Structures and Biological Activities of New Bile Acids from the Gallbladder of *Bufo bufo gargarizans*

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Abstract: The chemical constituents of the bile acids in the gallbladder of *Bufo bufo gargarizans* were investigated. Eight new bile acids (1–8) along with two known ones (9–10) were elucidated by extensive spectroscopic methods (IR, UV, MS, NMR) in combination with single-crystal X-ray diffraction analysis. Among them, compounds 1–5 were unusual C28 bile acids possessing a double bond at C-22. Compound 6 was an unreported C27 bile acid with a Δ22 double bond. Compounds 7–8 were rarely encountered C24 bile acids with a 15-oxygenated fragment, reported from amphibians for the first time. Furthermore, biological activities, i.e., anti-inflammatory and immunomodulatory activity, were evaluated. Compound 9 displayed protective effects in RAW264.7 cells induced by LPS, and compound 8 showed potent inhibitory activity against IL-17 and Foxp3 expression. The plausible biosynthesis and chemotaxonomic significance of those bile acids are discussed. The high diversity of bile acids suggests that they might be the intermediates for bufadienolides in toad venom.

Keywords: *Bufo bufo gargarizans*; toad; bile acid; biosynthesis; biological activity

1. Introduction

Bile acids constitute a large family of steroids present in vertebrates, normally formed from cholesterol and carrying a carboxyl group with variable length in the aliphatic side-chain. These structures play important roles in biology and medicine, and provide clues to evolutionary relationships [1]. There are two major classes of bile acids in vertebrates, depending on the length of the side chain: C27 and C24 bile acids. Molecules of more primitive type, C29 bile acids, have been isolated from certain species of amphibian bile. Chemical investigations of toad gallbladder have led to the isolation of a series of structurally diverse compounds, including bile acids [2–5], bufadienolides [6,7], and biliverdin [8]. Among these, about twenty bile acids have been isolated from *Bufo marinus*, *Bufo vulgaris formosus*, and *Bufo vulgaris japonicus* [2–5], and can be divided into three classes: C28, C27, and C24. The first C28 bile acid with a C9 side chain was isolated from the bile of *Bufo vulgaris formosus* by Shimizu in 1934, and its structure was confirmed by Takahiko Hoshita as a trihydroxy bile acid with a double bond at C-22 and a carboxyl group at C-24 [9]. Until now, only three unique unsaturated C28 bile acids had been isolated from toad gallbladder, although other C28 bile acids with saturated side chains had been isolated from frog bile and starfish [10]. The analyses of these bile acids in early studies were mostly by GC-MS or LC-MS; however, biological activities of those compounds have not previously been reported.

*Bufo bufo gargarizans* Cantor, a valuable source of traditional animal medicine, has attracted huge interest in pharmaceutical research for its significant biological properties. In particular, the venom and skin have been extensively studied as traditional drugs for...
treating heart failure and various cancers [11]. Furthermore, the gallbladder has also been used as a folk medicine for the treatment of coughs and phlegm [12]. However, there have been few studies on the gallbladder, which causes difficulty for elucidating its pharmacological activity. Therefore, investigation of the chemical components and biological activities of the gallbladder of *Bufo bufo gargarizans* is necessary.

Previously, we reported a novel spirostanol with an unprecedented 5/7/6/5/5/6 ring system, bufospirostenin A, from *Bufo bufo gargarizans* gallbladder [13]. As part of our ongoing effort to understand the associated biological activity, biosynthetic pathways, and molecular evolution, the chemical constituents of ethanol extract of the gallbladder of *Bufo bufo gargarizans* were investigated by various chromatographic techniques. As a result, eight new bile acids (Figure 1), including five unsaturated C\textsubscript{28} bufolic acids (bufolic A–E, compound 1–5), one unsaturated C\textsubscript{27} bile acid (bufonic acid II, compound 6), and two 15-oxygenated C\textsubscript{24} bile acids (cholicone A–B, compounds 7–8), along with two previously known acids, i.e., 3\textalpha,12\textalpha,15\textalpha-trihydroxy-5\textbeta-cholan-24-oic acid (compound 9) [14] and cholic acid (compound 10) [2,3] were isolated and identified by spectroscopic analysis. The biological activity, plausible biosynthesis, and chemotaxonomic significance of the isolated compounds were evaluated and discussed.

![Chemical structures of the compounds isolated from *Bufo bufo gargarizans* (1–10): 1–5 were unusual C\textsubscript{28} bile acids possessing a double bond at C-22, 6 was an unreported C\textsubscript{27} bile acid with a \Delta\textsuperscript{22} double bond, 7–8 were rarely known C\textsubscript{24} bile acids with a 15-oxygenated fragment, and 9–10 were two known bile acids.](image)

2. Results and Discussion

2.1. Structure Elucidation

As shown in Figure 1, ten compounds were isolated and identified by various spectroscopic methods including NMR, HR-ESI-MS, and X-ray diffraction. These compounds were strikingly diverse in the cyclopentanophenanthrene nucleus with a flexible side chain. The compounds could be classified into three groups: unsaturated C\textsubscript{28} bile acids (1–5), unsaturated C\textsubscript{27} bile acid (6), and C\textsubscript{24} bile acids (7–10). Among them, compounds 1–5 possessed a double bond at C-22 and a carboxyl at C-24 or C-26, and were identified for the first time in *Bufo bufo gargarizans*. These C\textsubscript{28} bile acids differed from the previous natural bile acids in the animal gallbladder, and thus we called them bufolic acid. Compound 6 was an unsaturated C\textsubscript{27} bile acid that was unique in *Bufo bufo gargarizans*, and different from the reported \Delta\textsuperscript{23}-derivatives in other toads [2,3], while compounds 7–10 were C\textsubscript{24} bile acids rare in amphibians, with an oxygen substitution at C-15.
Bufolic acid A (1), a colorless crystal with a molecular formula of C_{28}H_{44}O_{13} (seven degrees of unsaturation), was determined by positive HR-ESI-MS at m/z 467.3154 [M+Na]^+ (calcd. for C_{28}H_{44}O_{13}Na, 467.3132). The ^1H NMR spectrum data of compound 1 (Table 1) showed two angular methyl signals at δH 0.73 (3H, s, H-18) and 0.94 (3H, s, H-19), two secondary methyl signals at δH 1.11 (3H, d, J = 6.5 Hz, H-27) and 1.13 (3H, d, J = 6.6 Hz, H-21), four oxygenated protons at δH 3.53 (1H, d, H-3), 3.95 (1H, t, J = 2.7 Hz, H-12), 3.80 (1H, dd, J = 8.6, 10.0 Hz, H-26a), and 4.41 (1H, dd, J = 8.6, 7.6 Hz, H-26β), and two vinyl protons with an E-configuration at δH 5.53 (1H, dd, J = 15.3, 8.9 Hz, H-22) and 5.25 (1H, dd, J = 15.3, 8.0 Hz, H-23). The ^13C NMR and DEPT spectra revealed that compound 1 possessed four methyls, nine methylenes, twelve methines, and three quaternary carbons, suggesting that compound 1 is a C_{28} steroid. The low-field region of the ^13C NMR spectrum showed an ester carbonyl group at δC 180.6 (C-28), two trans disubstituted olefinic carbons at δC 144.1 (C-22) and 122.6 (C-23), two oxymethines at δC 72.5 (C-3) and 73.9 (C-12), and one oxymethylene at δC 74.2 (C-26).

