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Cyanobufalins: Cardioactive Toxins from Cyanobacterial Blooms

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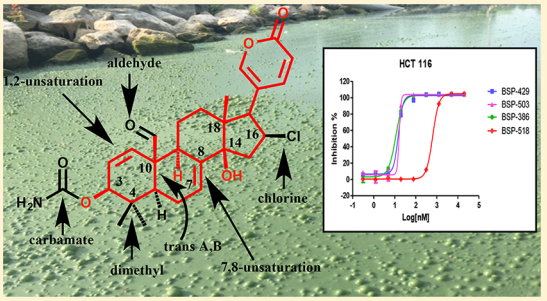
Supporting Information

ABSTRACT: Cyanobufalins A−C (**1**−**3**), a new series of cardiotoxic steroids, have been discovered from cyanobacterial blooms in Buckeye Lake and Grand Lake St. Marys in Ohio. Compounds **1**−**3** contain distinctive structural features, including geminal methyl groups at C-4, a 7,8 double bond, and a C-16 chlorine substituent that distinguish them from plant- or animal-derived congeners. Despite these structural differences, the compounds are qualitatively identical to bufalin in their cytotoxic profiles versus cell lines in tissue culture and cardiac activity, as demonstrated in an impedance-based cellular assay conducted with IPSC-derived cardiomyocytes. Cyanobufalins are nonselectively toxic to human cells in the single-digit nanomolar range and show stimulation of contractility in cardiomyocytes at sub-nanomolar concentrations. The estimated combined concentration of **1**−**3** in the environment is in the same nanomolar range, and consequently more precise quantitative analyses are recommended along with more detailed cardiotoxicity studies. This is the first time that cardioactive steroid toxins have been found associated with microorganisms in an aquatic environment. Several factors point to a microbial biosynthetic origin for the cyanobufalins.



Buckeye Lake (BL) and Grand Lake St. Marys (GLSM)[1] in Ohio are subject to recurrent, potent harmful algal blooms (HAB) that adversely impact water quality and public health.[2] In addition to the Schedule CCL-4 cyanotoxins, anatoxin-a, microcystin, saxitoxin, and cylindrospermopsin,[3] several other classes of secondary metabolites are known from these lakes.[4,5] We have initiated a program to mine the chemical diversity available in these HAB events for the discovery of novel chemical leads for anticancer drug discovery. Biomass was accumulated in kiloton amounts by leveraging proprietary harvesting technologies;[6] the constituents of the meta-metabolome were extracted, concentrated, fractionated, and tested for their effects on several cancer cell lines.

Meticulous refining of the most cytotoxic fractions led to the discovery of a new series of toxins in both lakes that are responsible for the observed cytotoxicity to cancer cell lines. These compounds are designated cyanobufalins A (**1**), B (**2**), and C (**3**) and are structurally related to the bufadienolides. Bufadienolides are a well-known class of cardioactive steroidal natural products, initially isolated from plants and toad skin[7] (e.g., bufalin **4**), and subsequently found in many other plants and animals, including humans.[8] Cyanobufalins are present at picomolar to nanomolar levels in the environment, but were detectable owing to their highly potent cytotoxicity. Moreover, cyanobufalins may represent a significant, previously unrecognized environmental health hazard because of their potent effects on cardiac cells. Cyanobufalins are the first examples of cardioactive steroid toxins isolated from microbial sources. In this report we present the isolation, structure elucidation, cytotoxicity, and cardioactivity of the cyanobufalins.

RESULTS AND DISCUSSION

Initially, small quantities of cyanobufalins A (**1**) and B (**2**) were isolated by chromatographic purification from the most potent cytotoxic fractions derived from microalgal biomass obtained from GLSM in May 2012. These compounds were later found in greater adundance in biomass harvested in 2014 from BL, about 130 miles southeast of GLSM in Ohio. Freeze-dried biomass was extracted with CH_2Cl_2/MeOH (2:1), and the extract was subjected to solvent partitions. Material of intermediate polarity was sequentially chromatographed over Diaion HP-20 and C18 resins. Compounds **1**, **2**, and **3**, which

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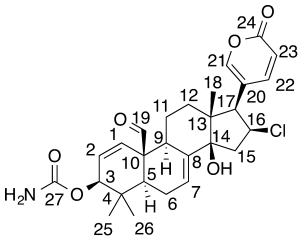

