JOURNAL OF NATURAL PRODUCTS

Article

Cite This: J. Nat. Prod. XXXX, XXX, XXX−XXX

pubs.acs.org/jnp

Isolation and Identification of Alkaloids from Poisons of Fire Salamanders (*Salamandra salamandra*)

Janosch Knepper,[†] Tim Lüddecke,[‡,§] Kathleen Preißler,[§] Miguel Vences,[§] and Stefan Schulz*[,†]

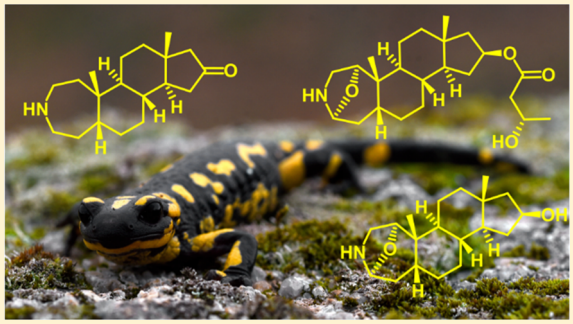
[†]Institute of Organic Chemistry, Technische Universität Braunschweig, Hagenring 30, 38106 Braunschweig, Germany

[‡]Animal Venomics Research Group, Fraunhofer Institute for Molecular Biology and Applied Ecology, Winchesterstraße 2, 35394 Gießen, Germany

[§]Zoological Institute, Technische Universität Braunschweig, Mendelssohnstraße 4, 38106 Braunschweig, Germany

Supporting Information

ABSTRACT: Fire salamanders (*Salamandra salamandra*) are conspicuously colored amphibians secreting a skin poison that contains unique steroid alkaloids such as samandarine (**1**) and samadarone (**2**), exhibiting toxic as well as antimicrobial activities. Because of their antipredatory and anti-infectious functions, alkaloids from *Salamandra* poison are of interest with regard to the threat that the lethal fungus *Batrachochytrium salamandrivorans* (*Bsal*) poses to salamanders. Nevertheless, reliable data on the biological activity of *Salamandra* alkaloids are scarce, in part due to the difficulty to obtain and study those substances. Thus, isolation of pure salamander alkaloids is an important task that might directly contribute to the understanding of *Bsal* infections. Here we present a noninvasive isolation procedure for samandarine (**1**) and *O*-acetylsamandarine (**3**), as well as for two new alkaloids, *O*-3-hydroxybutanoylsamandarine (**4**) and samanone (**6**), using HPLC. For the first time, high-field NMR data are presented for these alkaloids. Analysis using GC/MS and ESI$^+$-MS, provided important information on the structural variability of these salamander alkaloids.



European fire salamanders (*Salamandra salamandra*) are well known for their distinct black and yellow color pattern. This color pattern is assumed to act as an aposematic warning signal since the salamanders produce a defensive poison that is secreted by skin glands. These glands are distributed across the animal's dorsum and occur in concentrated form in a macroglandular system at the head, known as parotoid glands.

The first chemical investigations of fire salamander poison were performed by Zalesky in 1866, who isolated an amorphous base that was identified as an alkaloid and named samandarine.[1] Later studies on salamander poison were predominantly performed by Schöpf and Habermehl. They described the main constituents as steroid alkaloids and elucidated their absolute configurations.[2−11] Later the focus shifted toward functional and applied aspects of these compounds, such as biological properties, biosynthesis, and total synthesis. In this context, two main biological functions of the salamander alkaloids were discovered: First, they cause convulsions followed by lethal respiratory paralysis in various vertebrates.[12−15] Second, they possess antifungal and antimicrobial activity, but the mode of action of that phenomenon remained unresolved.[16−18] This second aspect is of special interest due to the lethal salamander plague, caused by the fungus *Batrachochytrium salamandrivorans* (*Bsal*), which led to the extinction of fire salamander populations in several parts of Europe.[19−22] In this regard an understanding of salamander toxins, especially their involvement in pathogen defense, is important, as discussed by us recently.[23]

Salamander alkaloids are not commercially available, as they are difficult to obtain in large quantities from the protected animals and even more difficult to synthesize. Earlier isolations were performed by manual extraction and crystallization methods, while no modern chromatographic isolation methods for this substance class are available.[24,25] However, for determination of the alkaloids' bioactivity and their potential in *Bsal* inhibition, it is necessary to produce larger quantities of purified alkaloids.