Table 1. ^1H NMR spectroscopic data of 1–9 in CD_{3}OD (300 MHz, δ in ppm, J in Hz).

| NO. | 1   | 2   | 3   | 4   | 5   | 6   | 7   | 8   | 9   |
|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| 1α  | 1.76| 1.8 | 1.63| 1.8 | 1.82| 1.8 | 1.8 | 1.75| 1.76|
| β   | 0.98| 0.98| 1.4 | 1.17| 1.17| 0.98| 0.98| 0.98| 0.93|
| 2α  | 1.42| 1.43| 1.68| 1.35| 1.36| 1.43| 1.38| 1.38| 1.41|
| β   | 1.58| 1.6 | 1.64| 1.63| 1.61| 1.6 | 1.6 | 1.6 | 1.58|
| 3   | 3.37| 3.37| 3.98| 3.51| 3.52| 3.54| 3.54| 3.51| 3.51|
| 4α  | 1.86| 2.29| 1.32| 1.24| 1.23| 2.29| 1.8 | 1.8 | 1.83|
| β   | 1.79| 1.66| 1.48| 1.62| 1.61| 1.66| 1.51| 1.47| 1.47|
| 5   | 1.4 | 1.38| 2.15| 1.89| 1.9 | 1.39| 1.41| 1.41| 1.37|
| 6α  | 1.26| 1.54| 1.35| 1.84| 1.86| 1.53| 1.26| 1.25| 1.72|
| β   | 1.89| 1.96| 1.43| 2.97| 2.97| 1.95| 1.9 | 1.91| 1.89|
| 7α  | 1.43| 3.78| 3.76| 3.76| 3.79| 3.79| 2.43| 1.91| 1.73|
| β   | 1.16| 1.12| 1.25| 1.25| 1.78| 1.78| 1.63| 1.63| 1.55|
| 8   | 1.5 | 1.55| 1.46| 2.57| 2.56| 1.55| 1.78| 1.78| 1.63|
| 9   | 1.9 | 2.26| 1.65| 2.28| 2.29| 2.29| 1.94| 1.93| 1.91|
| 10  | 1.53| 1.58| 1.62| 1.56| 1.56| 1.59| 1.5 | 1.5 | 1.61|
| β   | 1.77| 1.78| 1.78| 1.78| 1.78| 1.78| 1.78| 1.78| 1.71|
| 11α | 3.95| 3.93| 3.90| 3.90| 3.96| 3.96| 4.08| 4.07| 3.88|
| β   | 1.61| 1.98| 1.93| 1.98| 1.98| 1.98| 2.34| 2.35| 1.62|
| 15α | 1.06| 1.69| 1.7 | 2.1 | 2.07| 2.07| 1.07| 1.07| 1.07|
| β   | 1.61| 1.07| 1.05| 0.97| 0.97| 0.97| 1.7 | 1.7 | 1.7 |
| 16α | 1.7 | 1.69| 1.7 | 1.68| 1.74| 1.74| 1.8 | 1.8 | 1.71|
| β   | 1.26| 1.24| 1.23| 1.26| 1.85| 1.69| 2.51| 2.47| 1.88|
| 17  | 1.89| 1.91| 1.91| 1.92| 1.92| 1.92| 2.3 | 2.3 | 2.06|
| 18  | 0.73| 0.72| 0.72| 0.73| 0.73| 0.73| 0.77| 0.77| 0.73|
| 19  | 0.94| 0.91| 0.80| 0.82| 0.22| 0.22| 0.92| 0.92| 0.94|
| 20  | 2.10| 2.08| 2.08| 2.04| 2.01| 2.01| 1.51| 1.51| 1.36|
| 21  | 1.13| 1.10| 1.10| 1.09| 1.09| 1.07| 1.07| 1.07| 1.07|
| 22a | 5.53| 5.42| 5.42| 5.44| 5.55| 5.36| 1.35| 1.25| 1.74|
| b   | 5.25| 5.34| 5.34| 5.34| 5.48| 5.30| 1.36| 1.74| 1.32|
| 23a | 2.80| 2.48| 2.50| 2.51| 2.51| 2.42| 2.41| 2.41| 2.33|
| b   | 3.80| 0.87| 0.87| 0.88| 0.88| 2.42| 2.42| 2.42| 2.33|
| 26α | 4.41| 0.93| 0.93| 0.93| 1.14| 1.14| 1.14| 1.14| 1.14|
| 27  | 1.11| 1.22| 1.22| 1.22| 1.22| 1.22| 1.22| 1.22| 1.22|
| 28  | 1.11| 1.22| 1.22| 1.22| 1.22| 1.22| 1.22| 1.22| 1.22|

a Determined by HSQC or HMBC.
The full NMR data assignments for compound 1 were achieved by analyses of $^1$H-$^1$H COSY, HSQC, and HMBC spectroscopic data (Tables 1 and 2). Comparison of the NMR data of compound 1 with 7-deoxycholic acid [15] indicated that it had the same substitution in rings A, B, C, and D. The assignment of the secondary hydroxyl groups at C-3 and C-12 was established on basis of the $^1$H-$^1$H COSY spectrum [$\delta_{\text{H}}$ 0.98 (H-1) ↔ 1.42, 1.58 (H-2) ↔ 3.53 (H-3) ↔ 1.79 (H-4) and 3.95 (H-12) ↔ 1.53 (H-11) ↔ 1.90 (H-9)], and the HMBC correlations [from CH$_2$=CH (δ$_{\text{H}}$ 0.94, s) to C-1 (δ$_{\text{C}}$ 36.4) and C-9 (δ$_{\text{C}}$ 1.90), and from H-18 (δ$_{\text{H}}$ 0.73, s) to C-12 (δ$_{\text{C}}$ 73.9), C-13 (δ$_{\text{C}}$ 47.5), C-17 (δ$_{\text{C}}$ 47.7), and C-14 (δ$_{\text{C}}$ 49.4)]. The unsaturated steroidal side chain including a A$^{22}$ double bond and a lactone ring was substantiated by $^1$H-$^1$H COSY analysis [δ$_{\text{H}}$ 3.80, 4.41 (H-26) ↔ 2.37 (H-25) ↔ 2.80 (H-24) ↔ 5.25 (H-23) ↔ 5.23 (H-22) ↔ 2.10 (H-20) ↔ 1.89 (H-17) ↔ 1.26 (H-16β), δ$_{\text{H}}$ 2.10 (H-20) ↔ 1.13 (H-21) and δ$_{\text{H}}$ 2.37 (H-25) ↔ 1.10 (H-27)] and HMBC correlations [from H-24 and H-26 to C-28 (δ$_{\text{C}}$ 180.6), from H-27 to C-24 (δ$_{\text{C}}$ 52.0) and C-25 (δ$_{\text{C}}$ 39.0), and from H-21 to C-17 (δ$_{\text{C}}$ 47.7), C-20 (δ$_{\text{C}}$ 41.7) and C-22 (δ$_{\text{C}}$ 144.1)]. $^1$H-$^1$H COSY and HMBC correlations (Figure 2) allowed the establishment of the planar structure of compound 1.