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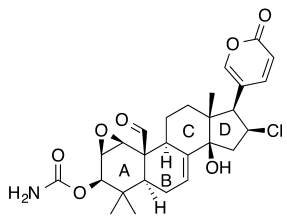

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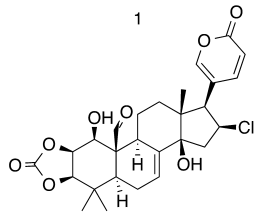

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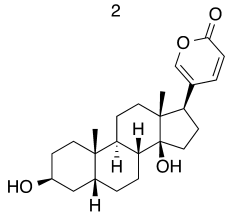

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are the most potent cytotoxic components in the biomass, were obtained in pure form by repeated HPLC steps. The structures of these compounds were elucidated by detailed analyses of MS and NMR data.

The molecular formula of **1** was determined by HRESIMS to be $C_{27}H_{32}ClNO_6$, implying 12 degrees of unsaturation. Initial analysis of the proton and carbon NMR spectra indicated the presence of an unsaturated heterocycle. The ^1H and ^{13}C chemical shifts of this moiety and their 2D NMR correlations (Table 1) displayed the typical features of a 2*H*-pyran-2-one or α-pyrone, which was supported by the UV absorption at λ_{max} 290 nm. Further examination of the NMR spectra revealed that **1** consisted of a steroid skeleton with the characteristic shielded methyl singlet at δ_H 0.60 for CH_3-18 and two others at δ_H 0.89 and 0.67 attached to C-4 at δ_C 36.4. The presence of the α-pyrone and steroid substructure drew our attention to the bufadienolides. Additional ^1H and ^{13}C NMR signals suggested that **1** contained a carbamate group (δ_C 157.3 and δ_H 6.65 and 6.38) and an aldehyde (δ_C 207.3 and δ_H 9.76). The latter appeared to replace the missing C-19 methyl group.

The COSY and TOCSY data identified four $^1H-^1H$ spin systems for the A/B/C/D fused cyclic moiety: (a) H-1 at δ_H 5.96, H-2 at 5.55 (both olefinic), and H-3 at 4.87; (b) H-5 at δ_H 1.95, H_2-6 at 2.48 and 2.29, and H-7 at 6.15 (olefinic); (c) H-9 at δ_H 2.26, H_2-11 at 1.70 and 0.94, and H_2-12 at 1.48 and 1.40; (d) H_2-15 at δ_H 2.99 and 1.94, H-16 at 4.97, and H-17 at 3.09. The two- and three-bond $^1H-^{13}C$ correlations in the HMBC spectrum (Figure 1) connected these spin systems and confirmed the steroid skeleton. The HMBC correlations also identified the locations of several functional groups on the skeleton. In particular, the correlations indicated that C-19 was an aldehyde, the carbamate group was linked to C-3, and a hydroxy group was attached to C-14. The α-pyrone was confirmed to be connected to C-17 by the HMBC correlations from H-17 at δ_H 3.09 to both C-21 at δ_C 151.8 and C-22 at 150.5. Finally, the Cl substituent required by mass spectrometric data had to be placed at C-16 (δ_C 60.2), as the only remaining available position. Table 1 and Figure 1 summarize the chemical shift and correlation data.

The relative configuration of **1** was determined by a NOESY spectrum, in which strong correlations indicate close proximities through space. The correlations between H_3-26 and H-19, H-19 and H-11b, and H-19 and H_3-18 (Figure 1) indicated that these protons were all β oriented. On the other hand, the cross-peaks between H-3 and H-5, H-5 and H-9, and H-9 and H-12a suggested that they were α. The NOESY data also required that **1** be oriented with A/B *trans* and C/D *cis* ring junctions and that the Cl group at C-16 is β. The structure of cyanobufalin A (**1**) was thus established as shown.

