VIP Pheromones

DOI: 10.1002/anie.201106592

Volatile Amphibian Pheromones: Macrolides from Mantellid Frogs from Madagascar

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Anuran amphibians (frogs) communicate mainly by acoustic, visual, and tactile signals. However, frogs and other amphibians can also use pheromones.[1,2] In some cases these have been chemically characterized; in frogs, newts, and salamanders they proved to be peptides or proteins, readily dissolving in water or spreading on the water surface.[2,3] These compounds are used by the males to attract females in water. An example is the 25 amino acid peptide splendipherin, which is used as a pheromone by males of the Australian tree frog, *Litoria splendida*.[4] In salamanders, proteins act as courtship pheromones, as shown for the red-legged salamander *Plethodon shermani* and related species.[5] Recently the sex pheromone of females of the Korean salamander *Hynobius leechii* has been identified as prostaglandin $F_{2\alpha}$,[6] which is also released into water. Male newts of the genus *Cynops* use 10 amino acid peptides such as sodefrin and silefrin to attract females, again in water.[7] Apart from these non-volatile cues, frogs have been shown to be able to respond to volatile pheromonal cues, but their chemical composition is still unknown.[8]

The Mantellidae are a very species-rich family of small frogs occurring in the rainforest of Madagascar.[9] A subfamily of mantellids, the Mantellinae, is characterized by particular glandular structures on the ventral side of the shanks of the males (Figure 1).[10] The function of these femoral glands is not known, but their sex-specific occurrence and their location might point to a use in pheromonal communication. Herein we present the surprising finding of nonpeptidic volatile compounds, being structurally related to volatile insect secretions and acting as pheromones, in these glands.

The femoral glands of five males of *Mantidactylus multiplicatus* were excised and extracted with dichloromethane. The individual extracts were separately analyzed by gas chromatography–mass spectrometry (GC-MS), and two major volatile components **A** and **B** were detected from each specimen (Figure 2). These compounds were accompanied by a less volatile part of the secretion that comprised diacetylated monoglycerides and to a lesser extent monoacylated diglycerides.

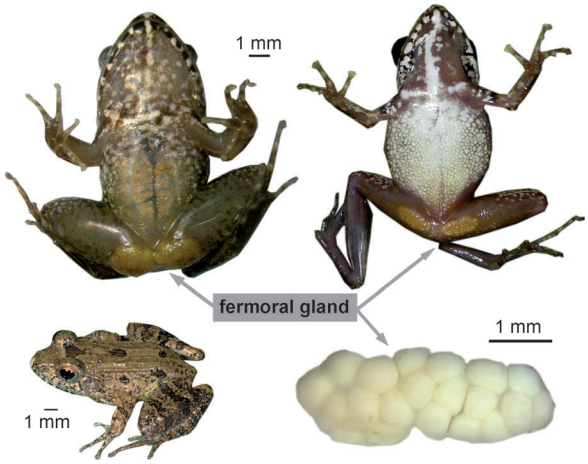

Figure 1. Ventral and dorsolateral view of a male *Mantidactylus multiplicatus* (left) and ventral view of a male *Gephyromantis decaryi* (right). Arrows point at femoral glands in the two species, and at the gland internal view of *G. decaryi* (bottom right).

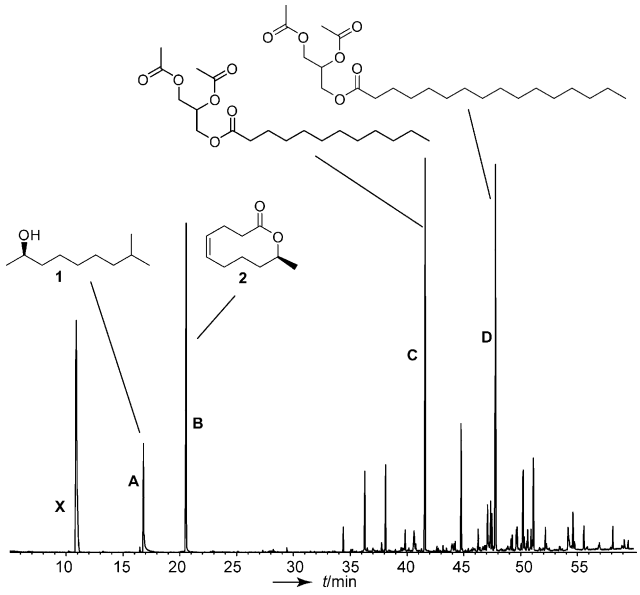

Figure 2. Gas chromatogram of a dichloromethane extract of a femoral gland of *Mantidactylus multiplicatus*. Two volatile major components **A** and **B** are accompanied by a less volatile complex secretion of glycerides. **C**: 3-(dodecanoyloxy)propane-1,2-diyl diacetate, **D**: 3-(palmitoyloxy)propane-1,2-diyl diacetate, **X**: artefact.