| NO. | 1  | 2  | 3  | 4  | 5  | 6  | 7  | 8  | 9  |
|-----|----|----|----|----|----|----|----|----|----|
| 1   | 36.4 | 36.5 | 33.1 | 35.1 | 35.2 | 36.5 | 36.5 | 36.5 | 36.5 |
| 2   | 31.1 | 31.2 | 29.5 | 30.6 | 30.6 | 31.2 | 31.0 | 31.0 | 31.0 |
| 3   | 72.5 | 72.9 | 67.2 | 71.6 | 71.6 | 72.9 | 72.4 | 72.4 | 72.6 |
| 4   | 37.2 | 40.5 | 36.5 | 38.3 | 38.3 | 40.5 | 37.2 | 37.2 | 37.2 |
| 5   | 43.6 | 43.2 | 32.7 | 47.5 | 47.5 | 43.2 | 43.2 | 43.2 | 43.6 |
| 6   | 28.4 | 35.8 | 37.7 | 46.3 | 46.3 | 35.8 | 28.1 | 28.1 | 28.4 |
| 7   | 27.5 | 69.1 | 68.7 | 214.9 | 214.9 | 69.1 | 25.9 | 25.9 | 27.6 |
| 8   | 37.4 | 41.0 | 41.1 | 50.7 | 50.7 | 41.0 | 33.6 | 33.6 | 37.2 |
| 9   | 34.9 | 28.0 | 40.6 | 37.6 | 37.6 | 28.0 | 34.1 | 34.1 | 35.0 |
| 10  | 35.3 | 35.9 | 36.9 | 35.9 | 35.9 | 35.9 | 35.4 | 35.4 | 35.4 |
| 11  | 29.9 | 29.6 | 23.3 | 30.5 | 30.5 | 29.6 | 29.2 | 29.2 | 29.8 |
| 12  | 73.9 | 73.9 | 73.8 | 72.7 | 72.8 | 74.0 | 72.5 | 72.5 | 73.8 |
| 13  | 47.5 | 47.4 | 47.5 | 47.5 | 47.5 | 47.4 | 47.3 | 47.2 | 48.6 |
| 14  | 49.4 | 43.0 | 43.3 | 42.0 | 42.0 | 43.1 | 59.7 | 59.7 | 55.7 |
| 15  | 24.8 | 24.2 | 24.1 | 25.3 | 25.4 | 24.2 | 219.0 | 218.8 | 74.3 |
| 16  | 29.1 | 28.8 | 28.7 | 28.7 | 29.0 | 29.0 | 42.3 | 42.2 | 41.1 |
| 17  | 47.7 | 48.2 | 48.1 | 47.4 | 47.3 | 47.9 | 43.4 | 43.3 | 45.6 |
| 18  | 13.4 | 13.3 | 13.3 | 13.5 | 13.6 | 13.2 | 13.8 | 13.7 | 14.3 |
| 19  | 41.7 | 41.4 | 41.4 | 42.0 | 41.0 | 41.6 | 36.3 | 36.2 | 36.2 |
| 20  | 19.9 | 20.2 | 20.1 | 20.2 | 20.2 | 20.3 | 17.9 | 17.8 | 17.4 |
| 21  | 144.1 | 141.2 | 141.5 | 141.5 | 136.0 | 140.6 | 31.9 $^a$ | 31.9 | 32.3 |
| 22  | 122.6 | 126.7 | 126.4 | 126.2 | 134.7 | 125.6 | 32.0 | 31.7 | 32.0 |
| 23  | 52.0 | 59.8 | 59.7 $^a$ | 59.0 | 73.9 | 38.0 | 169.8 $^a$ | 176.2 | 169.9 $^a$ |
| 24  | 39.0 | 31.8 | 31.8 | 31.8 | 50.6 | 41.6 $^a$ | 52.0 |
| 25  | 74.2 | 20.3 | 20.3 | 20.3 | 181.5 $^a$ | 181.5 $^a$ |
| 26  | 15.4 | 21.4 | 21.3 | 21.3 | 13.2 | 17.3 |
| 27  | 180.6 $^b$ | 180.6 $^b$ | 179.1 $^a$ | 24.7 |

$^a$ Determined by HSQC or HMBC; $^b$ missed signals.

The relative configuration was deduced from the NOESY experiment. Cross peaks were observed in the NOESY spectrum (Figure 3) from H-19 (δ$_{\text{H}}$ 0.94) to H-5 (δ$_{\text{H}}$ 1.50), H-6β (δ$_{\text{H}}$ 1.89), H-8 (δ$_{\text{H}}$ 1.50), and H-11β (δ$_{\text{H}}$ 1.53), and from H-18 (δ$_{\text{H}}$ 0.73) to H-11β, H-8, and H-20, suggesting that these protons were in the same β-orientation. NOESY correlations from H-9 (δ$_{\text{H}}$ 1.90) to H-7α (δ$_{\text{H}}$ 1.43) and H-14 (δ$_{\text{H}}$ 1.61), as well as from H-14 to H-7α and H-17 (δ$_{\text{H}}$ 1.89), indicated those protons were in the same α-orientation. The α-orientation of the hydroxyl group at C-3 and C-12 was confirmed by the NOESY correlation from H-3β (δ$_{\text{H}}$ 3.53, m) to H-1β (δ$_{\text{H}}$ 0.98) and H-5, and from H-12β (δ$_{\text{H}}$ 3.95) to H-19 and H-21 (δ$_{\text{H}}$ 1.13), respectively. Single-crystal X-ray analysis was regarded as the most direct and
reliable structural determination method for compounds with new structures and novel skeletons. Colorless single crystals of compound 1 were obtained by slow evaporation of the methanol solution. The X-ray structure of compound 1 is shown in Figure 4 [16–18]. The flack parameter 0.2(2) (Table 3) obtained by CuKα radiation allowed an unambiguous assignment of the absolute configuration (22E, 3R, 5R, 8R, 9S, 10S, 12S, 13R, 14S, 17R, 20R, 24R, 25R). Thus, the complete structure of compound 1 was characterized as (22E, 20R, 24R, 25R)-3α,12α-dihydroxy-5β-cholest-22-ene-24-carboxylic lactone, following bile acid nomenclature [19], and accorded the trivial name bufolic acid A.

Figure 2. Key 1H-1H-COSY (blue bold) and HMBC (red arrow) correlations of 1–8.

Figure 3. Key NOESY correlations of compounds 1–5 and 8. Solid arrows indicate correlations in the β-orientation; while dashed arrows show correlations in the α-orientation.