Table 1. NMR Data for Cyanobufalin A (1) in DMSO-d_6 (^1H 800 MHz, ^{13}C 200 MHz)

	δ_C	δ_H (J in Hz)	$^1H-^{13}C$ HMBC correlation	NOESY
1	129.4, CH	5.96, dd (10.5, 2.4)	3, 5, 9, 10	2, 3, 9, 11a, 11b
2	129.5, CH	5.55, dd (10.5, 1.7)	4, 10	1, 3, 11a
3	76.9, CH	4.87, t (2.0)	2, 4, 25, 26, 27	2, 5, 6b, 25
4	36.4, C			
5	46.9, CH	1.95, ovlp	3, 4, 6, 9, 10 26	3, 6a, 6b, 9, 25
6a	22.5, CH_2	2.48, m	5, 7, 8	5, 6b, 7, 19, 25, 26
6b		2.29, m	5, 7, 8, 10	5, 6a, 7, 25
7	122.0, CH	6.15, m	5, 9, 14	5, 6a, 6b, 14-OH, 25
8	139.5, C			
9	43.9, CH	2.26, m	11	5, 11a, 12a, 15a
10	51.9, C			
11a	23.0, CH_2	1.70, m	8, 9, 12, 13	1, 9, 11b, 12a, 12b, 18
11b		0.94, m	9, 12, 13	1, 11a, 12a, 12b, 18, 19
12a	37.8, CH_2	1.48, m	9, 11, 13, 14, 17, 18	9, 11a, 11b, 15a, 16, 17, 18
12b		1.40, m	9, 11, 13, 14, 17, 18	
13	50.6, C			
14	83.1, C			
15a	50.5, CH_2	2.99, dd (15.8, 9.8)	8, 17	9, 12a, 15b, 16
15b		1.94, ovlp	13, 14, 16, 17	OH-14, 15a,16
16	60.2, CH	4.97, td (9.5, 2.5)	14, 15, 17	12a, 15a, 15b, 17
17	56.8, CH	3.09, d (9.3)	12, 13, 14, 15, 16, 20, 21, 22	12a, 16, 21
18	17.2, CH_3	0.60, 3H, s	12, 13, 14, 17	11b, 12b, 19
19	207.3, CH	9.76, s	1, 10	6a, 11b, 18, 26
20	119.3, C			
21	151.8, CH	7.59, d (2.4)	17, 20, 22, 24	12a, 12b, 16, 17, 18
22	150.5, CH	8.25 dd (9.8, 2.5)	20, 21, 24	14-OH, 15b, 17, 18, 23
23	112.0, CH	6.25, d (9.8)	20, 24	22
24	161.6, C			
25	25.2, CH_3	0.89, 3H, s	3, 4, 5, 26	5, 6a, 6b
26	16.5, CH_3	0.67, 3H, s	3, 4, 5, 25	6a, 19
27	157.3, C			
NH_2		6.65, bs	27	NHat 6.38
		6.38, bs	27	NH at 6.65
14-OH		5.10, bs	14, 15	

The molecular formula of **2** was determined by HRESIMS to be $C_{27}H_{32}ClNO_7$, with one more oxygen than **1**. In the ^1H and ^{13}C NMR spectra (Table S1), the olefinic H-1/C-1 and H-2/C-2 signals in **1** were respectively changed to δ_H 3.65/δ_C 54.2 and δ_H 3.10/δ_C 56.6, indicative of an epoxide in **2**. The HMBC correlations (Figure 1) from H-1 at δ_H 3.65 to C-2 at δ_C56.6 and to C-5 at δ_C 40.8, from H-2 at δ_H 3.10 to C-3 at δ_C 75.8 and C-4 at δ_C 36.0, and from H-19 at δ_H 9.82 to C-1 at δ_C

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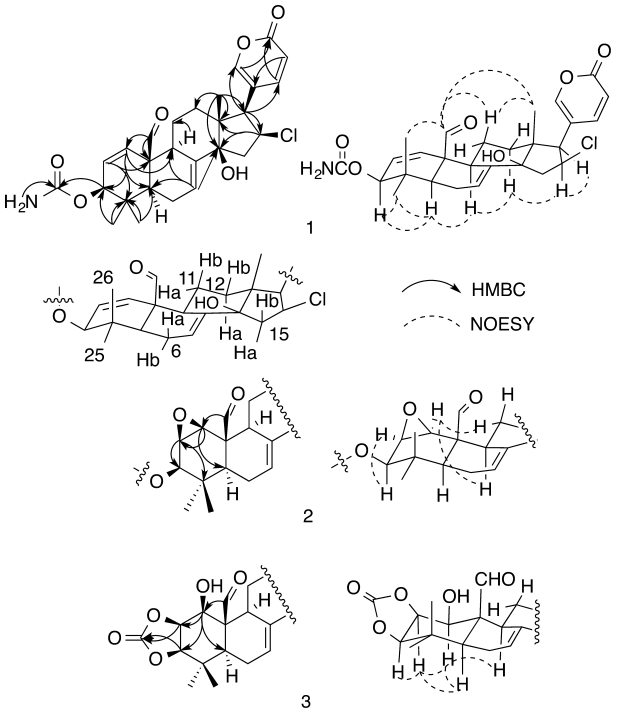

