 39 – Evolution of Mbg oxidised analogue levels with T3S3

The comparison between chromatograms of T3S3 and Flavo challenged with Mbg in culture tube as shown in Figure 40 illustrates the two step-process described above for the evolution of oMbg level. The peak of oMbg (**9**) dominates the ones of oRbg (**10**) and oBlin (**11**) in T3S3 chromatogram. In the first phase of the biodegradation, oMbg is the first oxidised analogue to emerge from the challenge with Mbg (**3**) resulting in the increase of its concentration through time. The oBlin peak dominates the other forms in Flavo chromatogram. The second phase develops when oMbg reaches its maximal concentration, keeps a dynamic rate of oxidoreduction and finally drops to the profit of the two other oxidised analogues.

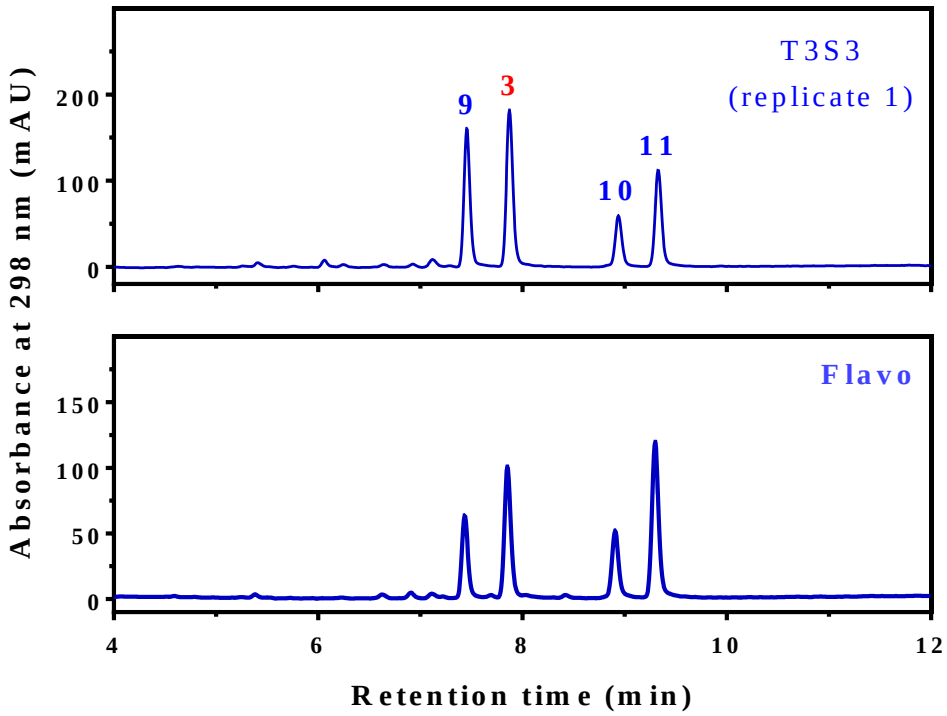

Figure 40 – Stacked chromatograms of T3S3 and Flavobacterium sp. degrading profiles on day 7

In the deep-well experiment, T3S3 hardly left any Mbg that it was challenged with. In addition, the strain was in the first phase of the biodegradation process as explained above. The development of the bacteria is linked to the medium but also container in which they grow and the deep-well seemed better suited T3S3 to degrade Mbg than the culture tubes.

a) Biotransformation

The selected and tested parotoid gland-associated strain assumed to be Gram+ through its transforming profile was T3S7 (4.2.5). The reduction of Mbg and formation of hydroxylated analogues could be compared with a CTRL+. Means (n = 3) and standard deviations are represented on both graphs given in Figure 41. Mbg levels from both Gram+ marked a downward trend while the opposite was observed for 11hMbg. The evolution of 17hMbg showed a difference between the two strains as the level was almost constant in T3S7 while it slightly increased in *Bacillus sp.*T3S7 showed better capacity in terms of reduction of Mbg and production of hydroxylated products than *Bacillus sp*. However, it was impossible to evaluate how better the transformation was as the standard deviations affected to the Mbg means translated a great dispersion of T3S7 values. One of the replicate had a level of Mbg significantly lower than the two others and explained the variability displayed. Even though those standard deviation values could be due to the inherent biological variability, the lack of Mbg injected to the culture tube at the start of the challenge is most likely the explanation to the results.

Although the rates of formation were slow in both cases, the emergence of hydroxylated analogues responded to what was described earlier. 11hMbg was the dominant figure and seemed to evolve independently from 17hMbg. It has to be noted that the reduction of Mbg and formation of hydroxylated products were critically more important after 5 days of challenge in deep-well (Figure 34) than after 7 days in culture tubes. As stated for the biodegradation, the development of bacteria is linked to the medium but also container in which they grow and the deep-well seemed better suited Gram+ to degrade Mbg than the culture tubes.