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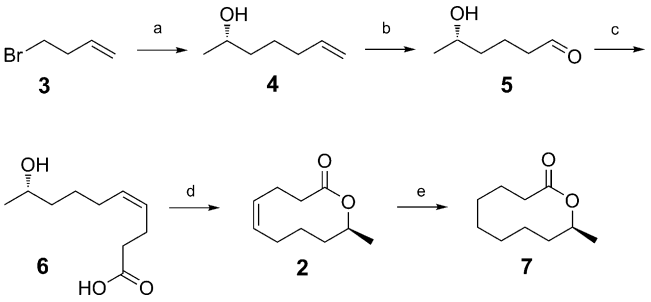
Supporting information for this article, including experimental procedures, is available on the WWW under http://dx.doi.org/10.1002/anie.201106592.

Compound **A** showed a mass spectrum similar to that of 2-decanol, but a variation in the gas chromatographic retention index, indicating 8-methyl-2-nonanol (**1**) as possible structure. The synthesis of **1** verified the proposal; both natural compound and synthetic material showed identical mass spectra and gas chromatographic retention indices (see the Supporting Information). The alcohol **1** was synthesized by coupling the tosylate of 5-hexen-1-ol with isopropylmagnesium bromide, followed by Wacker oxidation and LAH reduction. The enantiomers of the resulting *rac*-8-methyl-2-nonanol were separated by enzymatic resolution of the corresponding acetate. Chiral-phase gas chromatography revealed that the natural material has a *R* configuration (see the Supporting Information).

The mass spectrum of component **B** was identical to that of phoracantholide J (**2**) published in mass spectrometry databases. Already known synthetic routes to enantiomerically pure **2** were either quite long or used ring-closing metathesis (RCM) as the cyclization method,[11,12] which in our hands gave lower yields and lower stereoisomeric purity than reported. A new and short synthesis was therefore designed using the Corey–Nicolaou macrolactonization[13] method as a key step. The enantiomers of lactone **2** were synthesized as shown in Scheme 1 for the *S* enantiomer. The



Scheme 1. Synthesis of phoracantholides I (**7**) and J (**2**): a) 1. Mg, Et_2O, 2. CuCN, 3. (*S*)-propylene oxide, 0 °C, 12 h, 91 %; b) K_2OsO_4, $NaIO_4$, dioxane, H_2O, 0 °C, 2 h, 71 %; c) *n*BuLi, NaHMDS, $[Ph_3P(CH_2)_3COOH]Br$, THF, −78 °C to RT, 2 h, 69 %; d) 1. dipyridiyldisulfide, 2. $AgClO_4$, toluene, reflux, 14 h, 26 %; e) H_2, 10 % Pd/C, MeOH, 5 h, 75 %.

alcohol **4** was obtained by reaction of homoallylmagnesium bromide with (*S*)-propylene oxide. Lemieux–Johnson oxidation[14] of the double bond furnished the aldehyde **5** that gave preferentially the *Z*-configured hydroxyacid **6** by Wittig reaction with (3-carboxypropyl)triphenylphosphonium ylide ($Z/E = 95:5$). Finally, cyclization according to the Corey–Nicolaou method[13] yielded phoracantholide J (**2**), which is identical to the natural compound **B**. Compound **2** is a well-known defensive secretion constituent from the metasternal gland of Australian *Phoracantha synonyma* beetles,[11,15] where it occurs as *R* enantiomer. In contrast, the *S* enantiomer is produced by the frog, as the investigation using GC on a chiral phase revealed (see the Supporting Information). None of the two identified compounds were detected in the ventral skin of the frogs, which is devoid of the major gland complexes typical for the thighs.