Figure 1. 2D NMR correlations defining the structure and relative configurations of **1**−**3**.

54.2 confirmed the presence and location of the epoxide. NOESY correlations between H-1 and H-11a; H-1 and H-9; and H-2 and H-3 defined that the epoxide ring was β, as depicted in Figure 1. The remaining structure of cyanobufalin B (**2**) was identical to **1** according to the NMR and UV data.

The molecular formula of **3** was determined by HRESIMS to be $C_{27}H_{31}ClO_8$, with 12 degrees of unsaturation. The MS and NMR data (Table S2) indicated that **3** lacked the C-3 carbamate of **1** and **2**. Instead it contained a cyclic carbonate and a secondary hydroxy group. The coupling between H-1 at δ_H 4.59 and the OH at 5.87 in the COSY spectrum and the HMBC correlations (Figure 1) from H-19 at δ_H 9.73 and the OH at 5.87 to C-1 at δ_C 64.2 required that the hydroxy group was attached to C-1. Additional HMBC correlations from H-2 at δ_H 4.80 and H-3 at 4.58 to C-27 at δ_C 154.8 suggested that ring A and the carbonate formed a fused cyclic system at C-2 and C-3. The observation of several NOESY correlations required the relative configuration between these two rings to be *cis* and the hydroxy group at C-1 to be β, as illustrated in Figure 1. The remaining structure of cyanobufalin C (**3**) was identical to **1** and **2**, according to the NMR and UV data.

Cytotoxicity against Cell Lines. Compounds **1**, **2**, **3**, and **4** were tested against the cell lines listed in Table 2. Compounds **1** and **2** demonstrated potent and indiscriminate cytotoxicity against human cancer and normal cell lines derived from various tissues, mostly at single-digit nanomolar concentrations. Only the breast cancer cell line MCF-7 appeared somewhat less sensitive. Compound **3** was consistently 2 to 3 orders of magnitude less potent than **1**

Table 2. Concentration (nM) of Compound Causing 50% Reduction in the Viability (EC_{50}) of Cell Lines

		EC_{50} (nM)			
cell line	tissue type	1	2	3	4
BT-474[a]	breast	3.7	3.9	NT[d]	NT
SKOV-3[a]	ovary	6.0	6.1	530	10
PANC-1[a]	pancreas	2.1	2.4	NT	NT
HCT-116[a]	colon	6.0	5.9	610	11
HEP G2[a]	liver	5.8	3.2	1300	35
DU-145[a]	prostate	5.2	7.2	NT	NT
HT1080[a]	fibrosarcoma	5.4	5.4	NT	NT
MES-SA[a]	uterus	6.8	7.4	940	28
MES-SA/Dx5[a]	uterus	5.8	5.7	720	18
786-O[a]	kidney	2.3	2.6	350	7.5
Daudi[a]	myeloma	3.3	2.8	NT	NT
THP-1[a]	lymphoma	4.3	4.1	NT	NT
HeLa[a]	ovary	4.7	5.1	NT	NT
MCF-7[a]	breast	15	15	960	42
BxPC-3[a]	pancreas	5.7	5.5	1000	11
LoVo[a]	colon	5.6	6.0	NT	NT
LOX[a]	skin	3.7	3.2	NT	NT
U87-MG[a]	brain	5.7	6.4	610	14
NCI-N87[a]	gastric	5.8	6.3	NT	NT
A549[a]	lung	3.7	3.8	NT	NT
H1975[a]	lung	3.1	4.9	590	13.0
MV-4−-11[a]	myeloma	6.3	6.4	NT	NT
MBA-MB-468[a]	breast	7.0	8.4	NT	NT
HUVEC[b]	vein endothelium	9.7	7.7	800	12
MRC-5[b]	fibroblast lung	2.4	2.7	NT	NT
H22[c]	liver	4600	2400	NT	NT
S180[c]	sarcoma	>20 000	>20 000	>20 000	>20 000

[a]Human cancer cell lines. [b]Human normal cell lines. [c]Murine cancer cell lines. [d]NT, not tested.