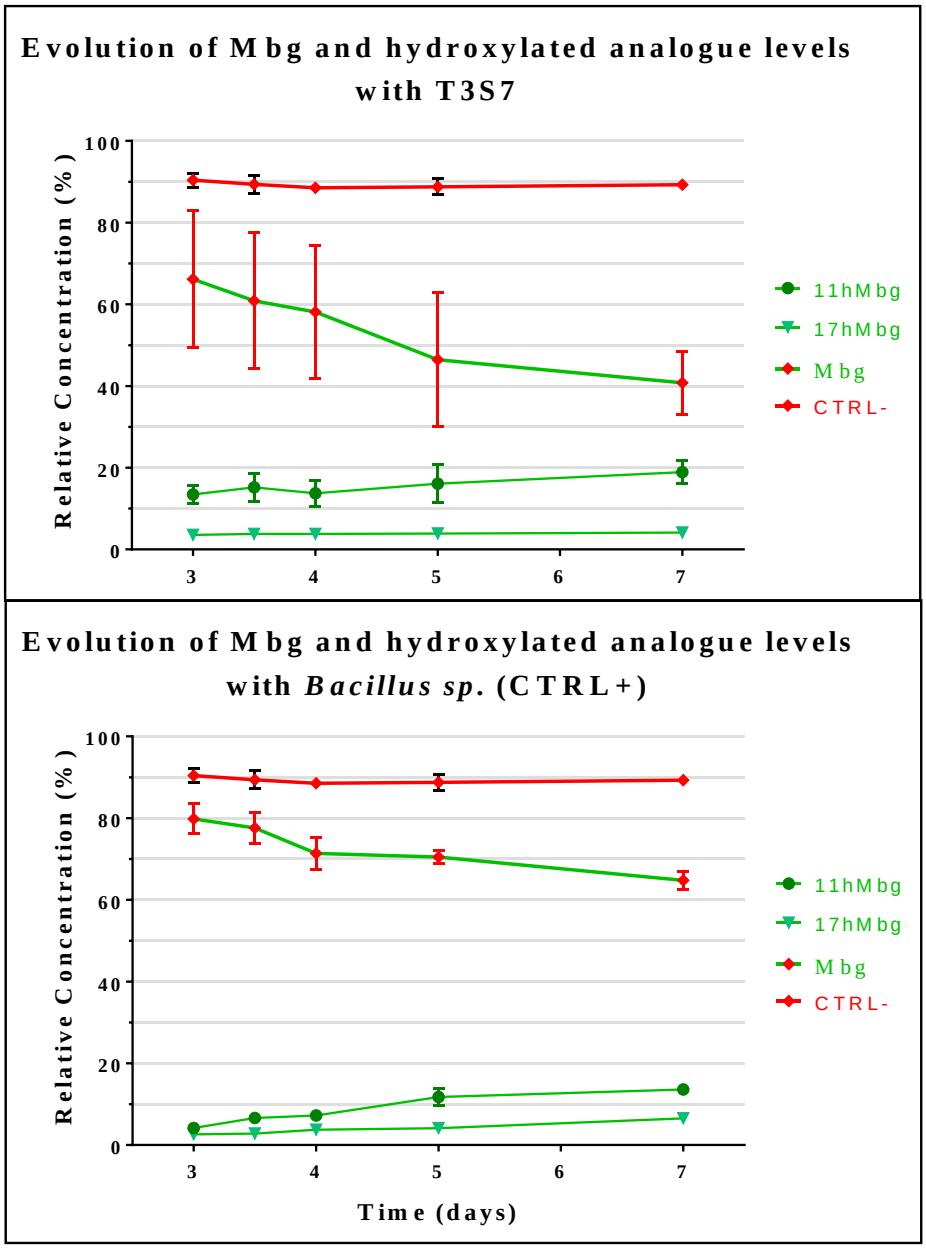

Figure 41 – Comparison between the evolution of Mbg and hydroxylated analogue levels with T3S7 and CTRL+

4.2.7. BIODEGRADATION/BIOTRANSFORMATION OF TELOCINOBUFOTOXIN

Bufotoxins are the storage form of bufadienolides. Mbt is the major bufotoxins within parotoid glands but co-exists with multiple forms as confirmed in Table 13. Dr Kamalakkannan hypothesised that bufotoxins evolved under the activity of Gram+ but also demonstrated that they were microbiologically stable against Gram- activity [83]. This contrasted with the bufagenins which were prone to microbial degradation. The new Gram- and Gram+ selected strains were, therefore, challenged with a solution assumed to contain Tbt. The results showed an activity of both Gram- and Gram+ but the CTRL- chromatogram demonstrated that the solution did not contain Tbt (A.5, A.5), its peak emerging round 7.1 min of retention time with the HPLC method. The results were, therefore, unexploited.

