Bioorganic & Medicinal Chemistry Letters 19 (2009) 1790–1792

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

Microbial biotransformation as a source of chemical diversity in cane toad steroid toxins

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ARTICLE INFO

Article history:
Received 11 December 2008
Revised 20 January 2009
Accepted 21 January 2009
Available online 27 January 2009

Keywords:
Biotransformation
Bufo marinus
Cane toad
Bufadienolide
Chemical ecology

ABSTRACT

The cane toad is an invasive pest that is rapidly colonising northern Australia. The cane toad parotoid gland secretes cardiotoxic steroids (bufadienolides) that are poisoning native predator species. This study reveals bufadienolide diversity within the secretions of Australian cane toads is different to cane toads from overseas, being far more structurally diverse than previously assumed. It is proposed that this variation is mediated by in situ bacterial biotransformation.

The cane toad, *Bufo marinus*, is an invasive pest that was introduced to multiple locations around the world as an ultimately unsuccessful biocontrol agent for beetle pests of sugar cane. In several locations, including Australia, Fiji and Hawaii, the species has had a significant ecological impact on native animal populations.[1] The cane toad and other members of the genus *Bufo* are renowned for their ability to produce and deploy cardiotoxic steroids (bufadienolides) as a form of chemical defence.[1] Bufadienolides are antagonists of Na^+/K^+-ATPase in much the same way as the plant derived cardenolides (such as digitalis)[2] and ingestion can lead to cardiac arrest and death. The prospect of fatal encounters with cane toads is enhanced by the presence of specialised parotoid glands that secrete high concentrations of bufadienolides in response to predatory attack. In Australia, the cane toad is a threat to native predator species such as fresh water crocodiles, marsupials, snakes and lizards, which are highly vulnerable to cane toad poisoning.[3–5]

Since its release in Australia in 1935,[6] the cane toad has advanced south along the eastern seaboard from Queensland into New South Wales, and west through the Northern Territory towards Western Australia – colonising >1 million km^2 and seriously impacting native predator populations.[1] To date, cane toad control has been limited to local and short term techniques such as hand collection and trapping.[7] In an attempt to broaden the control agenda, and identify more permanent solutions effective on a larger regional or national scale, we recently embarked on an analysis of cane toad chemical ecology.[8] This report describes one aspect of those investigations, namely an assessment of Australian cane toad bufadienolides, including the role played by microorganisms in diversifying and possibly enhancing bufadienolide toxicity.

Of the order of 100 bufadienolides have been described from toads of the genus *Bufo*, of which only 30 have been attributed to *B. marinus*,[9] and only four, marinobufagin (**1**), telocinobufagin (**2**), bufalin (**3**) and resibufogenin (**4**), have been reported from the cane toad parotoid gland secretion. More significantly, none of these reported chemical analyses was performed on cane toads sampled from the resident Australian population. Our investigations into the parotoid gland chemistry of cane toads sampled from locations at the eastern and western extremes of the Australian colonisation range revealed a bufadienolide composition dominated by **1**, with moderate levels of **2**, **3**, arenobufagin (**5**) and marinobufotoxin (**6**), lower levels of **4**, hellebrigenin (**7**), marinobufagin-3-pimeloyl-L-arginine ester (**8**), bufalin-3-pimeloyl-L-arginine ester (**9**) and bufalitoxin (**10**), and detectable levels of >30 minor bufadienolides (Fig. 1 and Scheme 1).[10] These studies reveal, for the first time, that Australian cane toad parotoid secretion chemistry is not identical to that reported from overseas cane toads,[9] and that the bufadienolide chemical diversity is far greater than previously assumed.