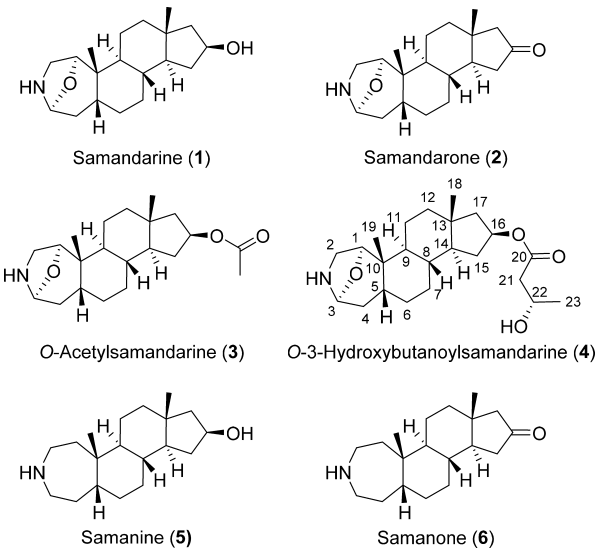
Here we developed a new isolation procedure based on HPLC and reevaluated the poison composition with modern methods. We isolated the four salamander alkaloids samandarine (**1**), *O*-acetylsamandarine (**3**), *O*-(*S*)-3-hydroxybutanoylsamandarine (**4**), and samanone (**6**), of which **4** and **6** have not been reported before as natural products.

RESULTS AND DISCUSSION

The skin secretion of 100 wild-caught individuals of *S. salamandra* from the Solling forest, Germany, was obtained via a noninvasive milking approach. Gentle application of

Received: January 22, 2019

DOI: 10.1021/acs.jnatprod.9b00065
J. Nat. Prod. XXXX, XXX, XXX−XXX



pressure by the thumbs on the parotoid glands and the tail root caused the release of the poison, which was poured into a glass container. The secretions were analyzed by HPLC coupled to heated electrospray ionization mass spectrometry (HESI-MS) after addition of MeOH. Chromatographic separation of most compounds was achieved as shown in Figure 1.

Eleven compounds (**A**–**K**) were detected by their $[M + H]^+$ ions, of which the major constituents were **A**, **D**, **H**, and **J**. Because the structures of only nine salamander alkaloids (Supporting Information, Figure S1) have been reported so far,[23] unknown compounds must have been present. Therefore, the compounds were isolated to clarify their structures and to obtain material for bioactivity testing. Purification of each compound was performed by scaling up the separation method to semipreparative HPLC conditions, which was complicated by lack of UV–vis activity of the target compounds.

By this method, 4 mg of compound **A** was isolated and identified as samandarine (**1**), already known as a major alkaloid of fire salamander poisons.[1] The molecular formula was determined as $C_{19}H_{31}NO_2$ by HR-ESI-MS. GC/EI-MS analysis showed a spectrum similar to the published one of **1** (Figure S3).[10] As no high-field NMR data of **1** have been reported so far, NMR data of the sample were obtained confirming the structure. The ^{1}H NMR spectrum (Table 1) showed signals at 5.44 and 4.49 ppm that were assigned to H-1 and H-3 of the ether bridge, while H-16 appeared at 4.31 ppm. COSY correlations led to four spin systems, H-1–H-2, H-3–H-4–H-5–H-6–H-7, H-9–H-11–H-12, and H-14–H-15–H-16–H-17, as shown in Figure 2. No coupling to other hydrogens was observed for the two methyl groups, implying their localization at a quaternary carbon. Characteristic ^{13}C signals were observed for both oxa-carbons, C-1 and C-3, at 80.9 and 89.6 ppm. The signal of C-16 at 72.2 ppm indicated a CH-OH group. HMBC correlations (Figure 2) completed and confirmed the structure. Additional structural confirmation was achieved by ^{1}H–^{15}N HMBC experiments showing couplings from the amine hydrogen to neighboring carbons, C-1 and C-4. The experimental NMR data were also highly similar to simulated spectra using the ACD/NMR Predictors software (ACD Laboratories). NOESY correlations confirmed the reported relative configuration of the salamander alkaloids, as most of the CH_2 protons were differentiated. The relative and absolute configuration of the main salamander alkaloids has been determined earlier, including X-ray crystallography.[26]

Compound **D** could not be isolated in pure form due to coeluting compounds. Although no EI-MS data were reported in the literature, the close similarity with the EI mass spectrum of **1**, differing mostly in the 2 amu lower molecular ion at m/z 303 (see Supporting Information Figure S4), the occurrence of ions characteristic for the N,O-acetal ring, m/z 56/57 and 85/86, and the known co-occurrence with **1** suggested this compound to be samandarone (**2**).[27]

Compound **J** proved to be *O*-acetylsamandarine (**3**), already known from fire salamander poisons,[25] although again no NMR data have so far been published. The molecular formula was determined to be $C_{21}H_{34}NO_3$ by HR-ESI-MS. The EI-mass spectrum (Figure S5) was similar to that of **1** (Figure S3 Supporting Information) with a molecular ion at m/z 347.

The ^{1}H and ^{13}C signals (Table 1) were assigned as for samandarine. Differences were found for C-16, which was detected at 76.1 ppm, typical for an ester. The ester carbon C_{20} was verified by a shift of 172.9 ppm. Additionally, HMBC couplings from C-20 to the hydrogens of the acetyl CH_3 group as well as to H-16 were detected. NOESY experiments revealed the same relative configuration as for **1**.

For compound **H** a molecular formula of $C_{23}H_{37}NO_4$ was determined by HR-ESI-MS. GC/EI-MS analysis revealed a spectrum shown in Figure 3a with M^+ at m/z 391, not matching with any of the known salamander alkaloids.