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Table 3. Change in Contractile Amplitude of Cardiomyocytes (%)

	delta amplitude (%)[a]							
	0.32 nM		1.6 nM		8 nM		40 nM	
time[b]	bufalin (4)	1	bufalin (4)	1	bufalin (4)	1	bufalin (4)	1
8 min	−3.0	−2.7	1.8	−1.9	2.8	3.9	8.5	2.6
33 min	−2.0	0.7	−0.6	−2.1	3.1	7.5	21	13
66 min	−4.4	−1.9	−2.7	2.6	10	14	−62	−94
4 h	12	19	11	9.7	34	−19	XX[c]	XX
12 h	17	23	8.9	3.3	35	−18	XX	XX
24 h	32	30	4.3	2.8	−22	−59	XX	XX
36 h	62	57	−2.9	11	7.6	XX	XX	XX
48 h	42	30	−10	−20	−32	XX	−84.3	XX

[a]Beating amplitude (amplitude of the contraction) is calculated from each negative peak to the following positive peak. The result is the average of all the amplitudes (whole peak) in one sweep plus/minus the standard deviation (see SI). [b]Elapsed time following initial dosing of test compounds. [c]XX = no measurement because cells ceased beating.

and **2**. These data are consistent with results obtained for **1** and **2** in the NCI 60-cell line panel (Tables S3, S4).[9] Notably, all of the compounds were several orders of magnitude less toxic to murine cell lines, as has been observed for other bufadienolides.[10]

Cardioactivity. In order to assess the effects of this new family of toxins on cardiac cells, cyanobufalin A (**1**) was compared to bufalin (**4**) in an impedance-based cellular assay conducted with human induced pluripotent stem cell (hIPSC)-derived cardiomyocytes.[11] This assay has been used as a diagnostic guide for cardiac liabilities in preclinical drug development.[12] In this system, cardiomyocytes are monitored in real time by 20 s impedance recordings referred to as the "cell-index", which is a surrogate measure of cell viability and attachment. Additionally, specific functional effects are assessed by monitoring changes in beating rate and contractility (amplitude of the impedance signal) of the cells over a period of 48 h. Bufalin concentrations of >40 nM were acutely toxic to the cells, as indicated by a steep drop in cell index following the initial dose (Figure S22). Cyanobufalin A was also acutely toxic to the cells at concentrations of 40 nM and greater, as well as showing acute toxicity upon the second dosing at 8 nM (Figure S23). Bufalin showed biologically significant effects on beating parameters at concentrations of 40 nM and lower. At 40 nM the beating rate and amplitude of contraction were increased within the first time points, followed by a slowing of beat rate and loss of amplitude after 1 h (Tables S5, S6; Figures S24, S25), owing to acute toxicity. As shown in Table 2, at concentrations of 8 nM and lower the effects on contractile amplitude were sustained for longer times. At the lowest concentration of 0.32 nM enhancement in contractile amplitude was maintained throughout the experiment. Cyanobufalin A showed a very similar profile of cardiac effects overall (Tables S7, S8; Figures S26, S27) and on contractility in particular (Table 3). The maximum enhancement in contractile amplitude elicited by **1** was observed after 36 h at the lowest concentration tested (0.32 nM). Overall cyanobufalin A mirrored the cardioactive effects of bufalin with some indication of greater toxicity.