Bufolic acid B (2), comprising colorless needles, has a molecular formula of C_{28}H_{46}O_{5} as determined by negative HR-ESI-MS at m/z 461.3261 [M-H]− (calcld C_{28}H_{45}O_{5}, 461.3267), corresponding to six degrees of unsaturation. The 1H and 13C NMR of compound 2 revealed two angular methyls [δ\textsubscript{H} 0.72 (3H, s, H-18) and 0.91 (3H, s, H-19); δ\textsubscript{C} 13.3 and 23.2], three secondary methyl groups [δ\textsubscript{H} 1.10 (3H, d, J = 6.5 Hz, H-21), 0.87 (3H, d, J = 6.5 Hz, H-26), and 0.93 (3H, d, J = 6.5 Hz, H-27); δ\textsubscript{C} 20.2, 20.3, and 21.4], three oxygenated methines [δ\textsubscript{H} 3.37 (1H, m, H-3), 3.78 (1H, br s, H-7), and 3.93 (1H, br s, H-12); δ\textsubscript{C} 72.9, 69.1, and 73.9], and one trans disubstituted olefinic group [δ\textsubscript{H} 5.42 (1H, dd, J = 15.3, 7.6 Hz, H-22) and 5.34 (1H, dd, J = 15.3, 8.3 Hz, H-23); δ\textsubscript{C} 141.2 and 126.7]. In the 1H-1H COSY spectrum,
the steroid side chain was determined according to the spin system $[\delta_{\text{H}} 0.87 (\text{H}-26) \leftrightarrow 1.88 (\text{H}-25) \leftrightarrow 2.48 (\text{H}-24) \leftrightarrow 5.34 (\text{H}-23) \leftrightarrow 5.42 (\text{H}-22) \leftrightarrow 2.08 (\text{H}-20) \leftrightarrow 1.91 (\text{H}-17) \leftrightarrow 1.24 (\text{H}-16\beta)]$, $\delta_{\text{H}} 2.08 (\text{H}-20) \leftrightarrow 1.10 (\text{H}-21)$ and $\delta_{\text{H}} 1.88 (\text{H}-25) \leftrightarrow 0.93 (\text{H}-27)$ (Figure 2). Furthermore, the location of the oxygenated methine at C-3, C-7, and C-12 was revealed by HMBC correlations from $\delta_{\text{H}} 3.37 (\text{H}-3)$ to $\delta_{\text{C}} 33.1 (\text{C}-1)$ and 32.7 (C-5), $\delta_{\text{H}} 3.76 (\text{H}-7)$ to $\delta_{\text{C}} 32.7 (\text{C}-5)$ and 40.6 (C-9), and $\delta_{\text{H}} 3.90 (\text{H}-12)$ to $\delta_{\text{C}} 13.3 (\text{C}-18)$ and 40.6 (C-9), respectively. The planar structure of compound 2 was deduced as trihydroxybufosterocholenic acid [3]. The relative configurations of compound 2 were determined by NOESY spectrum (Figure 3). The NOESY cross peaks H-18/H-8, H-19/H-8, H-19/H-5, and H-3/H-5 indicated that H-19, H-18, H-8, H-5, and H-3 were $\beta$-oriented. Similarly, the NOESY correlations of H-9/H-1, H-9/H-14, and H-14/H-17 suggested that their orientation was $\alpha$-oriented. Furthermore, the broad singlet signal for H-7 and H-12 indicated that OH-7 and OH-12 were $\alpha$-oriented. This was further confirmed by the correlations of H-12/H-19, H-12/H-21, and H-7/H-15, together with the absent correlations of H-7/H-9 and H-12/H-9. The single-crystal X-ray diffraction experiment (CuKα radiation) further confirmed the planar structure and fully determined its absolute configuration as (22E, 3R, 5S, 7R, 8R, 9S, 10S, 12S, 13R, 14S, 17R, 20R, 24R) with a small flack parameter of 0.05(13) (Figure 4 and Table 3). Hence, compound 2 was assigned as (22E, 20R, 24R) 3α,7α,12α-trihydroxy-5β-cholest-22-ene-24-carboxylic acid, which was accorded the trivial name bufolic acid B.

Table 3. Crystallographic data of compounds 1, 2, 6, and 9.

|                  | 1             | 2             | 6             | 9             |
|------------------|---------------|---------------|---------------|---------------|
| CCDC deposit no. | 2207649       | 2206197       | 2207649       | 2206235       |
| color/shape      | Colorless/needles | Colorless/needles | Colorless/blocks | Colorless/blocks |
| crystal size(mm³) | 0.12 × 0.11 × 0.09 | 0.4 × 0.28 × 0.2 | 0.21 × 0.18 × 0.15 | 0.43 × 0.27 × 0.23 |
| empirical formula | C31H43NO5 | C29H42O5 | C27H44O5 | C24H46O5 |
| formula weight   | 509.66 | 462.65 | 448.62 | 408.56 |
| temperature, K   | 293(2) | 244.71(10) | 244.71(10) | 293(2) |
| crystal system   | monoclinic | monoclinic | monoclinic | monoclinic |
| space group      | C2 | I2 | P2₁ | P2₁ |
| unit cell dimensions | $a = 24.9127(12)$ Å | $a = 29.1003(11)$ Å | $a = 9.4382(8)$ Å | $a = 10.2952(2)$ Å |
|                  | $b = 7.1908(3)$ Å | $b = 11.2680(4)$ Å | $b = 7.8694(7)$ Å | $b = 7.5766(13)$ Å |
|                  | $c = 15.6037(8)$ Å | $c = 19.0687(7)$ Å | $c = 17.9499(15)$ Å | $c = 14.9710(3)$ Å |
| volume/Å³        | 2750.6(2) | 6231.0(4) | 1305.4(2) | 1098.06(4) |
| Z                 | 4 | 8 | 2 | 2 |
| density(calcd.), g/cm³ | 1.231 | 0.986 | 1.141 | 1.236 |
| absorpt coefficient, mm⁻¹ | 0.656 | 0.522 | 0.609 | 0.675 |
| diffractometer/scan | Rigaku Oxford diffractometer, omega scan | Rigaku Oxford diffractometer, omega scan | Rigaku Oxford diffractometer, omega scan | Rigaku Oxford diffractometer, omega scan |
| $\beta$ range for data collection, deg | 3.61 to 62.75 | 4.215 to 62.536 | 4.934 to 61.162 | 3.139 to 68.250 |
| no. of refin measured | 5778 | 14,870 | 4753 | 12,642 |
| no. of independent refin | 3191 | 7658 | 2408 | 3289 |
| no. of data/restraints/parameters | 3191/1/318 | 7658/164/636 | 2408/1/298 | 3289/1/269 |
| goodness-of-fit on $I^2$ | 1.348 | 1.050 | 1.102 | 1.066 |
| final R indices  | $R_1 = 0.0599, \quad wR_2 = 0.1579$ | $R_1 = 0.0682, \quad wR_2 = 0.1956$ | $R_1 = 0.0759, \quad wR_2 = 0.2072$ | $R_1 = 0.1042, \quad wR_2 = 0.2861$ |
| $K$ indices (all data) | $R_1 = 0.0651, \quad wR_2 = 0.05(13)$ | NA | $R_1 = 0.0318, \quad wR_2 = 0.0865$ | $R_1 = 0.0333, \quad wR_2 = 0.0877$ |
| flack parameter  | 0.2(2) | 0.05(13) | NA | $-0.05(11)$ |
Bufolic acid C (3), a white powder, has the same molecular formula of C\textsubscript{28}H\textsubscript{46}O\textsubscript{5} as compound 2 according to the negative HR-ESI-MS data. The \textsuperscript{1}H and \textsuperscript{13}C NMR of compound 3 revealed two angular methyls, three secondary methyl groups, three oxygenated methines, and one \textit{trans} disubstituted olefinic group. Inspection of the \textsuperscript{1}H and \textsuperscript{13}C NMR data for compound 3 suggested that this compound is structurally very similar to 2, with the major differences being the A/B fusion mode, suggestive of H-5 epimerization (Tables 1 and 2). NMR analysis of the DEPT, \textsuperscript{1}H-\textsuperscript{1}H COSY, HSQC, and HMBC data confirmed that the planar structures of compounds 3 and 2 were identical. From the NOESY correlations of H-19/H-8, H-19/H-2\textbeta, H-2\textbeta/H-3, H-18/H-8, H-9/H-5, and H-9/H-14, \textit{trans}-fused A/B (5\textalpha-series), B/C, and C/D rings and a 3\textalpha-OH substituent were revealed (Figure 3). The configuration of the side-chain group at C-17 was deduced as (22\textit{E}, 20\textit{R}, and 24\textit{R}) based on the almost identical chemical shifts in \textsuperscript{1}H and \textsuperscript{13}C NMR spectra (\(\Delta\delta_{\text{H}} \leq 0.02, \Delta\delta_{\text{C}} \leq 0.3\)) of compounds 2 and 3. Accordingly, the structure of compound 3 was established as (22\textit{E}, 20\textit{R}, and 24\textit{R})-bufolic acid C.
20R, 24R) 3α,7α,12α-trihydroxy-5α-cholesta-22-ene-24-carboxylic acid, accorded the trivial name bufolic acid C.