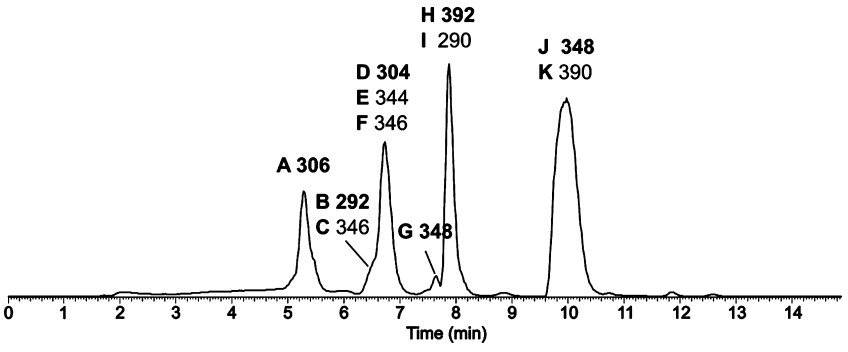


Figure 1. Base-peak chromatogram of salamander skin poison analyzed by HPLC/HESI-MS. The masses of $[M + H]^+$ ions are shown. Major components are shown in bold letters. The separation was optimized using a gradient composed of CH_3CN, H_2O, and 2% formic acid in H_2O. The CH_3CN content increased from 5% to 18.8% over 9 min, was constant for 3 min, and then increased again to 30% over 3 min, while a constant 0.1% formic acid concentration was used.. For details of the gradient, see Supporting Information Table S1.

DOI: 10.1021/acs.jnatprod.9b00065
J. Nat. Prod. XXXX, XXX, XXX–XXX

Table 1. ^{1}H and ^{13}C NMR Shifts of Samandarine (1), *O*-Acetylsamandarine (3), and *O*-3-Hydroxybutanoylsamandarine (4) in CD_3OD (^{1}H 600 MHz, ^{13}C 150 MHz)[a]

	1		3		4	
position	δ_C, type	δ_H (J in Hz)	δ_C, type	δ_H(J in Hz)	δ_C, type	δ_H(J in Hz)
1	80.8, CH	4.49, d (6.74)	80.7, CH	4.80, m	80.6, CH	4.45, d (6.71)
2	44.2, CH_2	3.40, m; 3.16, m	44.4, CH_2	3.34, m; 3.14, m	44.5, CH_2	3.33, m; 3.14, m
3	89.6, CH	5.44, s	89.6, CH	5.40, s	89.6, CH	5.38, s
4	30.8, CH_2	2.17, m; 1.39, m	31.2, CH_2	2.16, m; 1.37, m	31.3, CH_2	2.15, m; 1.35, m
5	34.5, CH	1.74, m	34.6, CH	1.75, m	34.6, CH	1.76, m
6	26.2, CH_2	1.85, tt (4.60, 21.01); 1.32, m	26.2, CH_2	1.85, m; 1.31, m	26.2, CH_2	1.85, m; 1.31, m
7	27.4, CH_2	1.51, m; 1.09, m	27.4, CH_2	1.48, m; 1.11, m	27.4, CH_2	1.49, m; 1.12, m
8	36.7, CH	1.49, m	36.7, CH	1.48, m	36.7, CH	1.49, m
9	42.5, CH	1.70, m	42.5, CH	1.72, m	42.5, CH	1.73, m
10	39.5, C		39.5, C		39.5, C	
11	21.6, CH_2	1.60, m; 1.39, m	21.6, CH_2	1.63, m; 1.39, m	21.6, CH_2	1.62, m; 1.39, m
12	40.2, CH_2	1.74, m; 1.18, dt (4.11, 12.85)	39.7, CH_2	1.76, m; 1.21, dt (3.84, 12.79)	39.7, CH_2	1.76, m; 1.21, dt (3.98, 13.04)
13	41.1, C		41.0, C		41.0, C	
14	54.7, CH	1.04, m	54.3, CH	1.09, m	54.4, CH	1.09, m
15	37.8, CH_2	2.17, m; 1.27, m	35.4, CH_2	2.28, m; 1.35, m	35.5, CH_2	2.29, m; 1.37, m
16	72.2, CH	4.31, m	76.1, CH	5.11, m	76.1, CH	5.15, m
17	51.8, CH_2	1.56, m; 1.49, m	49.0, CH_2	1.63, m; 1.58, m	49.6, CH_2	1.62, m
18	19.4, CH_3	0.95, s	18.8, CH_3	0.91, s	18.8, CH_3	0.92, s
19	17.6, CH_3	0.95, s	17.6, CH_3	0.95, s	17.6, CH_3	0.94, s
20			172.9, C		173.2, C	
21			21.2, CH_3	1.99, s	45.1, CH_2	2.39, m
22					65.7, CH	4.14, dq (1.30, 6.18)
23					23.4, CH_3	1.20, d (6.28)

[a]For atom numbering see structure block.