It has previously been demonstrated that bufadienolides can undergo biotransformation when exposed to cultures of bacteria[11–13] or plant cells.[14] Although these biotransformation studies lack ecological relevance, they do raise the possibility that in situ bacterial biotransformation could be a mechanism for chemical diversification within the parotoid gland. To test this hypothesis, we recovered bacterial isolates from swabs taken from the parotoid gland, ovary, tongue and stomach of a dissected mature

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doi:10.1016/j.bmcl.2009.01.064

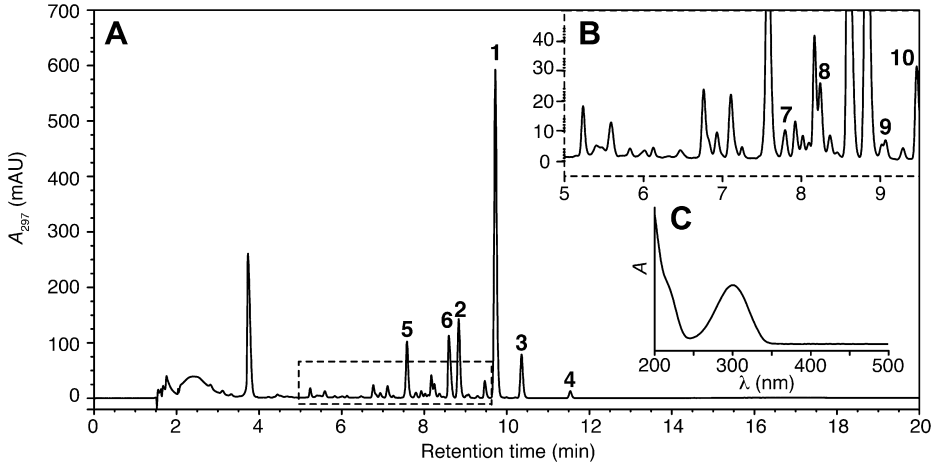
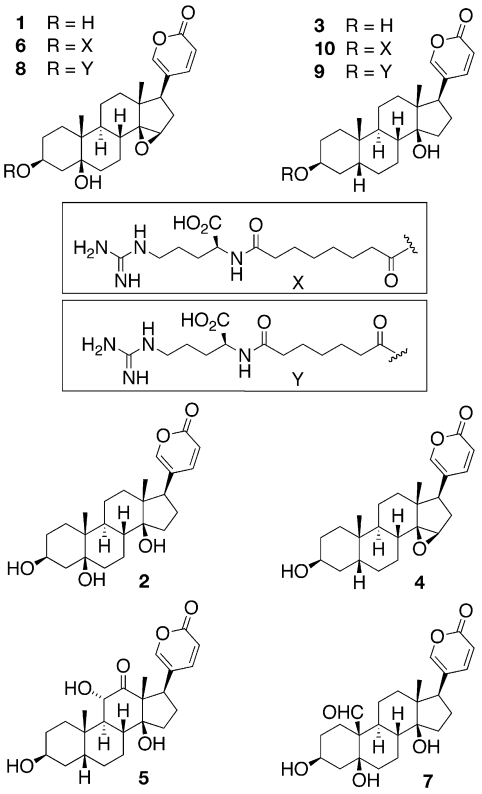


Figure 1. (A) HPLC trace (297 nm) of *n*-butanol soluble fraction of adult cane toad parotoid gland secretion. Peak numbers correspond to compound numbers in Scheme 1. (B) Expansion of chromatogram showing numerous minor components, each displaying a UV–vis spectrum typical of a bufadienolide. (C) UV–vis spectrum of a typical bufadienolide showing characteristic absorbance maximum at 297 nm.

female cane toad. Liquid cultures of these bacteria were incubated in the presence of **1**, followed by solvent extraction and analysis by HPLC-DAD-MS.[15] Three isolates that demonstrated biotransformation capability were identified as *Acinetobacter johnsonii* (from the ovary), and *Flavobacterium* sp. and *Comamonas testosteroni* (both from the parotoid gland). Of note, the Gram-negative bacterium *C. testosteroni* is known to grow on C_{19}–C_{27} steroids as a carbon and energy source.[16–18] Biotransformation products arising from a 96-h exposure of **1** to a liquid culture of *C. testosteroni* were analysed (Fig. 2) and identified by the application of HPLC-DAD-SPE-NMR and HPLC-DAD-MS, as 3-*epi*-marinobufagin (**11**),[19] 3-oxomarinobufagin (**12**),[20] $\Delta^{1,4}$-3-oxoresibufogenin (**13**)[21] and $\Delta^{1,4}$-3-oxobufalin (**14**)[22] (Scheme 2).[23]

