



Dramatic dietary shift maintains sequestered toxins in chemically defended snakes

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Unlike other snakes, most species of *Rhabdophis* possess glands in their dorsal skin, sometimes limited to the neck, known as nuchodorsal and nuchal glands, respectively. Those glands contain powerful cardiotoxic steroids known as bufadienolides, which can be deployed as a defense against predators. Bufadienolides otherwise occur only in toads (*Bufo*nidae) and some fireflies (*Lampyrinae*), which are known or believed to synthesize the toxins. The ancestral diet of *Rhabdophis* consists of anuran amphibians, and we have shown previously that the bufadienolide toxins of frog-eating species are sequestered from toads consumed as prey. However, one derived clade, the *Rhabdophis nuchalis* Group, has shifted its primary diet from frogs to earthworms. Here we confirm that the worm-eating snakes possess bufadienolides in their nuchodorsal glands, although the worms themselves lack such toxins. In addition, we show that the bufadienolides of *R. nuchalis* Group species are obtained primarily from fireflies. Although few snakes feed on insects, we document through feeding experiments, chemosensory preference tests, and gut contents that lampyrine firefly larvae are regularly consumed by these snakes. Furthermore, members of the *R. nuchalis* Group contain compounds that resemble the distinctive bufadienolides of fireflies, but not those of toads, in stereochemistry, glycosylation, acetylation, and molecular weight. Thus, the evolutionary shift in primary prey among members of the *R. nuchalis* Group has been accompanied by a dramatic shift in the source of the species' sequestered defensive toxins.

bufadienolides | chemical defense | firefly | nuchal glands | *Rhabdophis*

Chemically defended animals may either synthesize their toxins from nontoxic precursors or acquire them from an external source and store them for redeployment against a predator. Such sequestered defenses are widespread among nonvertebrates, but are less well known among vertebrates (1). The Japanese natricine colubrid snake *Rhabdophis tigrinus* sequesters potent cardiotoxic steroids known as bufadienolides (BDs) from toads (*Bufo*nidae) consumed as prey (2–4). The toads synthesize BDs from cholesterol in their integumentary glands (5), and *R. tigrinus* ingests the toxins and stores them in structures known as nuchal glands (NGs) located in the dorsal skin of the neck. When the snake is attacked, the NGs rupture and release the noxious toxins sequestered from toads. Several other species of Asian snakes possess either NGs or similar structures that extend the full length of the body (known as nuchodorsal glands, NDGs). A recent study confirmed that all species of snakes that possess either NGs or NDGs belong to a monophyletic lineage of Natricinae, and all are now placed in the genus *Rhabdophis* (6). The phylogeny further reveals that the basal species of *Rhabdophis* prey on anuran

amphibians, which thus appear to comprise the ancestral diet of this lineage (Fig. 1B).

Bufadienolides are a class of cardiotoxic steroids distinguished by the presence of a six-membered lactone ring at the C-17 position on the steroid nucleus (*SI Appendix*, Fig. S1 and Fig. 2), in contrast to the five-membered ring that characterizes the more familiar cardenolide compounds, such as digoxin and ouabain. Both BDs and cardenolides exert their pharmacological effects by inhibiting Na⁺/K⁺-ATPase (NKA), a ubiquitous cell-surface enzyme that transports sodium ions out of the cell and potassium ions in. Inhibition of NKA increases and prolongs contraction of cardiac muscle, rendering these compounds potent toxins against most vertebrate predators. However, mutations encoding for resistant paralogs of NKA have evolved in diverse animal groups

Significance

Animals protected by sequestered toxins must employ an alternative source of defensive chemicals when a shift in diet evolves. Snakes of the genus *Rhabdophis* possess defensive glands that store powerful defensive toxins known as bufadienolides. The ancestral prey of *Rhabdophis* consists of anuran amphibians, including toads (*Bufo*nidae) that are the source of sequestered bufadienolides. However, members of a derived clade of *Rhabdophis* consume nontoxic earthworms as their primary prey, yet their integumentary glands still contain bufadienolides. We demonstrate that snakes in the worm-eating clade obtain their defensive toxins primarily by consuming larval fireflies. Our finding represents a remarkable evolutionary example in which a shift from vertebrate to nonvertebrate prey provides the same chemical class of sequestered defensive toxins.