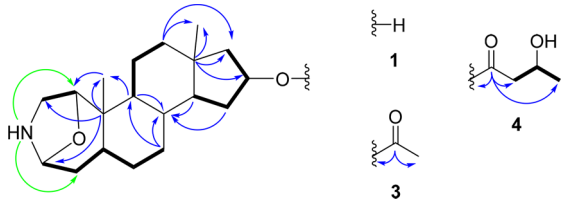


Figure 2. COSY (thick bonds), selected HMBC (blue arrows), and $^{1}H-^{15}N$ HMBC (green arrows) correlations of samandarine (**1**), *O*-acetylsamandarine (**3**), and *O*-3-hydroxybutanoylsamandarine (**4**).

Interestingly, the mass spectrum below m/z 288 was found to be very similar to that of compound **3**, revealing similarity in the ring systems of both. Therefore, the additional atoms are likely to be located outside the ring system. The ^{1}H and ^{13}C signals (Table 1) from C/H-1 to C/H-19 were almost identical to those of **3**, suggesting a modified acyl side chain. COSY, HSQC, and HMBC experiments revealed the presence of a 3-hydroxybutanoyl group, including a value of 65.7 ppm for the carbinol C-22 and an ester carbonyl at 173.2 ppm at C-20. While the configuration of the ring system was proven to be identical to those of **1** and **3**, the configuration of the hydroxybutanoyl group had to be elucidated. Therefore, transesterification was performed with trimethylsulfonium hydroxide (TMSH) in MeOH, resulting in the formation of methyl 3-hydroxybutanoate (MHB).[28] Chiral-phase GC using a Lipodex G phase showed that MHB had an *S*-configuration (Figure S6). The *R*-enantiomer of 3-hydroxybutyric acid predominates in animals, as it is formed during fatty acid biosynthesis, while fatty acid catabolism produces (*S*)-3-hydroxybutyric acid.[29−32] Compound **H** was therefore identified as the new salamander alkaloid *O*-(*S*)-3-hydroxybutanoylsamandarine (**4**).

Compound **I**, coeluting with **H** (**4**), was assigned to the only other mass in the poison extract not assignable to any known molecular ion. The molecular formula was determined to be $C_{19}H_{31}NO$ by HR-ESI-MS. GC/EI-MS analysis indicated a compound with m/z 289 and a retention index (RI) of 2574, whose spectrum is shown in Figure 3b. While the previous mass spectra were similar especially in the intensity differences from high to low amu, the spectrum of **I** was dominated by two intense signals, m/z 289 $[M]^+$ and 274 $[M - CH_3]^+$. No loss of H_2O was observed in the ESI-MS spectra, different from the samandarine-type compounds. MS^2 experiments with different ionization energies only led to either no fragmentation or complete degradation of the compound. Thus, two potential structures were proposed, i.e., an oxidized form of samanine (**5**) or a dehydroxylated form of samandarine (**1**). Given the spectral similarity to the mass spectrum of **5**[33] (Figure 3c), its oxidation product samanone (**6**) seemed to be the most likely structure. To verify this proposal, compound **I** was isolated from the fraction **H**/**I** by an additional separation, yielding about 1 mg of product (Table S2 and Figure S2 Supporting Information).

The NMR data supported again a samandarine-type skeleton, but lacking the oxygen bridge in ring A. COSY correlations confirmed four spin systems, H-1−H-2, H-3−H-4−H-5−H-6−H-7−H-8−H-9, H-11−H-12, and H-14−H-15 (Figure 4). Two methyl groups with no coupling to any other hydrogen were observed, suggesting that they are localized at a quaternary carbon. A keto group with a shift of 220.9 ppm was located at C-15 by HMBC, while no other oxygen-substituted carbons were present (Table 2). Further structure elucidation was performed using HMBC correlations that connected the