The results of our study demonstrate, for the first time, that cane toads harbour bacteria with the capacity to biotransform and diversify cane toad bufadienolides. The specific biotransformation products identified are not necessarily representative of those that would be produced in vivo as the environmental conditions and range of biotransforming microorganisms present within the toad are significantly different from those present in the laboratory. Nevertheless, this study provides an important proof of principle, highlighting a hitherto unexplored area of cane toad chemical ecology. We hypothesise that a broadening of the parotoid gland bufadienolide chemical diversity could lead to survival advantages by increasing the prospects for antagonism of a wider array of Na^+/K^+-ATPase isoforms. Genetic variability in Na^+/K^+-ATPase sensitivity to bufadienolides is exemplified by the observation that some species are highly susceptible to the effects of toad bufadienolides, while others (including the cane toad itself) appear to be immune.[7] Toxic secretions that antagonise a wider subset of Na^+/K^+-ATPase isoforms increase the probability that would-be predators will be susceptible and succumb. Binding constants for



Scheme 1. Bufadienolides **1–10** isolated from Australian cane toad parotoid gland secretions.

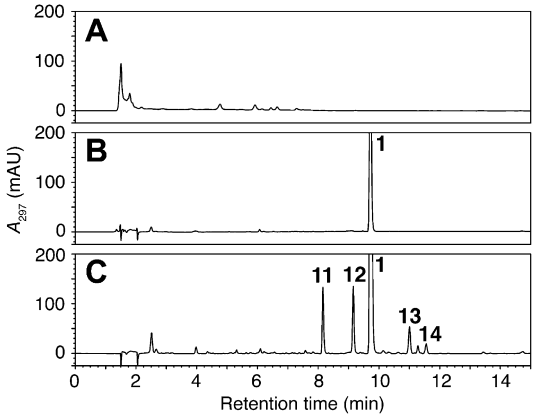
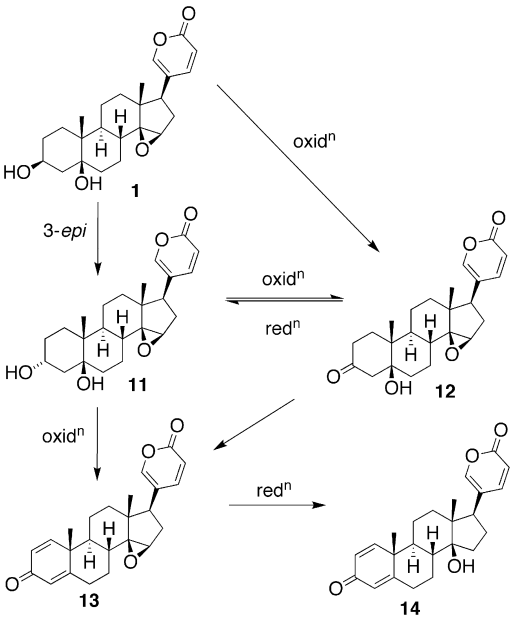


Figure 2. HPLC traces (297 nm) of ethyl acetate extracts obtained from (A) nutrient broth inoculated with *Comamonas testosteroni*; (B) uninoculated nutrient broth containing marinobufagin (**1**); (C) nutrient broth inoculated with *C. testosteroni* containing **1**; after incubation at 26.5 °C for 96 h. Peak numbers correspond to structures shown in Scheme 2.



Scheme 2. Marinobufagin (**1**) biotransformation products 3-epi-marinobufagin (**11**), 3-oxomarinobufagin (**12**), $\Delta^{1,4}$-3-oxoresibufogenin (**13**) and $\Delta^{1,4}$-3-oxobufalin (**14**), and a possible sequence of transformations.

bufadienolides against Na^+/K^+-ATPase can range over several orders of magnitude,[24] demonstrating that even minor bufadienolides can have ecological (toxic) significance against relevant species.