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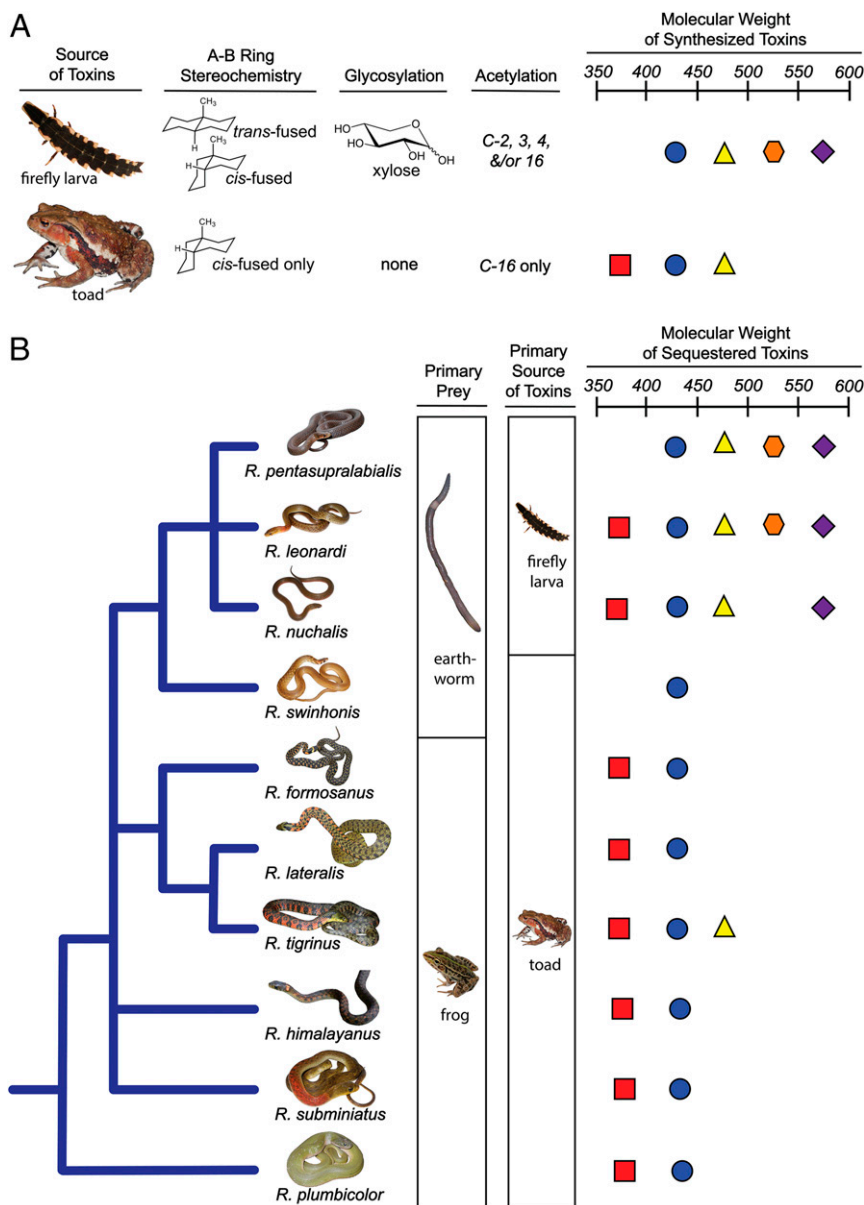


Fig. 1. Evolutionary shift in diet of *Rhabdophis* species is accompanied by a novel source of sequestered defensive compounds. (A) Chemical characteristics of the steroid toxins of fireflies and toads (25), which have been identified as the sources of sequestered defensive compounds in snakes of the genus *Rhabdophis*. Differences in stereochemistry, glycosylation, acetylation, and molecular weight between bufadienolides of toads and fireflies have been used to identify the primary source of toxins among species of *Rhabdophis* shown in B. Note that sulfated bufadienolides and suberoyl esters of bufadienolides without arginine, which are considered atypical, are excluded from the table of molecular weights. (B) Phylogenetic relationships among the 10 species of *Rhabdophis* for which data on defensive toxins (based on at least four individuals) are available. Anuran amphibians (frogs) comprise the ancestral diet of *Rhabdophis*, and the shift in prey from frogs to earthworms is followed by a shift in the primary source of toxins from toads to fireflies in the *R. nuchalis* Group, which contains *R. nuchalis*, *R. pentasupralabialis*, and *R. leonardi*. Red square, molecular weight ranging from 350 to 400; blue circle, molecular weight ranging from 401 to 450; yellow triangle, molecular weight ranging 451 to 500; orange hexagon, molecular weight ranging from 501 to 550; purple rhombus, molecular weight ranging from 551 to 600.