Subsequently, we used 109 live individuals of *M. multiplicatus* to evaluate whether the compounds **1** and **2** might influence their behavior. Individual frogs were placed in plastic containers with cotton cloths on either half, one of which was impregnated with either of the two compounds. Experiments were conducted during the breeding season with freshly collected specimens and under natural temperature conditions. Each frog was tested eight times with several hours of rest between experiments. On the basis of 800 series of seven photos automatically taken after 5 min intervals for all trials (see the Supporting Information), we measured frog activity as the number of body position changes. Relative to cotton cloths impregnated with water only, activity was reduced by exposure to the hexane solvent. However, relative to the hexane control, males showed a higher activity when exposed to compounds **1** and **2** (Figure 3). Furthermore,

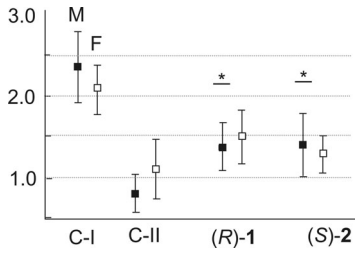


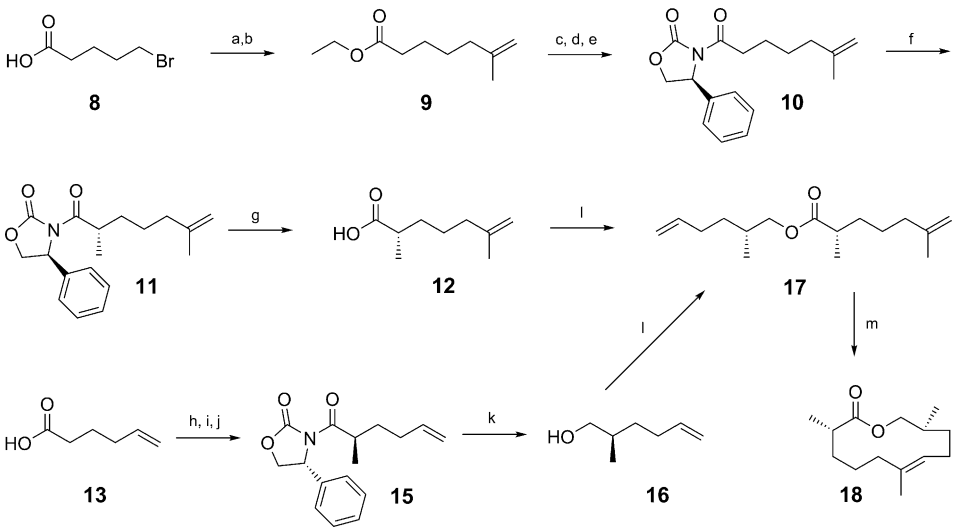
Figure 3. Activity (number of body position changes in 7 intervals of 5 min) in males (M) and females (F) of *M. multiplicatus* if exposed to water (C-I, control; males 100, females 216 series of observations), hexane (C-II, control; male 73, female 42) and hexane-dissolved compounds **1** (male 78, female 46) and **2** (male 36, female 123), indicating an increase of activity relative to hexane exposure (asterisks mark significant differences to C-II; $P < 0.05$).

males in their first change of sides in the container showed a significant preference for moving towards compound **1** (see the Supporting Information). Despite the apparent confounding effect of the solvent, these preliminary results support that compounds **1** and **2** are able to release changes in behavior of conspecific individuals. Therefore, they conform to the general definition of pheromones and might play a role in intraspecific communication, although their exact function is currently unknown.

Tropical amphibian species communities can be extremely species-rich. In Ranomafana National Park in Madagascar, over 100 mantelline species of often very similar morphology are known to co-occur.[9] Therefore, it was then tested whether in this area mantelline species contain the same or different compounds in their femoral glands. Analysis of glands from different species indicated that volatile compounds are widespread in mantellines, but occur in species-specific mixtures. In males of the closely related *Mantidactylus betsileanus* from three different localities, compound **2** was detected as well but alcohol **1** was absent. *M. femoralis* femoral glands contained the closely related macrolide, (*S*)-phoracantholide I (**7**), the hydrogenated analogue of **2**, that again is known from *Phoracantha* beetles in opposite configuration.[11,15] This compound was synthesized by hydrogena-

tion of lactone **2** (Scheme 1) and its absolute configuration was established by chiral-phase GC.