The realisation that bacteria may play a role in the chemical ecology of the cane toad—modifying toxicity and environmental impact—suggests an exciting new line of research for cane toad control. More detailed knowledge of the relationship between cane toads, bacteria and bufadienolides will, we believe, contribute to our ability to control the impact of cane toads on native predator species. For instance, more competitive strains of *C. testosteroni* (or other microorganisms) that either have no capacity for biotransformation, or alternatively over-transform all available bufadienolides to non-toxic analogues, could be used to supplant wild strains. Such a strategy is widely used with probiotics in aquaculture[25,26] and agriculture[27] and shifts in symbiotic microbial community structure due to invasive bacteria have been shown to be deleterious to coral populations in the field.[28] Alternatively, cane toads could be infected with bacteriophages specific to bufadienolide biotransforming bacteria, thereby down-regulating toxicity, and lessening environmental impact.[29] These outcomes would best be achieved through a multidisciplinary collaboration involving ecologists, chemists and microbiologists.

Acknowledgments

The authors thank Prof. Lindsay Sly and Dr. Feras Lafi (SMMS, UQ) for 16S rRNA sequencing and the Queensland State Government and Invasive Animals CRC for research funding.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2009.01.064.

References and notes

1. Lever, C. *The Cane Toad: The History and Ecology of a Successful Colonist*; Westbury Academic and Scientific: York, UK, 2001.
2. Chen, K. K.; Kovarikova, A. *J. Pharm. Sci.* **1967**, *56*, 1535.
3. Letnic, M.; Webb, J. K.; Shine, R. *Biol. Conserv.* **2008**, *141*, 1773.
4. Crossland, M. R.; Alford, R. A. *Aust. J. Ecol.* **1998**, *23*, 129.
5. Burnett, S. *Pac. Conserv. Biol.* **1997**, *3*, 65.
6. Mungomery, R. *Cane Grow. Q. Bull.* **1935**, *3*, 21.
7. Molloy, K.; Henderson, W. *Science of Cane Toad Invasion and Control: Proceedings of the IA CRC/CSIRO/QLD NRM&W Cane Toad Workshop, June 2006, Brisbane*; Invasive Animals Cooperative Research Centre: Bruce, A.C.T., 2006.
8. Hayes, R. A.; Barrett, A.; Alewood, P. A.; Grigg, G. C.; Capon, R. J.. In *Chemical Signals in Vertebrates*; Hurst, J. L., Beynon, R. J., Roberts, S. C., Wyatt, T. D., Eds.; Springer: New York, 2008; Vol. 11, pp 409–417.
9. Steyn, P. S.; van Heerden, F. R. *Nat. Prod. Rep.* **1998**, *15*, 397.
10. *Isolation of bufadienolides:* Parotoid secretion was collected by manual compression of the parotoid gland of an adult cane toad (*B. marinus*), and was subsequently partitioned between equal volumes of *n*-butanol and water and the *n*-butanol layer was reduced to dryness in vacuo. The residue was separated by HPLC on a Zorbax SB-C_{18} column (150 × 4.6 mm; 5 μm), gradient of 10–100% MeCN–H_2O over 15 min, then 100% MeCN for 5 min (constant 0.01% TFA).
11. Zhan, J.; Guo, H.; Ning, L.; Zhang, Y.; Guo, D. *Planta Med.* **2006**, *72*, 346.
12. Zhan, J.; Liu, W.; Guo, H.; Zhang, Y.; Guo, D. *Enz. Microb. Tech.* **2003**, *33*, 29.
13. Zhan, J.; Zhang, Y.; Liu, W.; Guo, H.; Guo, D. *Biocatal. Biotransform.* **2003**, *21*, 141.
14. Ye, M.; Ning, L.; Zhan, J.; Guo, H.; Guo, D. *J. Mol. Catal. B: Enzym.* **2003**, *22*, 89.