(7–9), including *Rhabdophis* (10), allowing them to consume, and in some cases to store, cardiotoxic steroids.

In addition to toads, BDs occur in some fireflies (Lampyridae), in which they may be referred to specifically as lucibufagins (Fig. 1A) (11, 12). The females of some firefly species (so-called “femmes fatales”) obtain their BDs by aggressive mimicry, luring and consuming the males of chemically defended species, thereby acquiring toxins by sequestration (13). Other fireflies may synthesize their BDs, but the biosynthetic pathway in fireflies has not been identified (14). Toads and fireflies are the only animals presently known or suspected to synthesize these compounds.

Here we report a remarkable case in which a derived clade of *Rhabdophis* has shifted its primary diet from amphibians to earthworms and, concomitant with that dietary change, has shifted the source of its sequestered defensive toxins from toads to fireflies. Our data demonstrate that an evolutionary change in diet from vertebrate to nonvertebrate prey has been accompanied by the acquisition of the same class of sequestered compounds from a novel prey source, thereby preserving the predator’s chemical defense. Although some phytophagous insects and many poison frogs consume a diverse diet and sequester compounds from different plants or arthropods, respectively (15, 16), the dramatic

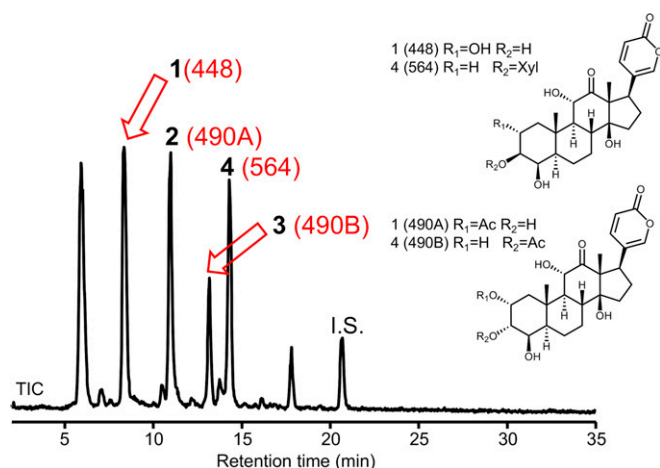


Fig. 2. Total ion chromatogram (TIC) of gland fluid from *R. pentasupralabialis* and structures of compounds 1 to 4. Major compounds showed ultraviolet (UV) spectral characteristics of bufadienolides (λ_{max} 295 nm, in CH_3OH/H_2O). "I.S." = internal standard (digitoxigenin).

shift reported here, from a vertebrate to an arthropod source of the same class of defensive compounds, represents an extraordinary evolutionary event.

Results and Discussion

Within *Rhabdophis* there exists a derived clade, the *R. nuchalis* Group, which contains *R. nuchalis*, *Rhabdophis pentasupralabialis*, and *Rhabdophis leonardi*. This species group has undergone a shift from the ancestral diet of amphibians to a diet dominated by earthworms (Fig. 1B) (6). Members of the *R. nuchalis* Group are smaller than other *Rhabdophis*, with less dramatic coloration and a narrower head. The latter feature generally reflects relatively shorter quadrate bones (17), a condition typical of snakes that

consume elongate prey, such as earthworms. The limited literature on the diet of the *R. nuchalis* Group suggests that these snakes mainly eat earthworms and slugs (18). We confirmed that the diet of wild *R. pentasupralabialis* consists primarily of earthworms. Examination of gut contents of 28 preserved specimens revealed 12 with remains of prey, eight of which included one or two earthworms (SI Appendix, Table S1). In laboratory feeding tests involving 10 individuals of *R. pentasupralabialis*, nine consumed earthworms, whereas none accepted ranid frogs and only one consumed a toad. Standard chemical preference tests of ingestively naive hatchlings showed a similar tendency: a strong chemical preference for earthworms, medium preference for toads, and no significant preference for ranid frogs (SI Appendix, Fig. S2 and Table S2).