4.2.8. PURIFICATION OF BIODEGRADATION/BIOTRANSFORMATION PRODUCTS

In order to isolate and purify biodegradation and biotransformation products, a scale-up experiment was designed. The pure compounds that were to be yield would have been used to characterised the oxidised and hydroxylated analogues hardly detectable on a classic analytical HPLC system. The case of the fungal strain was also investigated. T3S3, T3S7 as well as T1S12 were cultured in 100 mL NB. They were to be challenged and analysed as described in 3.2.8 but time went missing so it was finally decided to preserve the stock of Mbg and the experiment was discontinued for the biodegrading and biotransforming strain.

Worth a note, preliminary tests in culture tubes were conducted as to determine if T1S12 produced aspergillic acid in response to the presence of Mbg or not. The results showed that the strain independently produced it (A.6) and were, as for Flavo, more performant in deep-well or culture flask than in culture tubes.

4.2.9. TAXONOMIC DETERMINATION OF MICROORGANISMS

The sequencing results did not arrive at the time of completion of this Master Thesis and could not be included. The taxonomic characterisation could not, subsequently, take place. It has to be noted that electrophoresis gel revealed under UV light that the DNA from the road-killed cane toad parotoid gland-associated bacteria assumed to be Gram+ were effectively extracted and amplified by PCR only. As expected, this technique did not work for the strains assumed to be Gram- and the use of a DNA extraction kit was necessary. Those observations reinforce the assumptions made and support the fact that the degradation profile and oxidised analogues are related to Gram-, while the transformation profile and hydroxylated analogues are related to Gram+.

GENERAL DISCUSSION

The bufadienolides profile of parotoid glands in decomposition showed the stability of the bufagenins over time. This was observed on four different carcasses and could not be attributed to a coincidence. The profile generated on the day of arrival of the roadkill demonstrated that bufotoxins were still present at a certain level within the parotoid toxins, which contrasted with the results obtained in further batches. In living cane toads, bufadienolides are stored within parotoid glands as bufotoxins. On the contrary, the bufagenins level quickly shot up in roadkill and confirmed that the first step in the evolution of the bufadienolides profile was the hydrolysis of bufotoxins into bufagenins. This reaction was known to be mediated by the enzymatic activity of BtH [83] and be necessary to obtain the chemical composition affecting Australian native predators. The chemical composition of parotoid secretion and road-killed cane toads in decomposition were, therefore, close related to each other as confirmed by the analysis on the water extracts and parotoid secretions. The road-killed cane toads were known to remain toxic [82]. This toxicity could now be attributed to bufagenins and especially Mbg. Giving that a living cane toad is mainly composed of bufotoxins, the bufadienolides composition of roadkill suggested that they could affect more quickly an animal that would attempt to eat the carcass as there would be no hydrolysis step needed to obtain bufagenins.

The evolution of the bufadienolide profile was expected to undergo a second step where bufagenins would progressively be replaced with analogue products. Indeed, parotoid glands were known to shelter bacteria able to mediate the reactions leading to the apparition of bufagenin scaffolds. A distinction was made by Dr Kamalakkannan and used in this master thesis according to the type of bacteria catalysing the reaction: degradation for Gram- and transformation for Gram+ [83]. The biodegradation/biotransformation of Mbg operated by bacteria previously isolated on fresh parotoid glands helped describing the differences in both case.

The biodegradation is a reaction resulting in the formation of oxidised analogues. In the case of Mbg, it followed a sequence [78] where oMbg was the first to be formed under the activity of Gram-. The subsequent formations of oRbg then oBlin did not seem to need the mediation of bacteria. Two phases could be described depending on the oMbg level evolution: the increase and the decrease. The shift between those two phases appeared when the rate of formation of oMbg reached zero, overtaken by the ones of subsequent products. oBlin was the end product of the reaction at the end of the experiment. Gram- might benefit cane toads but doubts remained. Oxidised analogue structures are hardly different to bufagenins and raise questions as their toxicity towards Australian native predators. Bufagenins could only be qualified to be degraded if they would lose their inhibition activity against Na^+/K^+-ATPase receptors. If the oxidised analogues were toxic, Gram- would enhance the toad protection by diversifying the bufagenin forms as it was the case for the biotransformation (see below). If they were not, Gram- could protect cane toads from self-intoxication by keeping the Mbg content to a non-lethal level.