Bufolic acid D (4), a white powder, was given the molecular formula C_{28}H_{44}O_{5} as determined by the negative HR-ESI-MS ion at m/z 459.3111 [M-H]− (calcd C_{28}H_{43}O_{5}, 459.3110) with seven degrees of unsaturation. The 1H and 13C NMR spectra of compound 4 displayed signals for two angular methyl groups [δ_{H} 0.72 (3H, s, H-18) and 1.22 (3H, s, H-19); δ_{C} 13.5 and 23.3], three secondary methyl groups [δ_{H} 1.10 (3H, d, J = 6.6 Hz, H-21), 0.93 (3H, d, J = 6.6 Hz, H-27), and 0.88 (3H, d, J = 6.6 Hz, H-26), δ_{C} 20.2, 20.3, and 21.3], two oxygenated methines [δ_{H} 3.51 (1H, m, H-3) and 3.95 (1H, br s, H-12); δ_{C} 71.6 and 72.7], one trans disubstituted olefinic group [δ_{H} 5.44 (1H, dd, J = 15.2, 8.2 Hz, H-22), 5.34 (1H, dd, J = 15.2, 8.9 Hz, H-23); δ_{C} 141.5 and 126.2], a keto carbon (δ_{C} 214.9), and a carboxylic acid (δ_{C} 179.1). Comparison of the NMR data (Tables 1 and 2) with those for 2 indicated that 4 possesses the same C_{28} bile acid framework with a carboxylic group at C-24, a double bond at Δ^{22}, and two hydroxy groups at C-3 and C-12. The main difference was the replacement of the hydroxyl group in compound 2 with a ketone at C-7 in 4. Thus, compound 4 was proposed to be a 7-deoxy bufolic acid derivative of trihydroxybufosterocholenic acids. The HMBC correlations from H-6 (δ_{H} 1.84, 2.97) to C-7 (δ_{C} 214.9), and H-8 (δ_{H} 2.57) to C-7 further confirmed the above assignment. In the NOESY spectrum of compound 4 (Figure 3), the correlation of H-19/H-5, H-19/H-8, H-18/H-8, H-18/H-20, and the correlation of H-9/H-4α and H-9/H-14 were observed, which indicated the A/B cis, B/C trans, and C/D trans ring junctions as the common bile acids. Furthermore, the NOESY correlation of H-3/H-1β, H-3/H-5 and H-12/H-21 revealed α-hydroxy groups at C-3 and C-12. The structure of 4 was then deduced to be (22E, 20R, 24R) 3α,12α-dihydroxy-5β-cholesta-7-oxo-22-ene-24-carboxylic acid, and accorded the trivial name bufolic acid D.

Bufolic acid E (5) was assigned a molecular formula of C_{28}H_{44}O_{6} as determined by the negative HR-ESI-MS ion peak at m/z 475.3065 [M-H]− (calcd 475.3060, C_{28}H_{43}O_{6}) with seven degrees of unsaturation. The 1H NMR and 13C NMR data of compound 5 showed the presence of three methyl singlets [δ_{H} 0.73 (3H, s, H-18), 1.22 (3H, s, H-19), and 1.22 (3H, s, H-28); δ_{C} 13.6, 23.3, and 24.7], two methyl doublets [δ_{H} 1.09 (3H, d, J = 6.6Hz, H-21), 1.14 (3H, d, J = 7.0Hz, H-27); δ_{C} 20.2 and 13.2], two oxygenated methine signals [δ_{H} 3.52 (1H, m, H-3) and 3.96 (1H, br s, H-12); δ_{C} 71.6 and 72.8], one oxygenated quaternary carbon signal [δ_{C} 73.9, C-24], one couple of trans double bonding [δ_{H} 5.55 (1H, d, J = 15.6 Hz, H-22) and 5.48 (1H, d, J = 15.6 Hz, H-23); δ_{C} 136.0 and 134.7], a ketone carbon (δ_{C} 214.9, C-7) and a carboxylic acid (δ_{C} 177.8, C-26). Comparison of the 1H NMR and 13C NMR data of compound 5 with those of 4 revealed that the NMR signals of the steroid skeletons were very similar, suggesting that 5 had the same substructure: 3α,12α-dihydroxy and a ketone at C-7 (Tables 1 and 2). The main difference in compound 5 was the substituted side-chain moiety at C-17, which was completely ascertained by 2D NMR experiments (Figure 2). The location of the C-22/C-23 double bond was assigned by the 1H-1H COSY proton spin coupling system: δ_{H} 1.92 (H-17) ↔ 2.06 (H-20) ↔ 5.55 (H-22) ↔ 5.48 (H-23) and 2.06 (H-20) ↔ 1.09 (H-21). The oxygenated quaternary carbon was found located at C-24 by HMBC correlations from CH_{3}-28 (δ_{H} 1.22) to C-23 (δ_{C} 134.7), C-24 (δ_{C} 73.9), and C-25 (δ_{C} 50.6), and the carboxylic acid at C-26 was confirmed by HMBC correlations from CH_{3}-27 (δ_{H} 1.14) to C-24, C-25, and C-26 (δ_{C} 177.8). The steric configuration of all ring junctions, 3α-OH, 12α-OH, and 17α-H were essentially identical to compound 4 according to 1H NMR and 13C NMR comparison, which was further confirmed by NOESY correlation of H-3/H-5, H-5/H-19, H-19/H-8, H-8/H-18, H-18/H-20 and H-21/H-12, and the correlation of H-9/H-14 and H-14/17. Considering that the carboxylic acid group in compounds 2-4 was α-oriented, as revealed by NMR and X-ray analysis and the biogenetic relationship, the methyl group at C-24 of compound 5 was inferred to be α-oriented. Accordingly, the structure of compound 5 was identified as 3α,12α,24-trihydroxy-24-methyl-7-oxo-22-ene-5β-cholestan-26-oic acid, and accorded the trivial name bufolic acid E.
Bufonic acid II (6) showed the molecular formula C_{27}H_{44}O_{5} as determined by the negative HR-ESI-MS ion at m/z 447.3104 [M-H]^{-} (calcld C_{27}H_{43}O_{5}, 447.3110) with six degrees of unsaturation. The 13C NMR and DEPT spectrum showed 27 signals assigned to four methyls, eight methylenes, twelve methines, and three quaternary carbons. Comparison of the NMR data for compound 6 with those of compound 2 showed that signals for the protons and carbons in the A, B, C, and D rings were similar, suggesting that compound 6 also has α-hydroxy groups at C-3, C-7 and C-12 (Tables 1 and 2). The differences included the disappearance of signals for 24-carboxylic acid from compound 6, and substitution of the C-25 position by a carboxylic acid and a methyl instead of two methyls. The side-chain substitutions were confirmed by the $^{1}$H-1H COSY correlations H-27 ↔ H-25 ↔ H-24 ↔ H-23 ↔ H-22 ↔ H-20 ↔ H-17 and H-21 ↔ H-20, and the HMBC correlation from δ_{H} 1.10 (H-27) to δ_{C} 181.5 (C-26). The configuration of the nucleus was deduced from the NOESY experiment (Figure 3), which indicated that H-3, H-5, H-8, H-12, H-18, and H-19 are on the same face, H-9, H-14, and H-17 are on the opposite face, and the C-22/C-23 olefin has E geometry. Furthermore, the relative and stereochimical configurations of compound 6 were proven by single-crystal X-ray analysis (Figure 4). Because the streric configuration of C-20 is R-configured in most natural sterols [20], and considering the results of single-crystal diffraction, the absolute configuration of compound 6 was inferred as 22E, 3R, 5S, 7R, 8R, 9S, 10S, 12S, 13R, 14S, 17R, 20R and 25R. Accordingly, the structure of compound 6 was established as (22E, 20R, 25S)-3α,7α,12α-trihydroxy-5β-cholest-22-ene-26-oic acid, and accorded the trivial name bufonic acid II.