We analyzed fluid from the nucho-dorsal glands of *R. pentasupralabialis* and found that the fluid contains BDs. Earthworms, however, are not known to produce BDs; we tested several taxa and failed to detect the toxins. Thus, earthworms are a very unlikely source of BDs in the snakes. From the NDG fluid of *R. pentasupralabialis*, we identified and structurally characterized four bufadienolides not previously reported from *Rhabdophis* (Figs. 2 and 3). Compounds 1 (448) and 4 (564), however, had previously been isolated from a North American firefly, *Lucidota atra* (19). The other two, compounds 2 (490A) and 3 (490B), are novel BDs, both of which we also found in the firefly *Diaphanes lampyroides* from Taiwan. Importantly, bufadienolide xylosides, such as compound 564, had previously been reported among animals only from fireflies (Fig. 1A) (12, 19), further supporting the hypothesis that *R. pentasupralabialis* stores bufadienolides from fireflies. We also recovered each of these four BDs from at least one specimen each of *R. nuchalis* and *R. leonardi* (Fig. 3), both members of the *R. nuchalis* Group. These findings indicate that the shift in toxin source from toads to fireflies is a derived feature of the worm-eating lineage.

The hypothesis that these four compounds are sequestered from fireflies is further supported by the stereochemistry of the

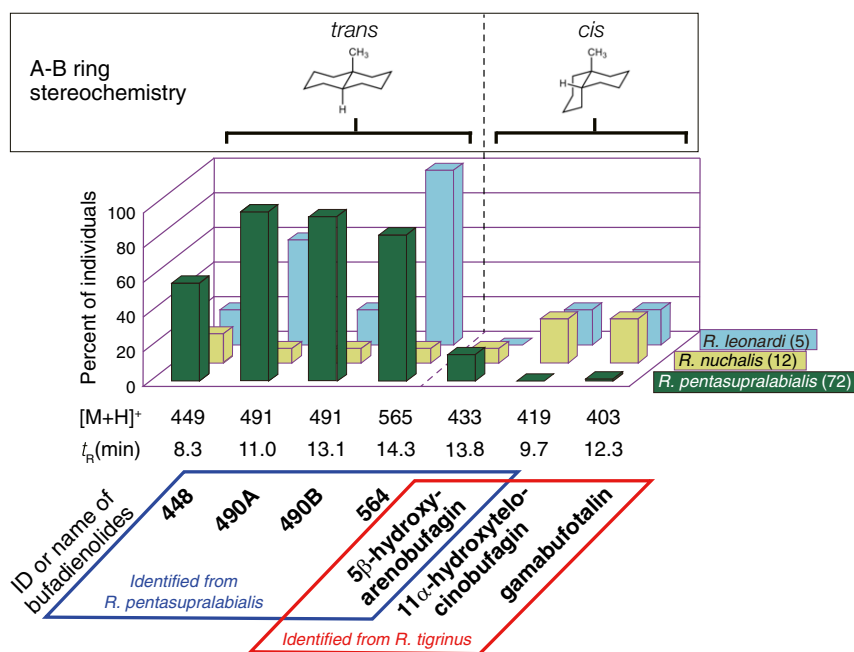


Fig. 3. Seven bufadienolides identified from *R. pentasupralabialis* and/or *R. tigrinus* based on NMR, and their observed percentage in three species of the *R. nuchalis* Group (*R. pentasupralabialis*, *R. nuchalis*, and *R. leonardi*). $[M+H]^+$ ions, retention time, and A-B ring stereochemistry are shown for each compound. Observed frequencies are based on either NMR or HPLC analysis. Numerals in parentheses after scientific names are sample size. Bufadienolides identified from *R. pentasupralabialis* are surrounded by a blue rhombus and those from *R. tigrinus* are surrounded by a red rhombus.

compounds. All bufadienolides identified from *R. pentasupralabialis* possessed a *trans*-fused A-B ring system. The BDs of fireflies include compounds with either *trans*- or *cis*-fused rings, whereas all BDs from toads (and those sequestered by *R. tigrinus* from toads) have a *cis*-fused A-B ring system (Fig. 1A) (2, 20–23). It is known from cardenolides, which target the same cell-surface protein (NKA), that both types of A-B ring junction are toxic, but compounds with a *cis* junction have a slightly stronger pharmacological effect than those with a *trans* junction (24). It is also significant that compounds 2 and 3 are acetylated at the C-2 and C-3 positions, respectively. Some firefly BDs are known to be acetylated at C-2, C-3, C-4, or C-16, whereas toad toxins are known to be acetylated only at C-16, if at all (Fig. 1A) (25).