In contrast, the biotransformation is a reaction mediated by Gram+ resulting in the formation of hydroxylated analogues. In the case of Mbg, only monohydroxylated analogues independently emerged and their rate of formation increased at a constant rate. The hydroxyl group seemed to have an affinity for the position 11 as the dominant transformation product was 11hMbg. Its rate of formation was certainly overrated in this master thesis as its peak certainly hid the 12hMbg described by Dr Kamalakkannan [86] in its fronting. 11hMbg was the only hydroxylated analogue detected within the parotoid glands in decomposition but remained at a very low level. The development and activity of Gram+ were limited in the parotoid gland conditions and no further change was brought to bufadienolides. The hydroxylated analogues are ecologically relevant as they are found in cane toad eggs and ovaries. They participate to the diversification of the bufagenin structures and increase the range of Na^+/K^+-ATPase isoforms that can be affected. Gram+, therefore, have a positive interaction with their host.

The isolation of bacteria from parotoid glands in decomposition confirmed the presence of strains able to degrade or transform bufagenins [78,86]. At least one Gram- and Gram+ that were able to mediate those reactions were found in each road-killed cane toads which supported the assumptions that bacteria play an ecological role for their host. The results showed great differences in the different profiles and nothing suggested that cane toads in decomposition were a better source of performant degrading/transforming strains than fresh individuals.

Road-killed cane toad parotoid gland-associated bacteria were able to degrade and transform bufagenins but did not perform *in situ*. A first reason that could explain the scission between the results from the natural and experimental substrates is the microbial competition. Bacteria that can be cultured were not representative of the total microbial population within parotoid glands. Although degrading/transforming strains were present, their growth would be limited by bacterial competition *in situ* which would prevent the effective degradation/transformation of bufagenins. A second hypothesis would be that bacteria need water to perform. Indeed, the microbiological experiments that helped assess the biodegradation/biotransformation were designed for liquid culture media. Contrariwise, the road-killed cane toad parotoid gland dried and adopted a cardboard like texture. Water is an essential agent in the activity of bacteria, as a molecular compound or solvent for example. Its evaporation could have limited the biotransformation and would explain why the detected level of 11hMbg stayed constant over time.

Referring to the three objectives pursued in this research project, the discussion developed above helps conclude that:

1) **A constant bufadienolide profile principally composed of bufagenins characterises road-killed parotoid glands in decomposition;**
2) **The microbial degradation and transformation differ in the type of bacteria required (*i.e.* Gram- vs Gram+), the chemical reactions involved (*i.e.* oxidation vs hydroxylation) and the emergence of the products (*i.e.* sequenced vs independent);**
3) **Parotoid glands are a confirmed source of bufagenin degrading and transforming bacteria.**

CONCLUSION & PERSPECTIVES

The cane toad chemical ecology is an area of study that recently led to the development of promising control methods. This research project intended to shed some light on the natural degradation and transformation of cane toad parotoid gland bufadienolides. Bufotoxins were known to be the common storage form of bufadienolides bringing chemical, physical and microbiological stability to cane toad toxins. Bufagenins, on the other hand, were known to be originated by an *ex situ* enzymatic hydrolysis after parotoid gland compression and be microbiologically vulnerable. The first assumption was, therefore, that the motor vehicle collision and the decomposition of the parotoid glands would trigger an *in situ* hydrolysis of bufotoxins. The second hypothesised that transformation and degradation of bufagenins mediated by bacteria would take place. Those reactions were seen as the key to understand what naturally happens to bufagenins once cane toads died. The general conclusion of this master thesis went in the opposite way of this hypothesis:

Although bacteria that are able to transform or degrade bufagenins are present within cane toad parotoid glands, they do not perform during the decomposition of the natural substrate and consequently, do not influence the bufadienolide content.

Consequently, road-killed cane toads keep their cardiotoxic agents for an enduring period of time. Roadkill parotoid glands contain essentially Mbg which means that their mixture could potentially inhibit Na^+/K^+-ATPase receptors more quickly. However, nothing suggests that it is the case at an ecological scale as Australian scavengers are part of the fauna immune to parotoid toxins [37–39]. That conclusion could also be specific to road-killed cane toads only and raises questions about the cause that lead to those results.