DOI: 10.1021/acs.jnatprod.9b00065
J. Nat. Prod. XXXX, XXX, XXX−XXX

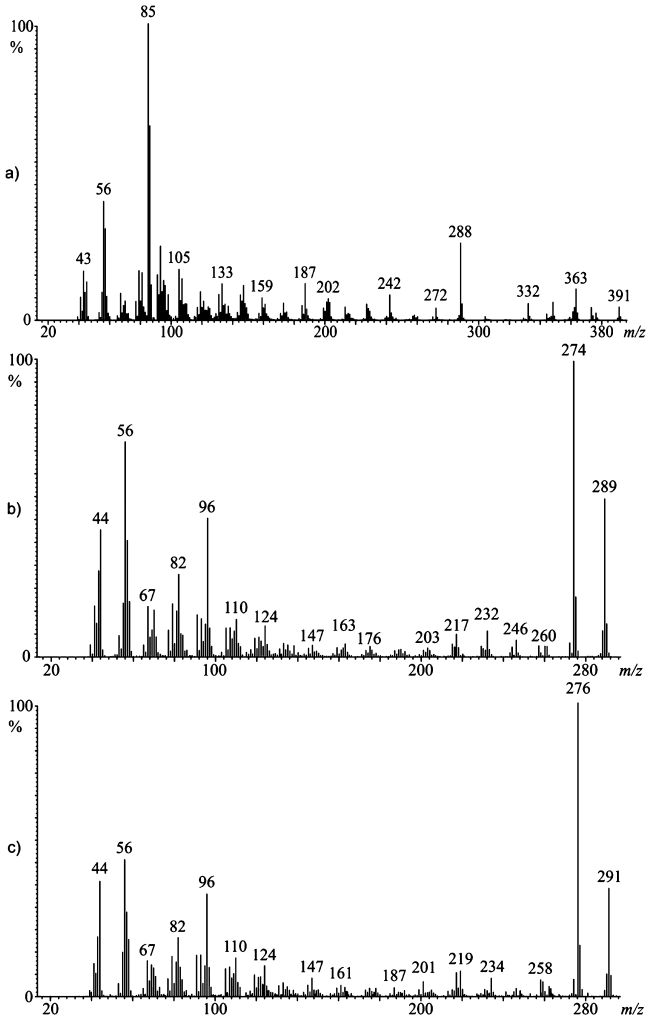


Figure 3. EI-mass spectra of (a) *O*-3-hydroxybutanoylsamandarine (**4**), (b) samanone (**6**), and (c) samanine (**5**).

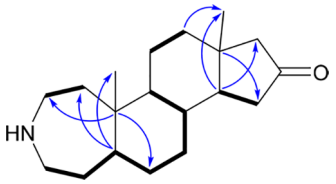


Figure 4. COSY (thick bonds) and characteristic HMBC (blue arrows) correlations of samanone (**6**).

structural parts of the individual H-spin systems. The NMR data showed high similarity, except near the C=O group, to the published data of synthetic samanine.[34] Therefore, compound **I** was identified as samanone (**6**), another new salamander alkaloid.

In the present study, we described a separation method that provided access to various salamander alkaloids and led to the identification of new structures occurring along with the previously known compounds. In other alkaloid-containing amphibians, the compound diversity of the alkaloid cocktail has been interpreted as one factor influencing overall toxicity[35] and thus one important component in the intertwined evolution of toxicity and aposematism.[36,37] Differently structured alkaloids might confer different biological activities, sometimes in complex interactions with other chemical compounds facilitating a delivery system.[38] Therefore, it is important to determine the exact composition of the alkaloid cocktail and other compounds produced by salamanders. Our work suggests that the inventory of steroid alkaloids in salamanders is not yet complete, and it remains to be experimentally determined which biological function the individual compounds exhibit alone or in combination with others.

By the experimental approach described here, relevant quantities of pure salamander alkaloids were obtained relying on a collection method less harmful and invasive for the animals than in previous protocols.[39,40] The isolated alkaloids will be used for bioassays to test their biological properties toward the pathogenic fungi *Batrachochytrium dendrobatidis* and *B. salamandrivorans*, as well as beneficial and pathogenic bacteria colonizing the skin.[41,42] A comparison of assays using these alkaloids alone and in biologically realistic mixtures of the entire salamander secretion may contribute to the understanding of the functional properties of the salamander defensive system.

Table 2. ^{1}H and ^{13}C NMR Shifts for Samanone (6) in CD_3OD (^{1}H 600 MHz, ^{13}C 150 MHz)[a]

position	δ_C, type	δ_H
1	38.9, CH_2	1.92, m; 1.69, m
2	41.9, CH_2	3.27, m; 3.15, m
3	48.9, CH_2	1.29, m
4	31.1, CH_2	1.94, m; 1.53, m
5	45.7, CH	1.74, m
6	29.1, CH_2	2.14, m; 1.54, m
7	28.1, CH_2	1.47, m; 1.05, m
8	36.8, CH	1.53, m
9	49.9, CH	3.35, m
10	38.1, C	
11	21.9, CH_2	1.53, m
12	39.1, CH_2	1.87, m; 1.50, m
13	40.1, C	
14	52.4, CH	1.64, m
15	40.1, CH_2	2.18, m; 1.96, m
16	220.9, C	
17	56.7, CH_2	2.04, m
18	18.3, CH_3	0.91, s
19	22.1, CH_3	1.08, s

[a]For carbon numbering see structure block.

EXPERIMENTAL SECTION

General Experimental Procedures. Optical rotations were measured using an MCP 150 polarimeter from Anton Paar. IR spectra were recorded with a Dani Instruments DiscovIR IR detector coupled to an Agilent Technologies 7890B gas chromatograph. NMR spectra were acquired with an AV II-600 (Bruker Biospin) instrument, operated at 600 MHz for ^{1}H and 150 MHz for ^{13}C. Tetramethylsilane (TMS, δ = 0 ppm) was used as an internal standard. Spin multiplicities of ^{13}C signals were determined via DEPT-135 measurements. High-resolution mass spectra of purified compounds were measured using an LTQ Orbitrap Velos (Thermo Scientific) via direct injection (DI). DI measurements were performed using a custom-made microspray device to allow flow rates of 1 μL·min^{-1} through a stainless-steel capillary (90 μm i.d.). Measurements were performed with a scan range from 50 to 2000 amu with a resolution of 100 000 fwhm at m/z 400. Typical spray voltages ranged between 2.3 and 2.8 kV for positive and between 1.7 and 2.5 kV for negative mode measurements. Additionally, the cation of tetradecyltrimethylammonium bromide (M = 256.29988 amu) was set as lock mass