The conclusions from our chemical studies are further supported by examination of the diet and behavioral prey preferences of *R. pentasupralabialis*. In addition to earthworms, we recovered eight larvae of lampyrine fireflies (*Diaphanes* sp.) from the stomach and/or intestine of five of the 12 preserved snakes with gut contents (SI Appendix, Table S1). In separate laboratory feeding tests, at least three of five *R. pentasupralabialis* voluntarily consumed larvae of a related lampyrine firefly (*Pyrocoelia* sp.). Taken together, our chemical, dietary, feeding, and behavioral studies indicate that *R. pentasupralabialis* feeds primarily on earthworms, but it also consumes lampyrine firefly larvae and sequesters its bufadienolides from those insects (Fig. 1B). The chemical, dietary, and behavioral studies also suggest that toads are retained as a minor component of the diet and a minor source of BDs.

The sequestration of defensive toxins has been most extensively studied in phytophagous insects, from which a large number of dietary compounds have been identified among diverse taxonomic orders (15). Speciation among insects that sequester dietary toxins often involves colonization of closely related host plants, and thus the ingestion of similar defensive compounds. This presumably reflects the presence of existing traits that confer resistance to those compounds and facilitate their uptake and storage (26). Nonetheless, prominent examples of dramatic host shifts in phytophagous insects are known, in which a lineage evolves to consume a distantly related host plant that supplies toxins similar chemically to the ancestral defensive compounds. For example, leaf beetles of the genera *Platyphora* and *Longitarsus* (Chrysomelidae) have undergone multiple shifts in host-plant families while retaining the same classes of sequestered defensive compounds (27, 28), as have sawflies (*Athalia*) (29) and some hemipterans (Lygaeinae) (30). Among vertebrates, the roughly 155 species of poison frogs, belonging to five families, are known to sequester more than 850 different lipophilic alkaloids from arthropod prey that include ants, beetles, millipedes, and oribatid mites (16, 31–33). Although these species may be “specialists” on small, alkaloid-rich arthropods, the geographic and temporal variation in diets, even within a species, suggests that they consume a fairly wide variety of such prey.

Among species of *Rhabdophis*, the shift in the primary diet, from frogs to earthworms, is not unusual. Earthworms are abundant, widespread, and elongate in shape, making them available to snakes with even limited gape. Consequently, they are eaten by many species of snakes belonging to diverse lineages (34). Indeed, some other natricine genera, such as *Thamnophis* (North America) and *Hebius* (Asia), include species that consume earthworms, often in addition to frogs and other prey (35, 36). Unlike earthworms, however, insects or other arthropods are consumed by relatively few species of alethinophidian snakes (17, 37). Thus, the shift in toxic prey observed within *Rhabdophis*, from the ancestral source of bufadienolides (toads) to a phylogenetically distant source of the same class of toxins (fireflies) (Fig. 1B), is remarkable.

The consumption of firefly larvae by members of the *R. nuchalis* Group is best explained as an adaptation to exploit a novel source

of bufadienolide toxins for defensive sequestration. That shift is even more unexpected considering the strong reliance of snakes upon chemosensory cues for the selection and location of prey. Given the distant relationship between toads and fireflies, the most likely behavioral path to that diet shift is through reliance upon the BDs themselves as a chemical cue. Certain insects employ plant-derived defensive toxins as feeding attractants, as in the case of pipevine swallowtail butterflies (Troidini and Zerythiini), for which aristolochic acids serve both functions (38). Indeed, the moderate response of *R. pentasupralabialis* to toad scent, and the consumption of one toad by a captive individual, suggest that members of the *R. nuchalis* Group retain at least some degree of the ancestral chemical preference for toads as prey (39). The relatively elongate shape and soft bodies of most firefly larvae may also enhance their general resemblance to earthworms. Although we suggest that chemical cues are probably of primary importance in identifying suitable prey, the general physical similarity of lampyrine larvae to earthworms may have played a secondary role in the early evolution of this diet shift by reducing the likelihood that the snakes would reject such larvae based on visual or tactile cues. Whatever the behavioral path underlying this shift in diet, we have demonstrated that *R. pentasupralabialis* consumes firefly larvae and possesses BDs identical with the lucibufagins of lampyrines. This strongly supports the hypothesis that the remarkable incorporation of firefly larvae in the diet of this species and other members of the *R. nuchalis* Group functions to preserve its chemical defense in the absence of a significant amphibian source of dietary bufadienolides.