The microbial competition within the parotoid gland was one of the reasons given to explain the conclusion. A study determining the phylogenetic diversity of the parotoid microbiome could better assess the microbial distribution within the glands and help answer that question. The loss of the optimal condition of growth could also be a critical part that led to the inactivity of parotoid gland-associated strains as water went missing. Carcasses of toads drying under the Australian sun were certainly an extreme type of substrate. Cane toads decomposing in waterbodies would be the antipode but could potentially favour the development of degrading and transforming bacteria. The results that would be yield could, therefore, help better understand how bufadienolides naturally evolve. This area of study could definitely bring key knowledge in the cane toad chemical ecology and would deserve more efforts.

The discovery of degrading strains brought once the hope that they could inspire the development of practical control solutions that would reduce the anuran toxicity. The road may still be long before to see the first detoxified cane toad, dead or alive, but, Rome was not built in a day and it is brick by brick that answers would be found and solutions would emerge to restore tranquillity to the Australian wildlife.

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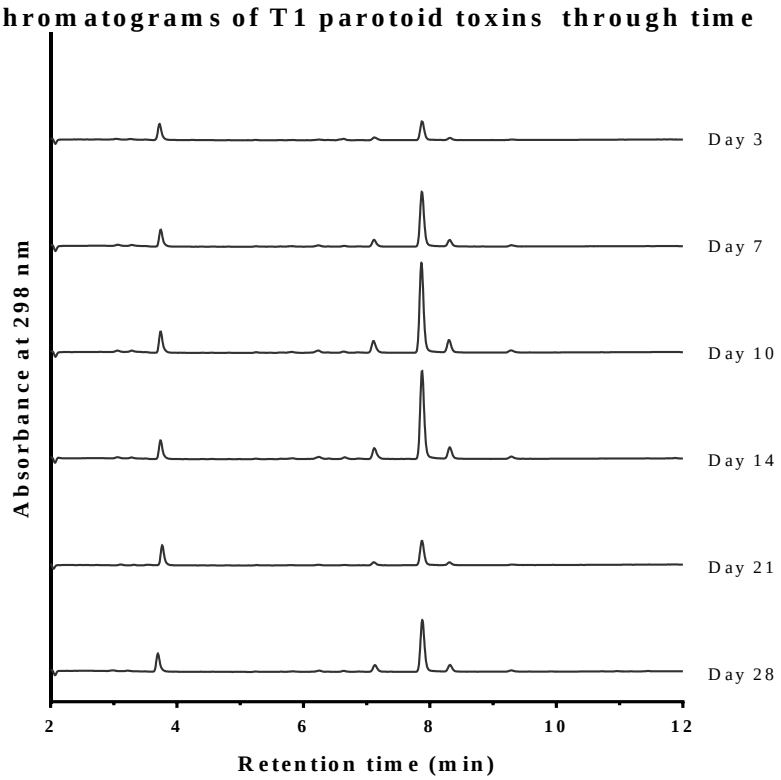
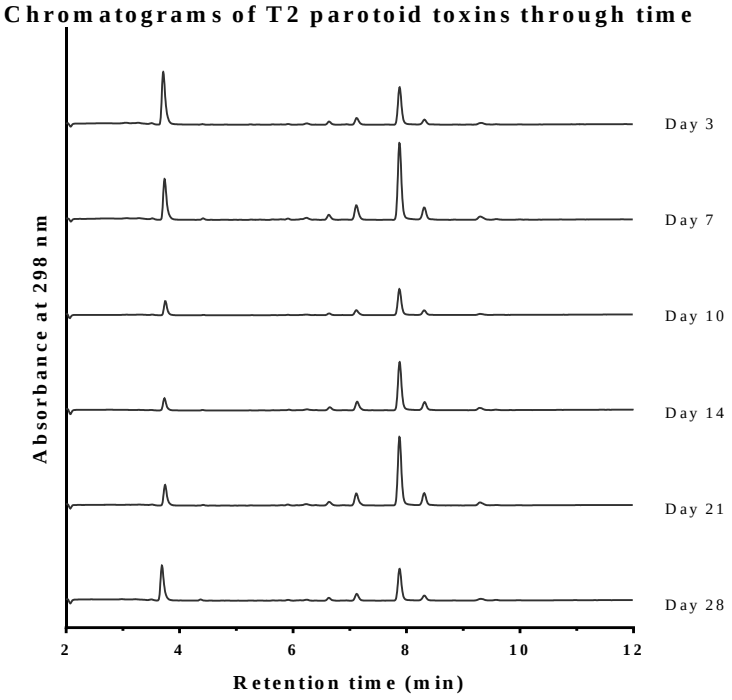
Appendix