DOI: 10.1021/acs.jnatprod.9b00065
J. Nat. Prod. XXXX, XXX, XXX−XXX

and was used as internal mass reference. An HPLC system consisting of an Accela photodiode array (PDA) Detector, Accela autosampler, and Accela 1250 pump was coupled to an LTQ XL mass spectrometer (from Thermo Scientific) for HPLC/HESI-MS analyses. Heated electrospray ionization was used with an enhanced scan range of 120 to 2000 amu. Gradient HPLC solvent programs consisted of LCMS-grade H_2O, CH_3CN, and 2% formic acid in H_2O. An Agilent Zorbax Eclipse Plus C18 (3.5 μm, 2.1 × 150 mm) column was used, which was kept at 30 °C. The PDA detector was set to a scanning range from 190 to 600 nm with 1 nm wavelength steps. Purification of compounds was performed via semipreparative HPLC-DAD using a Dionex Ultimate 3000 HPLC system consisting of a DAD, autosampler, pump, and automated fraction collector (Thermo Scientific). Gradient programs used HPLC-grade H_2O, CH_3CN, and 2% formic acid in H_2O with a flow rate of 4.73 mL·min^{-1}. A Nucleodur C18 HTec (5 μm, 10 × 250 mm) column was used. Analyses via GC/EI-MS were performed with a 7890A GC system coupled to a 5975 Series mass detector (Agilent Technologies) equipped with an Agilent HP-5MS capillary column (0.25 μm film, 0.25 mm × 30 m) and helium as the carrier gas. Ionization was performed via electron impact (EI) with 70 eV, and the following temperature program was used: isothermal at 50 °C for 5 min, followed by heating at 10 °C·min^{-1} up to 320 °C. Gas chromatographic retention indices were calculated from a homologous series of *n*-alkanes (C_8−C_{38}). Chiral-phase separations were performed with a 7890A GC-FID system (Agilent Technologies) using a Lipodex G column (50 m × 0.25 mm) and hydrogen as the carrier gas with the following temperature program: isothermal at 50 °C for 15 min, followed by heating at 25 °C·min^{-1} up to 220 °C.

Extraction and Isolation. The glandular secretion (1 g) was obtained by a noninvasive "milking" procedure applied to fire salamanders from three sites in the Solling area (geographical coordinates 51°53′25 N 9°36′17 E; 51°48′24 N 9°29′31 E; and 51°45′15 N 9°40′32 E). Each animal was taken into one hand with the head directed toward the thumb. Then the thumb of the controlling hand was placed on top of one of the parotoids and the thumb of the other hand underneath. A glass container was placed close to the parotoids, and by applying gentle pressure with both thumbs the skin poison was collected. As an alternative, the salamander's tail may serve as another source of poison. By applying gentle pressure on the root of the tail and then gliding up to the tail tip, secretions can be released. This procedure however causes a wider dispersion of poison droplets and therefore appears to be less accurate. We refer to the herein described method as "milking" (as suggested by Geßner and Craemer[43]) in analogy to the term often used for venom sampling in snakes and other venomous animals.

This secretion was extracted with 15 mL of MeOH for 20 min with regular mixing via soft shaking. The extract was filtered and diluted 1:10 in MeOH for method development and purification. Because salamander alkaloids cannot be detected via PDA, development of the method was performed via HPLC/HESI-MS, followed by scaling up to semipreparative HPLC for purification. Pure fractions of samandarine (**1**), *O*-acetylsamandarine (**3**), and *O*-3-hydroxybutanoylsamandarine (**4**) were obtained within the first purification step and analyzed. The solvent system used is shown in the Supporting Information Table S1. A second purification method resulted in the separation of samanone (**6**). Solvent gradient information is shown in the Supporting Information Table S2.

*Samandarine (**1**):* $[\alpha]^{20}_D$ +36 (*c* 0.4, MeOH); GC-IR ν_{max} 3431, 3321, 2938, 2853, 1480, 1451, 1379, 1339, 1279, 1162, 1114, 1042, 1017, 854, 830 cm^{-1}; ^{1}H and ^{13}C NMR data (CD_3OD), Table 1; HR-ESI-MS *m*/*z* 306.24309 $[M + H]^+$ (calcd for $C_{19}H_{32}NO_2$, 306.24330); EI-MS spectrum, Figure S3 Supporting Information.

*O-Acetylsamandarine (**3**):* $[\alpha]^{20}_D$ +42 (*c* 0.2, MeOH); GC-IR ν_{max} 3341, 3264, 2936, 2853, 1738, 1444, 1377, 1358, 1249, 1115, 1086, 1039, 1020, 979, 848, 831, 752 cm^{-1}; ^{1}H and ^{13}C NMR data (CD_3OD), Table 1; HR-ESI-MS *m*/*z* 348.25365 $[M + H]^+$ (calcd for $C_{21}H_{34}NO_3$, 348.25387); EI-MS spectrum, Figure S5 Supporting Information.