15. *Isolation and cultivation of microorganisms*. A mature female cane toad (*B. marinus*) was euthanised and dissected. Microbial cultures were obtained from the ovaries, tongue, stomach and parotoid gland secretion of the toad by rubbing the tissues directly onto agar plates containing the following medium: peptone (Sigma) 4 g/L, sodium chloride (Univar) 1.5 g/L and agar (Sigma) 18 g/L. The plates were incubated at 26.5 °C until bacterial growth was evident on all plates (48 h). Selected colonies from each plate (total of 22) were subcultured onto fresh agar plates and incubated at 26.5 °C for 48 h. A single colony from each isolate was then used to inoculate aliquots of nutrient broth (10 mL) of the following composition: D-glucose (Amresco) 10 g/L, yeast extract (BD BBL) 4 g/L, peptone (Sigma) 2 g/L, sodium chloride (Univar) 1.5 g/L. The inoculated broths were incubated at 26.5 °C with vigorous shaking (140 rpm) until visibly turbid (24 h). A solution of pure **1** (purified from cane toad parotoid secretion) in acetone (15 mg/mL; 30 μL) was added to each culture, and incubation was continued for a further 48 h at 26.5 °C with shaking. Cultures were extracted with ethyl acetate (3 × 3 mL) and the combined organic layers were dried under a stream of nitrogen at 40 °C, then under high vacuum. The extracts were dissolved in methanol to a concentration of 2 mg/mL, filtered through a 0.45 μm PTFE filter and analysed by HPLC-DAD. Extracts showing the biotransformation of **1** were further analysed by HPLC-DAD-MS.
16. Skowasch, D.; Möbus, E.; Maser, E. *Biochem. Biophys. Res. Comm.* **2002**, *294*, 560.
17. Marcus, P. I.; Talalay, P. *J. Biol. Chem.* **1956**, *218*, 661.
18. Talalay, P.; Dobson, M. M.; Tapley, D. F. *Nature* **1952**, *170*, 620.
19. Li, L.; Li, P.; Ye, M.; Zhong, L.; Guo, D. *Lett. Org. Chem.* **2004**, *1*, 176.
20. Shimada, K.; Miyashiro, Y.; Nishio, T. *Biomed. Chromatog.* **2006**, *20*, 1321.
21. Kamano, Y.; Kotake, A.; Hashima, H.; Inoue, M.; Morita, H.; Takeya, K.; Itokawa, H.; Nandachi, N.; Segawa, T.; Yukita, A.; Saitou, K.; Katsuyama, M.; Petit, G. *Bioorg. Med. Chem.* **1998**, *6*, 1103.
22. Ribar, B.; Argay, G.; Kalman, A.; Vladimirov, S.; Zivanov-Stakic, D. *J. Chem. Res. Synop.* **1983**, 90.
23. *Large-scale biotransformation*. *Comamonas testosteroni* was grown in liquid medium (100 mL) as described previously.[15] A solution of pure **1** in acetone (15 mg/mL; 667 μL, 10 mg) was added to the cultures (in triplicate) and incubation was continued at 26.5 °C with shaking for 96 h. Blank samples were also prepared containing broth and **1**, but without inoculation with bacteria. The broth was then extracted with ethyl acetate reduced to dryness in vacuo, dissolved in methanol to 2 mg/mL and analysed by HPLC-DAD-SPE-NMR and HR-ESI-MS. HPLC conditions were as follows: Zorbax SB-C_{18} column (150 mm × 4.6 mm; 5 μm), 1 mL/min gradient elution from 90% H_2O/MeCN to 100% MeCN over 15 min then hold for 5 min.
24. Akimova, O. A.; Bagrov, A. Y.; Lopina, O. D.; Kamernitsky, A. V.; Tremblay, J.; Hamet, P.; Orlov, S. N. *J. Biol. Chem.* **2005**, *280*, 832.
25. Kesarcodi-Watson, A.; Kaspar, H.; Lategan, M. J.; Gibson, L. *Aquiculture* **2008**, *274*, 1.
26. Das, S.; Ward, L. R.; Burke, C. *Appl. Microbiol. Biotechnol.* **2008**, *81*, 419.
27. Vanselow, B. A.; Krause, D. O.; McSweeney, C. S. *Aust. J. Agric. Res.* **2005**, *56*, 219.
28. Ritchie, K. B. *Mar. Ecol. Prog. Ser.* **2006**, *322*, 1.
29. Kutter, E. In *Practical Handbook of Microbiology*; Goldman, E., Green, L. H., Eds.; CRC Press: Boca Raton, 2009; pp 713–730.