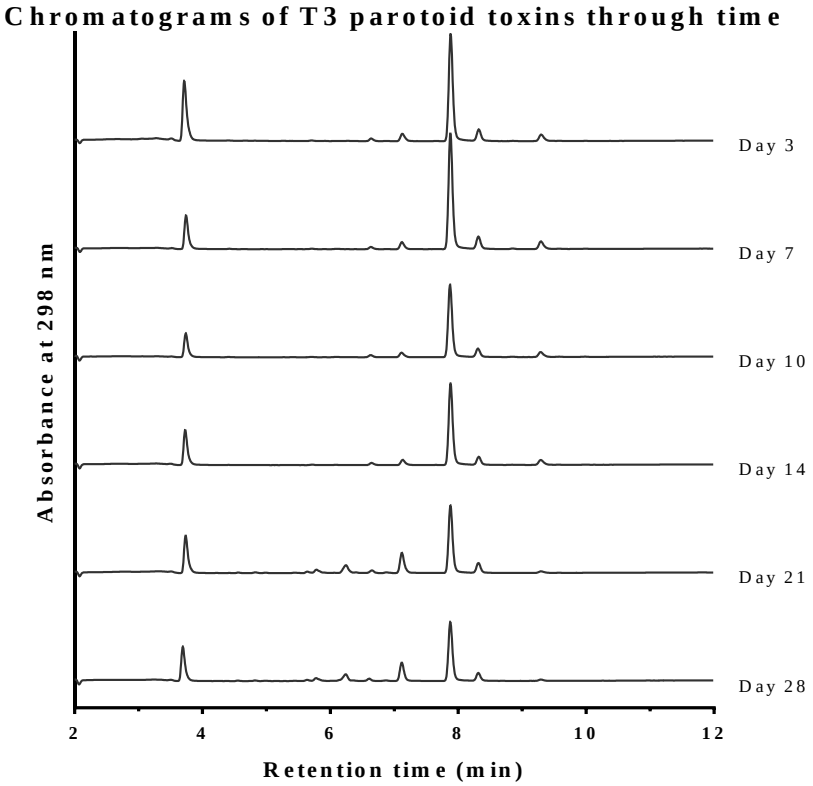
1. **Stacked chromatograms of T1 parotoid toxins through time**



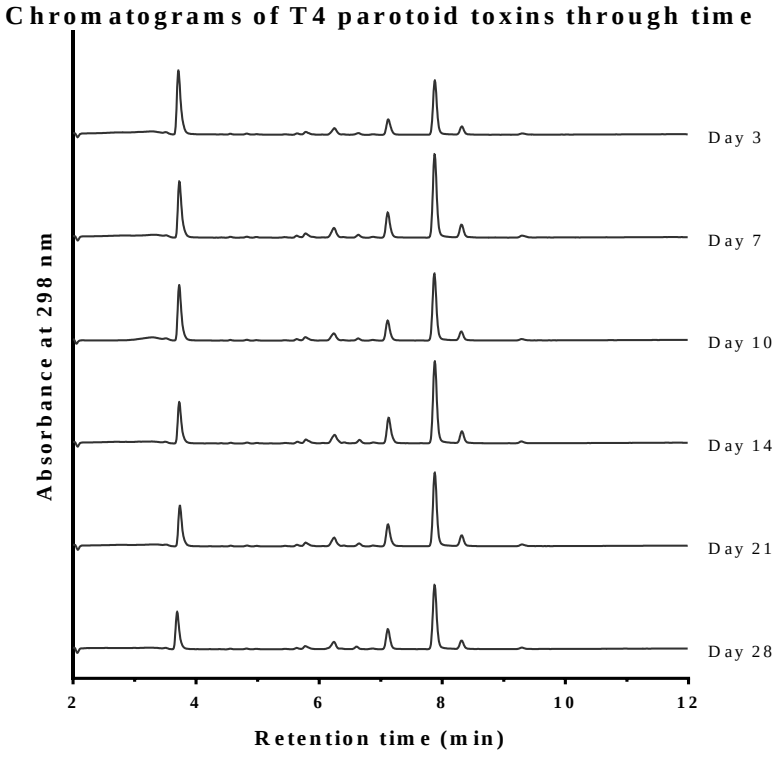
2. **Stacked chromatograms of T2 parotoid toxins through time**