*O-3-Hydroxybutanoylsamandarine (**4**):* $[\alpha]^{20}_D$ +40 (*c* 0.4, MeOH); GC-IR ν_{max} 3335, 2969, 2935, 2856, 1730, 1452, 1379, 1303, 1179, 1115, 1086, 1039, 1022, 999, 952, 915, 858, 831 cm^{-1}; ^{1}H and ^{13}C NMR data (CD_3OD), Table 1; HR-ESI-MS *m*/*z* 392.27986 $[M + H]^+$ (calcd for $C_{23}H_{38}NO_4$, 392.28008); EI-MS spectrum, Figure 3a.

*Samanone (**6**):* colorless semisolid; $[\alpha]^{20}_D$ −9.2 (*c* 0.1, MeOH); GC-IR ν_{max} 2960, 2931, 2875, 2860, 1726, 1601, 1582, 1465, 1381, 1283, 1263, 1126, 1076, 745 cm^{-1}; ^{1}H and ^{13}C NMR data (CD_3OD), Table 2; HR-ESI-MS *m*/*z* 290.24826 $[M + H]^+$ (calcd for $C_{19}H_{32}NO$, 290.24839); EI-MS spectrum, Figure 3b.

Transesterification of *O*-3-Hydroxybutanoylsamandarine (4). The absolute configuration of the 3-hydroxybutanoyl side chain was determined after transesterification of **4** with TMSH according to Schomburg et al.[28] A small amount of the compound was dissolved in 100 μL of CH_2Cl_2, and 100 μL of 0.25 M TMSH in MeOH was added. After 1 h, the mixture was diluted with CH_2Cl_2 and analyzed by GC/EI-MS.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jnatprod.9b00065.

Overview of salamander alkaloids identified so far, GC/EI-MS data for samandarine (**1**) samandarone (**2**), and *O*-acetylsamandarine (**3**), NMR spectra of all compounds, chromatograms of chiral-phase analysis, as well as purification gradients for semipreparative HPLC (PDF)

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: stefan.schulz@tu-bs.de.

ORCID

Stefan Schulz: 0000-0002-4810-324X

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

The research was funded by the German Ministry of Science and Education in the framework of the program ANOBIN. Fieldwork was performed in accordance with animal welfare (33.19-42502-04-18/2792) and under the permission of the administrative districts of Northeim (VI.3-NAT-1738/18) and Holzminden (366 3245 30 44/2018). We are grateful to E. Sanchez for valuable discussions on the subject and to S. Gippner, M. Dinis, M. Wagler, F. Goudarzi, R. Schmidt, U. Seidel, and S. Steinfartz for their much appreciated help during the fieldwork.

■ REFERENCES

(1) Zalesky, S. *Med. Chem. Untersuch. Hoppe-Seyler.* **1866**, *1*, 85−116.
(2) Schöpf, C.; Braun, W. *Liebigs Ann. Chem.* **1934**, *514*, 69−136.
(3) Schöpf, C.; Möller, O. W. *Liebigs Ann. Chem.* **1960**, *633*, 127−156.
(4) Wölfel, E.; Schöpf, C.; Weitz, G.; Habermehl, G. *Chem. Ber.* **1961**, *94*, 2361−2373.
(5) Habermehl, G. *Chem. Ber.* **1963**, *96*, 840−844.
(6) Habermehl, G. *Chem. Ber.* **1963**, *96*, 143−151.
(7) Habermehl, G. *Liebigs Ann. Chem.* **1964**, *679*, 164−167.
(8) Habermehl, G. *Chem. Ber.* **1966**, *99*, 1439−1442.
(9) Habermehl, G.; Haaf, G. *Chem. Ber.* **1965**, *98*, 3001−3005.