Discussion. Bufadienolides are known for their potent cardiac activity, which they exert through inhibition of Na^+/K^+-ATPase in heart tissue.[13,14] There have been some attempts to develop this class of compounds to treat cancer, although success has been limited.[15] The core structure of bufadienolides, exemplified by bufalin (**4**), contains a steroidal ring system linked at C-17 to an α-pyrone moiety. Approximately 400 natural analogues of this core structure are known from a range of plant and animal sources; however none have been reported to be produced by microorganisms. Several of the structural features found in the cyanobufalins are either exceptionally rare or unique, despite the extensive reports of bufadienolide analogues over the last 50 years. The geminal methyl groups at C-4, the C-7,8 double bond, and chlorine at C-16 are only reported in one other series of natural bufadienolides, the aegomycins.[16] Carbamate substitution at C-3 in **1** and **2** and the C-2/C-3 carbonate in **3** are features found only in the cyanobufalins.

These unique structural features prompted our assessment of how these modifications impacted the biological activities of the series. As the primary focus of the research was the discovery of anticancer drug leads, we first compared cytotoxicity profiles in tumor cell lines. In these tests **1** and **2** gave essentially the same profile as bufalin with about 2−5 times greater potency. No selectivity was observed for different tissue types (Table 2), and the results from the NCI 60-cell line panel (Tables S3, S4) revealed only generalized toxicity. Carbonate **3** was uniformly 1−2 orders of magnitude less potent against all cell lines, which is consistent with changes in potency found for certain other A-ring modifications.[10]

Next, in order to assess any potential differences in the mechanism of action and related cardiotoxicity between the cyanobufalins and the bufadienolides in general, we compared the activities of **1** and bufalin in cardiomyocytes. In all aspects, the cyanobufalins mirrored the activity of bufalin in cardiac activity. Therefore, our conclusion is that the cyanobufalins manifest their general cytotoxicity to cell lines and specific action on cardiac cells by inhibition of Na^+/K^+-ATPases just like other bufadienolides. Because the cyanobufalins did not exhibit any differentiation in biological profile compared to bufadienolide, no further work is planned to develop their potential as anticancer agents.

The biosynthetic origin of the cyanobufalins remains to be established. Accordingly, we have an ongoing meta-genomic mining project aimed at locating the biosynthetic genes for the cyanobufalins that will ultimately lead to the identification of the producing organism. We have consistently found these compounds associated with cyanobacterial biomass harvested months or years apart from GLSM or BL. In all cases these samples were dominated by filamentous cyanobacteria, primarily *Planktothrix* sp. (Supporting Information), suggesting a causative relationship. Our biomass sampling procedure selectively enriches the >10 μm fraction, which would include

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Planktothrix filaments. Although cyanobacteria have the biosynthetic capacity to make a wide range of terpenoids, as well as other secondary metabolites,[17] definitive evidence for sterol production remains to be established. The biomass from these lakes contains many other types of microorganisms, although at much lower abundance; hence the identity of the true producer remains ambiguous, pending genomic evidence. The structural uniqueness of cyanobufalins argues for an alternative biosynthetic process for the fused tetracyclic ring system distinct from other bufadienolides. The C-4 methyl groups and the C-7,8 double bond are conserved in all analogues of these compounds, including aegomycins, but are found in no other bufadienolides. Notably, the aegomycins were isolated from a marine sponge (*Mycale crassissima*),[16] which poses the possibility of their production by microbial symbionts.[18] Additional support for a microbial source of **1**−**3** includes the chlorine substitution at an unactivated carbon (C-16), which suggests the enzymatic action of a nonheme Fe^{II}/α-ketoglutarate/O_2-dependent halogenase.[19] These enzymes are responsible for the chlorinations in the cyanobacterial metabolite barbamide[20] and welwitindolinone, fischerindole, and hapalindole, cyanobacterial indole terpenoids.[21] Collectively these findings support a microbial source for the cyanobufalins, consistent with the natural histories of saxitoxin[22] and pederin-like compounds.[23]

This is the first reported example of the occurrence of natural cardioactive steroid derivatives in the aquatic environment. In terms of environmental and human health impact, the presence of the cyanobufalins in the aquatic environment is significant. Both GLSM and BL are known to have microcystin levels above regulatory thresholds. The presence of compounds that can cause synergistic damage to heart tissue (by ATP-ase inhibition) could enhance exposure risk. The minimal estimated combined concentrations of **1**, **2**, and **3** at 170 pM corresponds to approximately 50% of the maximally effective concentration tested of **1** in the cardiomyocyte assay (0.32 nM). Given that the estimated level of cyanobufalins represents the lower limit, it is entirely posssible that cardioactive concentrations exist in the environment. Thorough quantitative studies of these compounds in lakes such as BL and GLSM, including assessment of their "free" or unbound concentrations in the water, are needed in order to assess the possibility of any public health risk.