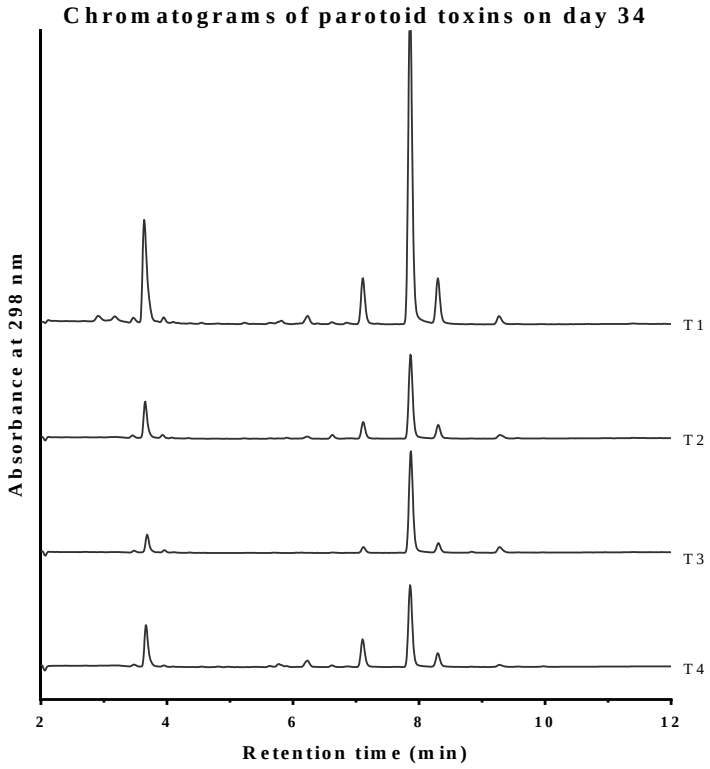
3. Stacked chromatograms of T3 parotoid toxins through time



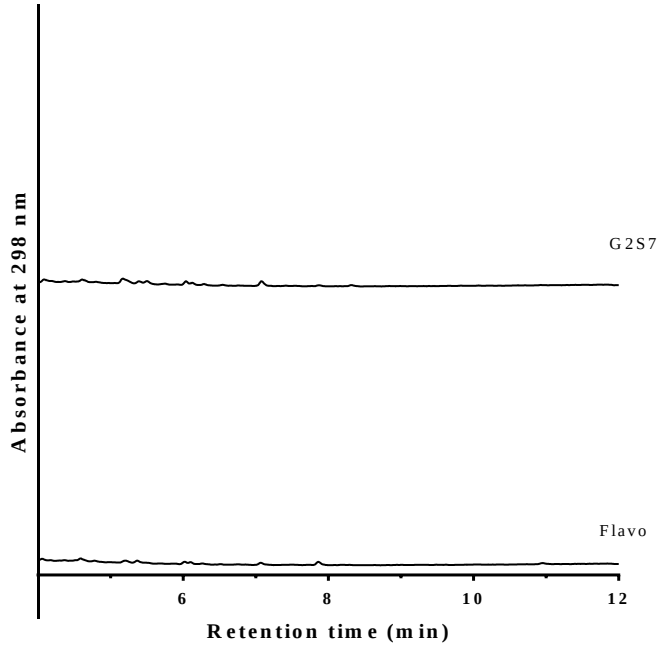
4. Stacked chromatograms of T4 parotoid toxins through time



5. **Stacked chromatograms of parotoid toxins on day 34**



6. **Stacked chromatograms of *Bacillus sp.* and *Flavobacterium sp.* challenged with MbgPal on day 3**



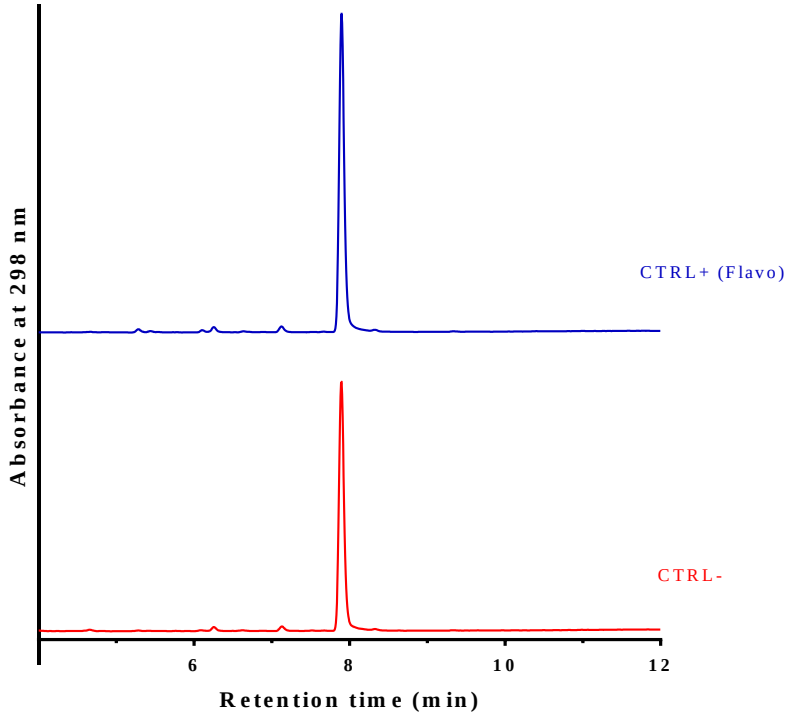
7. QTOF results: main oxidised analogues from the challenge of T3S3 with Mbg