DOI: 10.1021/acs.jnatprod.9b00065
J. Nat. Prod. XXXX, XXX, XXX−XXX

(10) Habermehl, G.; Spiteller, G. *Liebigs Ann. Chem.* **1967**, *706*, 213−222.
(11) Habermehl, G.; Haaf, A. *Liebigs Ann. Chem.* **1969**, *722*, 155−161.
(12) Günter, M. *Lacerta.* **1926**, *1*, 3−4.
(13) Gessner, O.; Möllenhoff, P. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **1932**, *167*, 638−653.
(14) Hostalka, G. *Wild und Hund.* **1984**, *87* (10), 54−55.
(15) Paulitsch, P. *Wild und Hund.* **1984**, *87* (14), 35.
(16) Habermehl, G. In *Mertensiella Vol. 4, Biology of Salamandra and Mertensiella*; Greven, H.; Thiesmeier, B., Eds.; DGHT: Bonn, 1994; pp 209−214.
(17) Habermehl, G.; Preusser, H.J. Z. *Z. Naturforsch., B: J. Chem. Sci.* **1969**, *24b*, 1599−1601.
(18) Preusser, H. J.; Habermehl, G.; Sablofski, M.; Schmall-Haury, D. *Toxicon* **1975**, *13*, 285−288.
(19) Martel, A.; Spitzen-Van der Sluijs, A.; Blooi, M.; Bert, W.; Ducatelle, R.; Fisher, M. C.; Woeltjes, A.; Bosman, W.; Chiers, K.; Bossuyt, F.; Pasmans, F. *Proc. Natl. Acad. Sci. U. S. A.* **2013**, *110*, 15325−15329.
(20) Sabino-Pinto, J.; Bletz, M.; Hendrix, R.; Perl, R. G. B.; Martel, A.; Pasmans, F.; Lötters, S.; Mutschmann, F.; Schmeller, D. S.; Schmidt, B. R.; Veith, M.; Wagner, N.; Vences, M.; Steinfartz, S. *Amphibia-Reptilia.* **2015**, *36*, 411−416.
(21) Spitzen-Van der Sluijs, A.; Martel, A.; Asselberghs, J.; Bales, E. K.; Beukema, W.; Bletz, M. C.; Dalbeck, L.; Goverse, E.; Kerres, A.; Kinet, T.; Kirst, K.; Laudelout, A.; Marin da Fonte, L. F.; Nöllert, A.; Ohlhoff, D.; Sabino-Pinto, J.; Schmidt, B. R.; Speybroeck, J.; Spikmans, F.; Steinfartz, S.; Veith, M.; Vences, M.; Wagner, N.; Pasmans, F.; Lötters, S. *Emerging Infect. Dis.* **2016**, *22*, 1286−1288.
(22) Stegen, G.; Pasmans, F.; Schmidt, B. R.; Rouffaer, L. O.; Van Praet, S.; Schaub, M.; Canessa, S.; Laudelot, A.; Kinet, T.; Adriaensen, C.; Haesebrouck, F.; Bossuyt, F.; Bert, W. *Nature* **2017**, *544*, 353−356.
(23) Lüddecke, T.; Schulz, S.; Steinfartz, S.; Vences, M. *Naturwissenschaften* **2018**, *105*, 56.
(24) Schöpf, C.; Braun, W. *Liebigs Ann. Chem.* **1934**, *514*, 69−136.
(25) Habermehl, G. *Liebigs Ann. Chem.* **1964**, *679*, 164−167.
(26) Schöpf, C. *Experientia* **1961**, *17*, 285−328.
(27) Mebs, D.; Pogoda, W. *Toxicon* **2005**, *45*, 603−606.
(28) Müller, K.-D.; Husmann, H.; Nalik, H. P.; Schomburg, G. *Chromatographia* **1990**, *30*, 245−248.
(29) Berg, J.; Tymoczko, J.; Stryer, L. *Streyer Biochemie, Der Fettsäurestoffwechsel. Stryer Biochemie.*; Springer: Berlin Heidelberg, 2013; pp 660−661.
(30) Stuart, J. A.; Ooi, E. L.; McLeod, J.; Bourns, A. E.; Ballantyne, J. *Biol. Bull.* **1998**, *195*, 12−16.
(31) Lafferty, R. M. U.S. Patent 4,211,846, 1980.
(32) Rietschel, E. T. *Eur. J. Biochem.* **1976**, *64*, 423−428.
(33) Cristofoli, W. A.; Benn, M. *J. Chem. Soc., Perkin Trans. 1* **1991**, *1*, 1825−1831.
(34) Walter, A. C.; Benn, M. *J. Chem. Soc., Perkin Trans. 1* **1991**, *1*, 1825−1831.
(35) Summers, K.; Clough, M. E. *Proc. Natl. Acad. Sci. U. S. A.* **2001**, *98*, 6227−6232.
(36) Santos, J. C.; Cannatella, D. C. *Proc. Natl. Acad. Sci. U. S. A.* **2011**, *108*, 6175−6180.
(37) Preißler, K.; Gippner, S.; Lüddecke, T.; Krause, E. T.; Schulz, S.; Vences, M.; Steinfartz, S. *J. Zool.* **2019**, DOI: 10.1111/jzo.12676
(38) Raaymakers, C.; Verbrugghe, E.; Hernot, S.; Hellebuyck, T.; Betti, C.; Peleman, C.; Claeys, M.; Bert, W.; Caveliers, V.; Ballet, S.; Martel, A.; Pasmans, F.; Roelants, K. *Nat. Commun.* **2017**, *8*, 1495.
(39) Schöpf, C.; Braun, W. *Justus Liebigs Ann. Chem.* **1934**, *514*, 69−136.
(40) Habermehl, G. *Mertensiella.* **1994**, *4*, 209−214.
(41) Sanchez, E.; Bletz, M. C.; Duntsch, L.; Bhuju, S.; Geffers, R.; Jarek, M.; Dohrmann, A. B.; Tebbe, C. C.; Steinfartz, S.; Vences, M. *Microb. Ecol.* **2017**, *73*, 455−465.
(42) Bletz, M. C.; Kelly, M.; Sabino-Pinto, J.; Bales, E.; Van Praet, S.; Bert, W.; Boyen, F.; Vences, M.; Steinfartz, S.; Pasmans, F. *Proc. R. Soc. London, Ser. B* **2018**, *285*, 20180758.
(43) Geßner, O.; Craemer, K. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **1930**, *152*, 229−237.

DOI: 10.1021/acs.jnatprod.9b00065
J. Nat. Prod. XXXX, XXX, XXX−XXX