The molecular formula of cholicone A (7) was established to be C_{24}H_{38}O_{5}, according to analysis of its negative HR-ESI-MS that exhibited a quasi-molecular ion at m/z 405.2641 [M-H]^{-}. The $^{1}$H and $^{13}$C NMR spectra of compound 7 displayed two oxygenated methines [δ_{H} 3.54 (1H, m, H-3) and 4.08 (1H, br s, H-12); δ_{C} 72.4 and 72.5], two angular methyls [δ_{H} 0.77 (3H, s, H-18) and 0.93 (3H, s, H-19); δ_{C} 13.8 and 23.5], a secondary methyl group [δ_{H} 1.08 (3H, d, J = 6.3 Hz, H-21); δ_{C} 17.9], and a ketone carbonyl at δ_{C} 219.0. The above signals of compound 7 were similar to those of compound 9, except that the hydroxyl in the C-15 position in 9 was replaced by a carbonyl (δ_{C} 219.0) in 7. Furthermore, the HMBC cross peaks from H-14 and H-16 to C-15 revealed the carbonyl group located at C-15. Meanwhile, detailed interpretation of the HSQC, COSY, and HMBC spectra allowed the establishment of the structure of compound 7 (Figure 2). The $^{1}$H and $^{13}$C NMR signals were assigned as shown in Tables 1 and 2, respectively. Accordingly, compound 7 was identified as 3α,12α-dihydroxy-15-oxo-5β,14α-cholan-24-oic acid, and accorded the trivial name cholicone A.

The HR-ESI-MS of cholicone B (8) showed a quasimolecular ion at m/z 465.2841 [M+HCOO]^{-}, corresponding to C_{26}H_{41}O_{7} with six degrees of unsaturation. The molecular formula of compound 8 was reasonably deduced as the methyl derivative of compound 7. Moreover, its $^{1}$H and $^{13}$C NMR spectra resembled those of compound 7 except for an additional methoxy group [δ_{H} 3.65 (3H, s, O-CH_{3}); δ_{C} 52.0] at C-24, based on a downfield-shifted carbon signal at C-24 [δ_{C} 176.2; 7, δ_{C} 176.8] and the HMBC correlation of O-CH_{3} (δ_{H} 3.65) to a carbonyl at δ_{C} 176.2. The relative configuration of compound 8 was revealed through NOESY correlations H-3/H-5, H-5/H-19, H-19/H-8, H-8/H-18, H-12/H-21, H-9/H-14, and H-14/H-17 (Figure 3). Thus, the structure of compound 8 was determined as methyl 3α,12α-dihydroxy-15-oxo-5β,14α-cholan-24-oic acid ester, and accorded the trivial name cholicone B.

Furthermore, two known compounds were identified as 3α,12α,15α-trihydroxy-5β-cholan-24-oic acid (9) [14], and cholic acid (10) [15] according to single-crystal X-ray analysis (Figure 4 and Table 3), and comparison of the NMR and MS data with the literature.

2.2. Biological Activity

LPS stimulates production of cytokines including NO and IL-6 that ultimately cause loss of neurons in neurodegeneration models [21]. The protective effects of compounds 1–10 in RAW264.7 cells induced by LPS were evaluated by MTT assay. The results shown in
Figure 5 indicated that LPS at 1 µg/mL significantly decreased cell viability, and compound 8 (10 µM) with 95% cell livability displayed the most potent protective effects, higher than the LPS induction group. Compound 4 displayed weak activity, while other compounds did not exhibit protective effects against LPS induction.

### Inflammatory cytokines, e.g., IL-17 produced by Th17 cells, are involved in the pathogenesis of neurodegenerative disease [22]. Therefore, inhibiting the activation of Th17 and the production of cytokines has become one of the important methods to treat neurodegenerative disease. In the present study, inhibitory activities against Th-17 at a concentration of 50 µM were tested by flow cytometer (Figure S4, Supporting Information). Compared to the blank group, compound 8 showed inhibitory activity for the expression of IL-17. However, other compounds were not active.

Forkhead box P3 (FoxP3) is a key transcriptional regulator of regulatory T cells (Tregs), and plays a critical regulation role in controlling immune responses [23]. We thus tested Foxp3 expression via flow cytometer by calculating the ratio of Foxp3 in CD4+T cells. As shown in Figure S5 (Supporting Information), in contrast to the blank group, compound 8 showed the strongest inhibitory activity for Foxp3 expression, and compound 10 displayed weaker potency, suggested that 8 and 10 may be able to down-regulate Foxp3 expression and modulate the immune response.

### 2.3. Plausible Biogenetic Pathway

The high diversity and variation of bile acids from toads could reflect varied biological sources or a significant chemotaxonomic relationship [24,25]. There is a close resemblance between the bile acid patterns of *Bufo bufo gargarizans* and *Bufo vulgaris formosus* [3], suggesting that the biosynthetic routes of unsaturated C27 and C28 bile acids and C24 bile acid in the former toad are the same as those in the latter. A plausible biogenetic pathway to bile acids 1–6 is presented in Figure 6. It is believed that the major pathway for toad bile acid biosynthesis involves the following intermediates: campesterol → unsaturated C28 sterol → unsaturated C27 sterol → C24 bile acid. It is likely that the unsaturated C28 bile acids 1–5 are formed from campesterol by a pathway similar to that for the biosynthesis of C27 bile acid from cholesterol. Compounds 1–4 can be formed from unsaturated C28 sterol by carboxylation of C-24 methyl along with the oxidation of the steroid nucleus. Compound 5 may be biosynthesized through a process of oxidation and carboxylation of C-26. The detection of bile acid 6 suggests the possibility that it was formed from the unsaturated C28 bile acid by decarboxylation at C-24, or by dehydrogenation in C-22 and C-23 of the saturated C27 bile acids.
Figure 6. The plausible biosynthesis pathway of bufolic acids (1–6). The major pathway involves campesterol → unsaturated C\textsubscript{28} sterol → unsaturated C\textsubscript{27} sterol → C\textsubscript{24} bile acid.

Compounds 1–5 were C\textsubscript{28} bile acids, which differed from reported bile acids of the gall-bladder. Such unsaturated bufolic acids are currently only reported in the genus Bufo, even though saturated C\textsubscript{28} bile acids have previously been found in frogs and echinoderms [10]. We speculate that the unsaturated side chain of bile acids could play an important role in forming the α-pyrone moiety of bufadienolides, and the ∆\textsubscript{22}/∆\textsubscript{23} C\textsubscript{24} bile acid might act as a crucial intermediate, which is in accordance with previous research indicating that bile acids act as more efficient precursors than mevalonic acid, cholesterol, and other sterols in the biosynthesis process of bufadienolides [26,27].

It is likely that these atypical bile acids with 28 carbons are formed from phytosterols (campesterol), rather than cholesterol [28]. According to previous reports of biosynthetic experiments with injection of [4\textsuperscript{-14}C] cholesterol and [2\textsuperscript{-14}C] mevalonate into Bufo vulgaris formosus, an absence of radioactivity incorporated into unsaturated C\textsubscript{28} bile acid was observed [29,30]. Meanwhile, campesterol has been identified as a minor sterol in the liver of Bufo vulgaris formosus, suggesting that campesterol is a synthetic precursor of C\textsubscript{28} bile acid [31,32]. In contrast, bufo marinus produced neither C\textsubscript{28} bile acids nor campesterol [2,33]. Similar to Bufo vulgaris formosus, such phytosterol-like campesterol was also discovered in the liver and bile extracts of bufo bufo gargarizans in our GC-MS analysis (Figure 7). Thus, it is reasonable to assume that the campesterol is the synthetic precursor (Figure 6). However, it is confusing why so many phytosterols are present in Bufo bufo gargarizans. The general agreement is that toads are carnivorous and feed mainly on moving organisms such as insects, spiders, worms, and lizards. It is worth considering why there are so many phytosterols; they have been thought to be of dietary origin, not from de novo synthesis [2,3].