EXPERIMENTAL SECTION

General Experimental Procedures. Optical rotations were measured using a Jasco P-2000 polarimeter. UV spectra were measured using a Beckman Coulter DU-800 spectrophotometer. 1D and 2D NMR data were acquired at 298 K on a Bruker 800 MHz NMR spectrometer equipped with a triple resonance (TXI) cryoprobe. Samples were dissolved in ca. 0.6 mL of DMSO-d_6 with deuterium serving as the lock nucleus. LC-MS was performed using a Waters 1525 system equipped with the 2767 sample manager and a ZQ mass spectrometer. Preparative HPLC separations were performed using an Agilent 1100 with a G1361A binary pump. Cytotoxicity against cell lines was determined by a standard MTT assay (Supporting Information). Bufalin (**4**) was obtained from Ontario Chemicals, Inc., ON, Canada.

Biomass Collection. Grand Lake St. Marys. Cyanobufalins A (**1**) and B (**2**) were originally isolated from biomass harvested from GLSM in west-central Ohio, USA, between May 15 and May 24, 2012, using the Biosortia SLS system.[6] Analysis of the water source showed that the major microbial species present was *Planktothrix agardhii*, at a density of 4×10^9 cells/L, within a complex consortium containing at least 14 other microbial species (Ohio EPA report for May 8, 2012, Supporting Information). The sample was obtained from water entering the Celina, Ohio, water treatment plant.

Buckeye Lake Harvest 2014. Biomass was harvested from BL in central Ohio, USA, between October 18 and November 17, 2014, using the Biosortia SLS system. Microscopic analysis of the water source revealed that the major microbial species present were of the filamentous cyanobacterial genus *Planktothrix*. A total volume of approximately 5 million liters of lake water was processed to obtain a concentrated paste of 1280 kg, 15% solids, over the course of 32 days (612 h). Immediately after concentration, the biomass was frozen and stored at −20 °C in 2-gallon freezer bags.

Extraction. Freeze-dried biomass (70 kg) was extracted with a 2:1 mixture of CH_2Cl_2/MeOH (3 × 200 L). The combined extract was concentrated to a viscous oil (5.1 kg). The concentrated extract was then washed with hexane (4 × 150 L) to remove the most nonpolar material, leaving an intermediate-polarity fraction (2.5 kg).

Bulk Separation on HP-20. A portion of the intermediate-polarity extract (500 g) was processed on Diaion HP-20. The HP-20 column (5 kg) was sequentially eluted with H_2O, H_2O mixed with increasing proportions of MeOH, and finally 100% MeOH. Those materials eluting in the range from 80% to 85% MeOH were collected and concentrated, yielding a complex mixture of metabolites (31 g).

Fractionation. The HP-20-derived mixture (31 g) was initially separated by low-resolution reversed-phase chromatography on bulk C18 (Polygoprep 60-50 C18 C-content: 12%; pore width: 60 Å, particle size: 40−63 μm; supplier: Macherey-Nagel GmbH & Co. KG, Düren, Germany). The column (50 × 250 mm) was eluted with MeOH/H_2O (each with 0.1% formic acid (FA)) in a linear gradient ranging from 50% MeOH to 80% at a flow rate of 100 mL/min. The range of components that eluted between 63% and 67% MeOH was collected to give a partially purified mixture (6.32 g). This material was further fractionated by preparative HPLC (2 × 3.16 g) on a LichrosphereSelectB, 250 × 50 mm, 10 μm column eluted with MeOH/MeCN, 1:1, and H_2O (each with 0.1% FA) in a gradient from 40% to 50% at 80 mL/min over 58 min. Components that eluted between 45% and 47% were collected in seven sequential fractions that yielded simplified mixtures: fr15 (162 mg), fr16 (188 mg), fr17 (174 mg), fr18 (85 mg), fr19 (125 mg), fr20 (58 mg), fr21 (58 mg). These fractions were used to isolate the pure cyanobufalins.