Materials and Methods

Study Species. *R. pentasupralabialis* is a medium-sized natricine snake distributed in Sichuan and Yunnan Provinces, China. It was originally described as a subspecies of *R. nuchalis* (40) and subsequently was elevated to a full species by Zhao (41). Mori et al. (42) demonstrated the presence of nucho-dorsal glands in *R. pentasupralabialis*, as also described for *R. nuchalis* by Smith (43). Additional chemical data are available for *R. nuchalis* and *R. leonardi*, both of which are members of the *R. nuchalis* Group, and for *Rhabdophis formosanus*, *Rhabdophis himalayanus*, *Rhabdophis lateralis*, *Rhabdophis plumbicolor*, *Rhabdophis subminiatus*, *Rhabdophis swinhonis*, and *R. tigrinus*, all of which have nucho- or nucho-dorsal glands (see SI Appendix, Supplementary Data for sample sizes and localities).

Prey Preference and Feeding Tests. Thirty individuals of *R. pentasupralabialis* were hatched from eggs collected from a communal nest in Jiulong, Sichuan Province, China on 6 August 2014, and were used for feeding tests to examine whether this snake voluntarily eats toads (Bufonidae), nontoxic frogs (Ranidae), or earthworms. The hatchlings, weighing 1.8 to 2.5 g, were maintained in individual plastic cages (ca. 137 × 76 × 84 cm), with a small dish filled with water and paper substrate, in a laboratory of the Chengdu Institute of Biology, Chinese Academy of Sciences, at an air temperature of 24 °C and a 14:10 d:night light cycle (light: 0600 to 2000 h). The snakes were divided equally into three groups, each of which consisted of five males and five females, and were used for feeding trials 2 to 3 wk after hatching. Each individual was offered either earthworms (*Eisenia andrei*), toads (*Bufo gargarizans*), or frogs (*Pelophylax nigromaculatus*). Toads and frogs were either collected in the field or purchased at a local market and were frozen until offered as food. Toads and frogs were thawed before feeding and were cut into small pieces so the hatchlings could swallow them. Our previous studies confirmed that *R. tigrinus* readily eats pieces of thawed toads and frogs in captivity (44). Earthworms were purchased at pet shops and were offered alive. Prey was placed in the cage and left for ~24 h. If the food was not eaten after 24 h, it was removed. Feeding trials were conducted twice, and the number of individuals that consumed each prey type was recorded.

For the firefly feeding test, we used five adult *R. pentasupralabialis* (snout-vent length, 485 to 605 mm, two males and three females) collected in Xingou, Ya'an, Sichuan Province, China in August 2016. Each snake was kept in a plastic cage (ca. 40 × 60 × 40 cm) in a laboratory at Leshan National University, at an air temperature of 25 °C, humidity of 70 to 80%, and 14:10 d:night light cycle (light: 0600 to 2000 h). Firefly feeding trials were conducted from late August to early September, and no food was offered for at least 1 wk before the trials. We introduced three larvae of

Pyrocoelia sp. (collected in Jiangxi Province) into the cage and left them for ~72 h. A video camera (HIKVISION, DS-7908N-E4) equipped with infrared lighting was set above the cage to record the snake for subsequent analysis.

A chemical response test was conducted on 5 September 2014 using 19 ingestively naive hatchlings to determine whether *R. pentasupralabialis* exhibits an innate preference for earthworms and/or toads. Preparation and presentation of chemical stimuli followed a well-established protocol for testing chemical discrimination by the vomeronasal organ in Squamata (45, 46). Cotton swabs bearing surface stimuli from potential prey were presented to the snakes. The animals used for chemical stimuli included frogs (*Fejervarya fujianensis*, Ranidae); toads (*B. gargarizans*, Bufonidae); lizards (*Gekko subpalmatus*, Gekkonidae); and two species of earthworms (*Amyntas aspergillum*, Megascolecidae, and *E. andrei*, Lumbricidae). These species were selected because they or their close relatives are sympatric with *R. pentasupralabialis* or other members of the *R. nuchalis* Group. Lampyrid firefly larvae were unavailable for use in this test. Distilled water and cologne (Shower Fresh, Ocean Citrus, Mandom; diluted to 10% with distilled water) were used as controls, water as the solvent for prey odors and cologne as a detectable but biologically irrelevant odor.