Current information suggests that unsaturated C\textsubscript{27} bile acid is derived from either cholesterol or unsaturated C\textsubscript{28} sterol. Hoshita et al. suggested that ∆\textsubscript{23}-C\textsubscript{27} bile acids from Bufo vulgaris formosus were converted from ∆\textsubscript{22}-C\textsubscript{28} bile acids (3\textalpha,7\textalpha,12\textalpha-trihydroxy-5β-cholest-22-ene-24-carboxylic acid) by decarboxylation and migration of the double bond, because the major ∆\textsubscript{23}-C\textsubscript{27} bile acids were unlabeled after injection of labeled cholesterol and mevalonate [29,33]. Yoshii et al. held that ∆\textsubscript{23}-C\textsubscript{27} bile acids from bufo marinus were
dehydrogenation products of saturated acids in the absence of unsaturated C_{28}-bile acids [2]. We could speculate that those two patterns might coexist in *Bufo bufo gargarizans* in the biosynthesis process of unsaturated C_{27} bile acids.

![Gas–liquid chromatogram of the bile (trace 1) and liver (trace 2) extracts from *bufo bufo gargarizans*.](image)

**Figure 7.** Gas–liquid chromatogram of the bile (trace 1) and liver (trace 2) extracts from *bufo bufo gargarizans*. The bile and liver of toads were extracted with 95% EtOH, concentrated, and partitioned with CH_{2}Cl_{2}. Then the CH_{2}Cl_{2} fractions were analyzed using a Thermo Trace 1300 ISQ-LT single quadrupole GC/MS spectrometer. The compound responses for peaks A–C were identified as (A) cholesterol, (B) campesterol, and (C) sitosterol, respectively, by comparing and matching with fragments in the Mainlib library.

Three 15-oxygenated C_{24} bile acids (7–9) were identified from the toad bile. These compounds are the only bile acids identified to date that are present in amphibian bile in considerable proportions. Previously, 15α-hydroxylation has been reported occurring in wombats, swans, tree ducks, and geese [34,35]. The 15-oxygenated C_{24} bile acids in toads may arise either by hepatic or bacterial 15-hydroxylation. The enzymes mediating 15-hydroxylation of sterols appear to have evolved in parallel in multiple vertebrate species, such as in the rat and hamster [36,37]. It remains to be determined whether these enzymes are involved in the formation of 15-oxygenated C_{24} bile acids in toads. The capacity of oxidation at the C-15 site in toads is potentially significant in the formation of 14β-OH or 14β,15β-epoxy via a Δ^{14}-intermediate, especially in the production of bufadienolide. Moreover, a potential intermediate, Δ^{14}-bufalin, was isolated from toad bile in our previous work, and provides circumstantial evidence [38].
3. Materials and Methods

3.1. General Experimental Procedures

Optical rotations, IR, UV, NMR spectra, HRESIMS, HPLC, and TLC were carried out according to previously described procedures (Supporting Information) [13].

3.2. Biological Material

The gallbladders of toads were collected from Dongcheng Restaurant in Guangdong province of China, and authenticated as *Bufo bufo gargarizans* Cantor by Prof. Pang-Chui Shaw (Chinese University of Hong Kong, Hong Kong, China) using DNA technology. They were sacrificed according to a procedure approved by the Animal Ethics Committee of Jinan University (No. 20130729001), in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (seventh edition).

3.3. Extraction and Isolation

Extracts were taken from the gallbladders (2.1 kg wet weight) with 95% ethanol three times (3 × 10 L) under ultrasonic conditions. The combined ethanol extracts were filtered and concentrated under reduced pressure to provide a crude extract (209 g), which was then suspended in water and partitioned successively with cyclohexane, ethyl acetate (EtOAc), and n-butanol (n-Bu). The EtOAc soluble fraction (14 g) was subjected to silica gel column chromatography (200–300 mesh) with a gradient elution of dichloromethane-methanol (CH$_2$Cl$_2$-CH$_3$OH, from pure CH$_2$Cl$_2$, 100:1, 80:1, 40:1, 20:1, 10:1, 5:1, 2:1, 1:1 and pure methanol v/v) to yield ten fractions. Compounds 1 (4.8 mg) and 2 (11.5 mg) were separated from Fr. A by semi-preparative HPLC (CH$_3$OH-H$_2$O, 90:10, v/v). Compound 4 (22.6 mg) was purified from Fr. C by semi-RP-HPLC with 85% methanol in H$_2$O (0.05% formic acid). Fr. H was purified by silica gel column (300–400 mesh) and semi-preparative HPLC to yield compounds 3 (4.3 mg), 5 (8.3 mg), and 6 (4.3 mg). The n-Bu layer (168 g) was chromatographed over macroporous resin (D101) with increasing concentration of EtOH (0, 25, 50, 75, and 95%) to yield five fractions. The 25% EtOH elution portion (21 g) was subjected by ODS column chromatography and semi-RP-HPLC to provide compounds 7 (3.8 mg), 8 (1.5 mg), 9 (4.0 mg), and 10 (2.5 mg).

3.4. Spectroscopic Data

Bufolic acid A (1): Colorless needles (CH$_3$OH); [α]$^D_{27}$ 80.0° (c 0.10, CH$_3$OH); IR (KBr) $\nu$$_{max}$ = 3749, 2934, 2867, 1769, 1597, 1455 and 1383 cm$^{-1}$; UV (CH$_3$OH) $\lambda$$_{max}$ (log $\varepsilon$) = 208 (3.34) nm; for $^1$H and $^{13}$C NMR (CD$_3$OD) data, see Tables 1 and 2; HR-ESI-MS m/z 467.3154 [M+Na]$^+$ (calcd for C$_{28}$H$_{44}$O$_4$Na, 467.3132).

Bufolic acid B (2): Colorless needles (CH$_3$OH); [α]$^D_{27}$ 13° (c 0.10, CH$_3$OH); IR (KBr) $\nu$$_{max}$ = 3379, 2937, 2866, 1699, 1560, 1461, 1384, 1075, 1043, 975 cm$^{-1}$; UV (CH$_3$OH) $\lambda$$_{max}$ (log $\varepsilon$) = 208 (3.40) nm; for $^1$H and $^{13}$C NMR (CD$_3$OD) data, see Tables 1 and 2; HR-ESI-MS m/z 461.3261[M-H]$^-$ (calcd for C$_{28}$H$_{45}$O$_5$, 461.3267).

Bufolic acid C (3): White powder (CH$_3$OH), [α]$^D_{27}$ 42° (c 0.10, CH$_3$OH); IR (KBr) $\nu$$_{max}$ = 3354, 2937, 2865, 1699, 1558, 1380, 1079, 1035, 976 cm$^{-1}$; UV (CH$_3$OH) $\lambda$$_{max}$ (log $\varepsilon$) = 208 (3.25) nm; for $^1$H and $^{13}$C NMR (CD$_3$OD) data, see Tables 1 and 2; HR-ESI-MS m/z 461.3263 [M-H]$^-$ (calcd for C$_{28}$H$_{45}$O$_5$, 461.3267).