In several other species of *Mantidactylus*, and of other mantellid genera such as *Gephyromantis*, volatile compounds occurred in species-specific mixtures, in agreement with the hypothesized function of these compounds in intraspecific communication and recognition. Often these compounds have a macrolide structure, and they are currently under structural investigation. The secretion of *Gephyromantis boulengeri* contained essentially only one major component **E**. The glandular extract was directly analyzed by several NMR spectroscopy experiments (see the Supporting Information). From these data and the HR-GC-MS data, which are consistent with the molecular formula $C_{14}H_{24}O_2$, the structure **18** was proposed. Compound (2*S*,6*E*,10*R*)-**18** was then synthesized in enantiomerically enriched form to confirm the proposal and allow determination of the absolute configuration of the natural compound **E** (Scheme 2). A key step in the synthesis was a ring-closing-metathesis to form the macrocyclic ring. 5-Bromopentanoic acid (**8**) was transformed into the ester and coupled with 2-propenylmagnesium bromide by Li_2CuCl_4 catalysis.[16] The resulting ester **9** was transformed into the Evans amide **10**. Alkylation with NaHMDS/MeI yielded **11** in excellent yield and 99% diastereoselectivity. Cleavage of the auxiliary furnished the chiral acid **12**. Again, an Evans approach furnished the alkylated Evans amide **15** starting from 5-hexenoic acid **13**. Reduction with LAH gave the alcohol **16** that was coupled with **12** to form the RCM precursor **17**. The RCM proved to be difficult, and several conditions and catalysts were tried. Best results were obtained with the Stewart–Grubbs catalyst in refluxing toluene in presence of C_6F_6.[17] Nevertheless, a difficult-to-separate mixture of compounds, including the *E* and *Z* isomers as well as smaller ring products, were formed by isomerization of the double bonds prior to ring closure. The other three enantiomers were obtained in an identical manner by using appropriate methylated precursors **12** and **16**. Pure **18** was obtained by isolation with semi-preparative HPLC. Chiral-phase GC revealed that the enantiomer (2*S*,6*E*,10*R*)-**18** is indeed compound **E**. This compound proved to be a new natural product for which we propose the name gephyromantolide A. While compounds **1**, **2**, and **7** are metabolites of the fatty acid biosynthetic pathway, on first sight compound **18** shows a typical terpenoid branching. Nevertheless, it lacks one carbon compared to sesquiterpenes and it therefore might be as well derived from fatty acid metabolism. The methyl branches are probably introduced by methylmalonate building blocks replacing usual malonate building blocks during biosynthesis.



Scheme 2. Synthesis of gephyromantolide A (**18**) from *Gephyromantis boulengeri*: a) H_2SO_4, EtOH, reflux, 6 h, 84%; b) Li_2CuCl_4, isopropenylmagnesium bromide, THF, 0°C, 12 h, 91%; c) KOH, EtOH, H_2O, reflux, 4 h, 84%; d) $SOCl_2$, Et_2O, 0°C, 12 h, 84%; e) (*S*)-4-phenyloxazolidin-2-one, *n*BuLi, THF, −78°C to RT, 12 h, 91%; f) NaHMDS, MeI, THF, −78°C to RT, 12 h, 95%; g) KOH, MeOH, H_2O, reflux, 4 h, 82%; h) $SOCl_2$, Et_2O, 0°C, 12 h, 91%; i) (*R*)-4-phenyloxazolidin-2-one, *n*BuLi, THF, −78°C to RT, 12 h, 88%; j) NaHMDS, MeI, THF, −78°C to RT, 12 h, 93%; k) $LiAlH_4$, Et_2O, RT, 86%; l) **16**+**12**, DMAP, EDC·HCl, CH_2Cl_2, 0°C, 2 h, 92%; m) Stewart–Grubbs catalyst II, C_6F_6, toluene, 80°C, 3 h, 15%.

In conclusion, we have established the occurrence of volatile compounds to be linked to male-specific glands and that frogs alter their behavior upon experimental exposure to them. This strongly suggests that amphibians use volatile pheromones, a trait not reported before from this vertebrate group. Furthermore, mantelline frogs produce species specific mixtures of compounds, often including macrolides, but also a variety of other components. In most frogs, acoustic communication is certainly the predominant mechanism to attract females over wider distances. However, in highly diverse species assemblages, chemical communication with volatile compounds may constitute a hitherto underrated means to distinguish conspecifics in closer vicinity. This also emphasizes the potential of species-specific pheromones in species formation and evolution of these amphibians.

Received: September 16, 2011
Revised: November 18, 2011
Published online: January 20, 2012

Keywords: amphibians · chemical communication · chiral-phase gas chromatography · macrolides · pheromones

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