Approximately 40 min prior to the experiment, the cages were moved to a testing table, and the water dish and paper substrate were removed. Each cage was separated by brown cardboard to eliminate visual stimuli from snakes in adjacent cages. Approximately 10 s before each trial the cover of a cage was gently removed, and the tip of a cotton swab was presented 1 to 2 cm anterior to the snout of the snake. Immediately before each trial the tip of the cotton swab was either rolled over the external surface of the animals wetted with distilled water or dipped into the control fluids. The number of tongue flicks directed to the swab was counted for 60 s after the first tongue flick was observed. If no tongue flick was made within 30 s after the presentation of the swab, the tip of the swab was gently touched to the snout of the snakes three times. If the snake did not flick its tongue for 60 s, the trial was terminated. If the snake bit the cotton swab, the trial was immediately terminated, and the number of tongue flicks and latency to bite, from the first tongue flick until the bite, were recorded. A freshly prepared swab was used for each snake. The order of presentation of the stimuli was randomized and counterbalanced. An interval of at least 20 min was maintained between trials using the same individual.

The tongue-flick attack score (TFAS) was calculated to compare the response of snakes among the chemical stimuli (see ref. 47 for the rationale for this index). If a snake does not bite, its TFAS is the number of tongue flicks. If it bites, TFAS is the largest number of tongue flicks by that individual in response to any stimulus plus 60 minus the latency time to bite (in seconds). TFAS was compared using a Friedman test, followed by pairwise multiple comparisons using a Wilcoxon signed rank test. In multiple comparisons, we did not strictly follow Bonferroni correction criteria for the adjustment of the statistical significance level because of the conservative nature of this correction and recent debates concerning the use of Bonferroni adjustments (e.g., refs. 48–50). Instead, we present the results for the levels $P < 0.01$, $P < 0.005$, and $P < 0.001$ for multiple comparisons, leaving the interpretation of significance to the reader. Statistical analyses were conducted using JMP 8.0.2.

All animal care and experimental procedures were approved by the Chengdu Institute of Biology Animal Care and Use Committee.

Analysis of Gut Contents. Gut contents were examined in 28 *R. pentasupralabialis*, collected by a local hunter in Xingou, Ya'an, Sichuan Province, China in June and August 2016. Snakes were preserved in 70% ethanol as soon as possible after collection, to prevent further digestion of prey. The digestive tract (from stomach to cloaca) was removed from each preserved specimen and dissected. Undigested contents were removed, preserved in 70% ethanol, and identified to the lowest possible taxonomic level.

Extraction and Isolation of Bufadienolides. Twenty-nine individuals of *R. pentasupralabialis* were collected in Xingou, Ya'an, Sichuan, China, and their nucho-dorsal gland fluid was collected. To collect the fluid, we placed a section of a laboratory tissue (Kimwipe) over the dorsal surface of neck and body and gently squeezed until the glands ruptured through the skin. The tissues were extracted two times with CH₃OH and the extract was dried in vacuo. The dried extract from mixed samples (91 mg) was redissolved in CH₃OH and filtered through a 0.45 μm membrane filter (DISMIC-13HP, Tokyo Roshi Kaisha, Ltd).

The extracts of gland fluid were fractionated into 20%, 30%, 40%, 50%, and 100% CH₃OH in H₂O (80 mL each) by a Sep-Pak C18 Cartridge (5 g, Waters Corp). The CH₃OH-H₂O (30:70) fraction was then subjected to preparative high performance liquid chromatography (HPLC) using a reversed-phase column (Mightysil RP-18 GP 4.6 mm internal diameter [I.D.] × 250 mm, Kanto Chemical Co., Inc.), eluted with 30% CH₃OH in H₂O at a flow rate of 1.0 mL/min. Compound 1 (3.0 mg) was isolated at $t_R = 8.3$ min. A portion of CH₃OH-H₂O (40:60) was also subjected to preparative HPLC using the same column, eluted with 40% CH₃OH in H₂O at a flow rate of 1.0 mL/min. Compounds 2 (1.6 mg) and 3 (2.8 mg) were isolated at $t_R = 11.0$ and 13.1 min, respectively. A portion of CH₃OH-H₂O (50:50) was also subjected to preparative HPLC using the same column, eluted with 40% CH₃OH in H₂O at a flow rate of 1.0 mL/min. Compound 4 was isolated at $t_R = 14.3$ min (2.9 mg). The respective compounds 1 to 4 were tentatively designated as compounds 448, 490A, 490B, and 564 (Fig. 2). HPLC was carried out using a Shimadzu system (LC-20AD pumps, CTO-20A column oven, and SPD-20A UV-VIS detector, Shimadzu). Peaks were monitored by UV absorbance at 295 nm, and the column temperature was maintained at 40 °C.