Compound	Calculated mass (m/z) $(M+H)^+$	Measured mass (m/z) $(M+H)^+$	ΔmDa	Molecular Formula
3-oxo marinobufagenin	398.2093	398.2088	0.51	C24 H30 O5
$\Delta^{1,4}$ -3-oxobufalin	380.1988	380.1984	0.37	C24 H28 O4

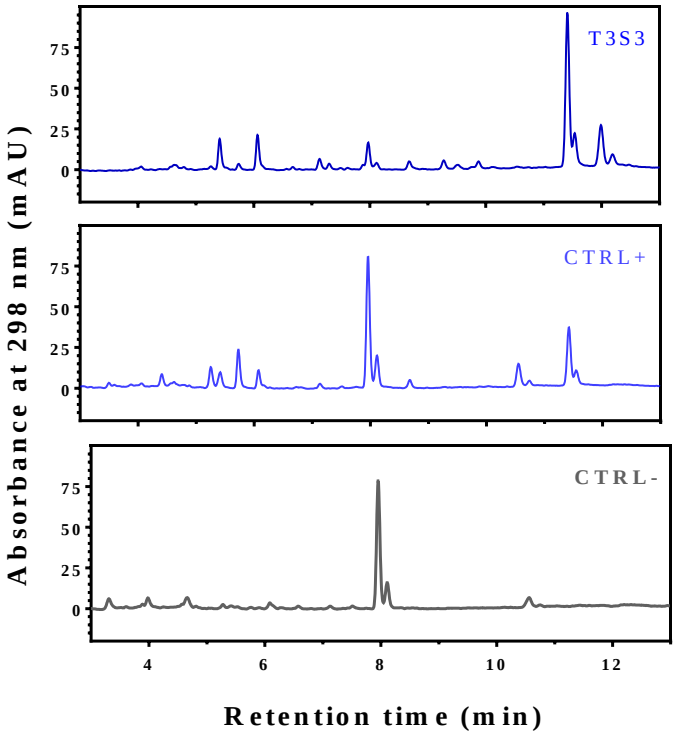
8. QTOF results: main hydroxylated analogues from the challenge of T3S7 with Mbg

Compound	Calculated mass (m/z) $(M+H)^+$	Measured mass (m/z) $(M+H)^+$	ΔmDa	Molecular Formula
11α-hydroxymarinobufagenin	416.2199	416.2201	-0.3	C23 H26 N7 O
17α-hydroxymarinobufagenin	416.2199	416.2201	-0.3	C23 H26 N7 O

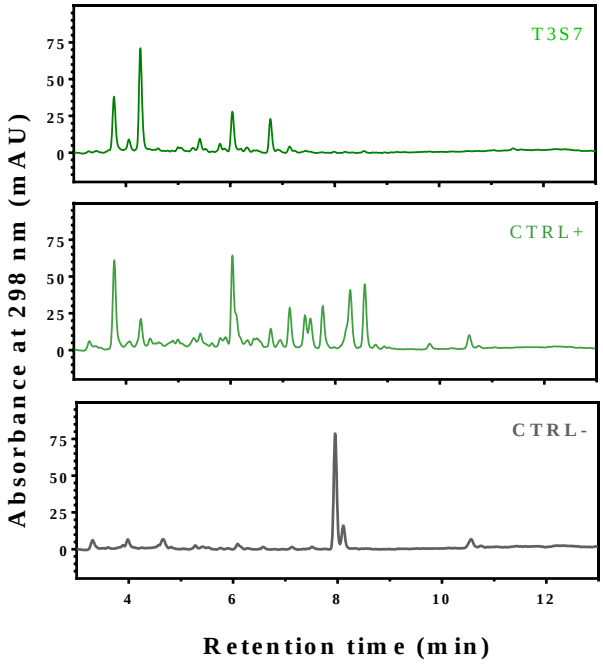
9. Stacked chromatograms of CTRL+ Flavo and CTRL- on day 5



10. **Stacked chromatograms of biodegrading strains challenged with unknown compounds (originally assumed to be Tbt)**



11. **Stacked chromatograms of biotransforming strains challenged with unknown compounds (originally assumed to be Tbt)**



12. **Stacked chromatograms of T1S12 in different culture containers, in presence or not of Mbg**