Isolation of Cyanobufalins 1, 2, and 3. C18 fractions 16, 17, 18, 19, and 20 were combined and further purified by two steps of reversed-phase preparative HPLC.

The first stage was done with a YMC column [ODS-A 30 × 250 mm, 10 μm, 120 Å] and a mobile phase consisting of 68% H_2O and 32% MeCN each modified with 0.05% FA. Cyanobufalin B (**2**; t_R 38.5 min; m/z 518), cyanobufalin C (**3**; t_R 43.0 min; m/z 519), and cyanobufalin A (**1**; t_R 49.0 min; m/z 502) were isolated in semipurified form as verified by LC-MS. Final purifications were carried out by repeated HPLC separations using a semipreparative YMC-ODS column (250 × 10 mm, 5 μm) using a 3 mL/min flow rate and isocratic solvent system of 68% H_2O and 32% MeCN each modified with 0.05% FA. Purified **2** (t_R 30.0 min, 15 mg), **3** (t_R 34.0 min, 5 mg), and **1** (t_R 37.0 min, 16 mg) were isolated.

*Cyanobufalin A (**1**):* white powder; $[\alpha]^{23}_D$ +9.9 (MeOH, *c* 0.10); UV (MeOH) λ_{max} (log ε) 202 (4.0) 290 (3.6) nm; ^{1}H (800 MHz) and ^{13}C NMR (200 MHz, DMSO-d_6), Table 1; HRESIMS m/z 502.1998 $[M + H]^+$ (calcd for $C_{27}H_{33}ClNO_6$,502.1986).

*Cyanobufalin B (**2**):* colorless oil; $[\alpha]^{23}_D$ +12 (*c* 0.08, MeOH); UV (MeOH) λ_{max} (log ε) 201 (4.0) 290 (3.5) nm; ^{1}H (800 MHz) and ^{13}C NMR (200 MHz, DMSO-d_6), Table S1; HRESIMS m/z 518.1937 $[M + H]^+$ (calcd for $C_{27}H_{33}ClNO_7$, 518.1946).

*Cyanobufalin C (**3**):* white powder; $[\alpha]^{23}_D$ −3.3 (*c* 0.20, MeOH); UV (MeOH) λ_{max} (log ε) 202 (4.0) 290 (3.5) nm; ^{1}H (800 MHz) and ^{13}C NMR (200 MHz, DMSO-d_6), Table S2; HRESIMS m/z 541.1602 $[M + Na]^+$ (calcd for $C_{27}H_{31}ClO_8Na$, 541.1605).

Estimation of Cyanobufalin Content in Buckeye Lake. BL water processed during the harvest was determined to have total solids at 0.038 g/L. On the basis of this density, the 14 kg equivalent of dried biomass used to isolate **1**, **2**, and **3** would have required 3.7×10^5 L of lake water. The isolated quantity of **1**−**3** requires the minimal

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concentrations of **1**: 4.3×10^{-8} g/L (86 pM), **2**: 4.1×10^{-8} g/L (79 pM), and **3**: 5.0×10^{-8} g/L (9.7 pM) in BL.

Cytotoxicity Testing. Compounds were tested in a standard cell proliferation assay (MTS kit, Promega, cat. no. G3581) in dilution series to obtain EC_{50} values by Sundia MediTech Co. Ltd., Shanghai, China (Supporting Information).

Cardioactivity Testing. Compounds were tested in dilution series from 0.32 nM to 5 μM in an impedance-based cellular assay using hIPSC-derived cardiomyocytes in xCELLigence RTCA Cardio plates from ACEA Biosciences, Inc.[11] Cardiomyocytes (Cor.4U) were obtained from Ncardia, Koln, Germany, who also performed the assays. In this assay test compounds are dosed at two time points, 24 h apart, following the establishment of stable beating cell cultures in 96-well plates.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jnatprod.8b00736.

GLSM whole water phytoplankton enumeration; HRMS procedures; general NMR procedures; tabulated NMR data for compounds **2** and **3**; ^{1}H and ^{13}C NMR spectra for compounds **1**–**3**; cytotoxicity (MTT) assay for guiding fractionation and purification; NCI 60-cell data for **1** and **2**; Sundia cytotoxicity procedure; cardiomyocyte assay results (PDF)

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Notes

The authors declare no competing financial interest.

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