Ten individual larvae of the firefly *D. lampyroides* (Lampyridae) were collected in Sun Link Creek, Nantou County, Taiwan and were dipped in CH₃OH, and then collectively disrupted using a bead beater (Beads Crasher μT-12, Taitec Corporation) with stainless beads (0.5 mm diameter) to provide a pooled sample. Soluble fractions were collected by centrifugation (6,000 rpm × 5 min) and dried in vacuo. The extract (47.7 mg) was redissolved in CH₃OH and filtered through a 0.45 μm membrane filter (DISMIC-13HP, Tokyo Roshi Kaisha, Ltd). Additionally, 14 earthworms (2 of megascolecoid species, 3 of lumbricid species, and 9 of unidentified species) collected in Jiulong, Sichuan Province, China, where *R. pentasupralabialis* syntopically occurs, and 3 earthworms obtained from a pet dealer in China (unidentified species sold as fishing bait) were analyzed individually for their chemical components using the same methods as for the firefly larvae.

Liquid Chromatography/Mass Spectrometry (LC/MS) Analysis. LC/MS was carried out using an LC/MS-2010 equipped with Prominence HPLC system (Shimadzu) in atmospheric pressure chemical ionization (APCI) positive ion mode. A reversed-phase column (Mightysil RP-18 GP 2.0 mm I.D. × 50 mm, Kanto Chemical Co., Inc.) was eluted (0.2 mL/min) with a gradient of 20% (0 to 2 min), 20 to 55% (2 to 20 min), and 55 to 100% (20 to 35 min) CH₃OH in H₂O (0.1% HCOOH). The column temperature was maintained at 40 °C. The MS was operated with nebulizer gas flow of 2.5 L/min, APCI voltage of 1.9 kV, temperature of 400 °C, curved desolvation line (CDL) temperature of 250 °C, and heat block temperature of 200 °C.

Exact mass analysis was performed by a nano-liquid chromatography (Ultimate 3000, DIONEX)-mass spectrometry system (LTQ Orbitrap Velos, Thermo Fisher Scientific). Extracts (5 μL) were injected and separated by reversed-phase chromatography using a monolithic C18 column (0.075 mm I.D. × 1000 mm, Kyoto Monotech Co., Ltd.) at a flow rate of 280 nL/min. The MS¹ resolution was set to 60000 (at mass-to-charge ratio 400), MS² to 30000. The gradient was 5% (0 to 10 min), 5 to 50% (10 to 40 min), 50 to 95% (40 to 60 min), and 95% (60 to 70 min) CH₃OH in H₂O (0.1% HCOOH). MS² analysis was performed using Top3 data-dependent acquisition mode with dynamic exclusion set to 15 s. The column temperature was maintained at 40 °C. The MS was operated with electrospray ionization (ESI) (positive) voltage of 1.8 kV, with a capillary temperature of 350 °C. The higher energy collision-induced dissociation (HCD) collision energy was set to 30%. Reference standards of 490A and 490B identified from gland fluids from *R. pentasupralabialis* and extracts of firefly larvae (*D. lampyroides*) and the earthworms were analyzed with the nano-liquid chromatography-mass spectrometry system.

Nuclear Magnetic Resonance (NMR)-Spectroscopic Analysis. NMR spectra were measured with a Bruker AV-400 III Spectrometer (400 MHz), using tetramethylsilane as an internal standard. Each sample was dissolved in CD₃OD, and ¹H, ¹³C, hydrogen-hydrogen correlation spectroscopy (H-H COSY), heteronuclear single-quantum correlation (HSQC), heteronuclear multiple-bond coherence (HMBC), and nuclear overhauser effect and exchange spectroscopy (NOESY) spectra were acquired. Data for ¹H-NMR are recorded as follows: chemical shift (δ, ppm), multiplicity (s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; br, broad), integration, and coupling constant (Hz).

Data Availability. All data are contained in the main text and supporting information, except for the data of molecular weight of toxins of snakes and fireflies, which will be available from the corresponding author on reasonable request.

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