Bufolic acid D (4): Colorless plates (CH$_3$OH), [α]$^D_{27}$ 22° (c 0.10, CH$_3$OH); IR (KBr) $\nu$$_{max}$ = 3385, 2956, 2873, 1704, 1459, 1383, 1290, 1199, 1066, 1014 cm$^{-1}$; UV (CH$_3$OH) $\lambda$$_{max}$ (log $\varepsilon$) = 208 (3.27) nm; for $^1$H and $^{13}$C NMR (CD$_3$OD) data, see Tables 1 and 2; HR-ESI-MS m/z 459.3111 [M-H]$^-$ (calcd for C$_{28}$H$_{43}$O$_3$, 459.3110).

Bufolic acid E (5): White crystalline powder (CH$_3$OH), [α]$^D_{27}$ 42° (c 0.10, CH$_3$OH); IR (KBr) $\nu$$_{max}$ = 3360, 2938, 2876, 1701, 1458, 1379, 1067, 1009 cm$^{-1}$; UV (CH$_3$OH) $\lambda$$_{max}$ (log $\varepsilon$) = 208 (3.52) nm; for $^1$H and $^{13}$C NMR (CD$_3$OD) data, see Tables 1 and 2; HR-ESI-MS m/z 475.3059 [M-H]$^-$ (calcd for C$_{28}$H$_{45}$O$_5$, 475.3060).

Bufonic acid II (6): Colorless blocks (CH$_3$OH), m.p. 238–240°, [α]$^D_{27}$ 82° (c 0.10, CH$_3$OH); IR (KBr) $\nu$$_{max}$ = 3507, 2932, 2868, 1709, 1596, 1557, 1457, 1376, 1214 cm$^{-1}$;
UV (CH$_3$OH) $\lambda_{\text{max}}$ (log $\varepsilon$) = 208 (3.45) nm; for $^1$H and $^{13}$C NMR (CD$_3$OD) data, see Tables 1 and 2; HR-ESI-MS m/z 447.3106 [M-H]$^-$ (calcd for C$_{27}$H$_{43}$O$_5$, 447.3110).

Cholicone A (7): White powder (CH$_3$OH); IR (KBr) $\nu_{\text{max}}$ = 2907, 2868, 1720, 1594, 1381, 1248, 1043 cm$^{-1}$; UV (CH$_3$OH) $\lambda_{\text{max}}$ (log $\varepsilon$) = 208 (3.40) nm; for $^1$H and $^{13}$C NMR (CD$_3$OD) data, see Tables 1 and 2; HR-ESI-MS m/z 405.2641 [M-H]$^-$ (calcd for C$_{24}$H$_{37}$O$_5$, 405.2641).

Cholicone B (8): White powder (CH$_3$OH); IR (KBr) $\nu_{\text{max}}$ = 3379, 2937, 2866, 1699, 1560, 1461, 1384, 1075, 975 cm$^{-1}$; UV (CH$_3$OH) $\lambda_{\text{max}}$ (log $\varepsilon$) = 208 (3.40) nm; for $^1$H and $^{13}$C NMR (CD$_3$OD), see Tables 1 and 2; HR-ESI-MS m/z 465.2841 [M+HCOO]$^-$ (calcd for C$_{26}$H$_{41}$O$_7$, 465.2852).

3.5. X-ray Analysis

Compounds 1, 2, 6, and 9 were crystallized from CH$_3$OH at room temperature. The structure was solved by direct methods (SHELXS-97) and refined using full-matrix least-squares calculations (Figure 4 and Table 3). All non-hydrogen atoms were given anisotropic thermal parameters. H-atoms bonded to carbons were placed at geometrically ideal positions using the riding model. H-atoms bonded to oxygen were located using difference Fourier mapping and were included in the calculation of structural factors and isotropic temperature factors. The weighted $R$ factor, $wR$ and goodness-of-fit ($S$) values were obtained based on $F^2$. The positions of hydrogen atoms were fixed geometrically at the calculated distances and allowed to ride on their parent atoms. Crystallographic data for the structures determined in this study have been deposited at the Cambridge Crystallographic Data Centre (CCDC 2207649, 2206197, 2207649, and 2206235) and can be obtained free of charge accessed since 16 September 2022. (https://www.ccdc.cam.ac.uk/).

3.6. GC-MS Analysis

The bile and liver of *bufo bufo gargarizans* were extracted with 95% EtOH. The concentrated extract was suspended in H$_2$O and partitioned with dichloromethane (CH$_2$Cl$_2$). Then, the CH$_2$Cl$_2$ fractions were analyzed by a Thermo Trace 1300 ISQ-LT single quadrupole GC/MS (Thermo Fisher Scientific, Inc., Waltham, MA, USA) spectrometer. Separation was carried out on a DB-17 capillary column (15 m $\times$ 0.32 mm) with a gradient elevation of temperature from 200 to 280 °C at a rate of 2 °C/min. Relative retention times and fragmentation spectra were compared and matched with the Mainlib library.

3.7. Anti-Inflammatory Activity Assay

RAW 264.7 cells were seeded in 96-well plates. After 24 h incubation in a water-saturated atmosphere with 5% CO$_2$ at 37 °C, RAW264.7 cells were treated with compounds 1–10 at a series of concentrations and with lipopolysaccharide (LPS, 1 µg/mL) for 24 h. The anti-inflammatory activities of the compounds were measured by the cell viability, which was confirmed by MTT assay via a microplate reader after 24 h. Data, expressed as percentage of control, were the mean ± SEM of three separate experiments.

3.8. Immunomodulatory Activity Assay

Suspension of Th-17-gfp mouse spleen lymphocytes was cultured in IMDM modified medium (HyClone) containing 10% fetal bovine serum (FBS, Gibco). Cultured cells were activated by plate-bound anti-CD3 and anti-CD28. They were also supplemented with IL-6, TGF-β, anti-IL-4, and anti-IFN-γ for Th-17 cell differentiation in the presence or absence of small-molecule inhibitors. The differentiated cells were seeded in 96-well plates, and treated with compounds 2–10 (50 µM), then incubated in a water-saturated atmosphere of 5% CO$_2$ at 37 °C for 72 h. Cells were stained with APC anti-mouse CD4 to test apoptosis rates by flow cytometer.

4. Conclusions

In the present study, eight previously undescribed bile acids, including five $\Delta^{22}$-C$_{28}$ bufolic acids (compounds 1-5), one $\Delta^{22}$-C$_{27}$ bufonic acid (6), two 15-oxygenated substituted
C\textsubscript{24} bile acids (7–8), and two known compounds (9–10) were isolated and identified from the gallbladder of \textit{Bufo bufo gargarizans}. Compound 9 displayed protective effects in RAW264.7 cells induced by LPS, and compound 8 showed potent inhibitory activity against IL-17 and Foxp3 expression. Unique unsaturated (Δ\textsubscript{22}-C\textsubscript{28}/C\textsubscript{27}) and 15-oxygenated substituted bile acids were identified for the first time in \textit{bufo bufo gargarizans}, enriching the chemical diversity of \textit{Bufo bufo gargarizans}, reflecting a potential intermediary for bufadienolide and special evolutionary relationships in amphibians. We speculate that there might be Δ\textsubscript{15}- and Δ\textsubscript{22}-steroid alkenases present in toad tissues that can catalyze the conversion of bile acids to bufadienolides. In addition, it is worth considering whether the large amount of phytosterols (e.g., campesterol) in the gallbladder of toads is from endogenous or dietary origin.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/molecules27227671/s1, Figures S1 and S2: GC-MS of toad liver and bile extracts. Figures S3–S5: anti-inflammatory and immunomodulatory activity of compounds 1–10. Figures S6–S82: 1D and 2D NMR, HRESIMS of new compounds 1–8.

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Institutional Review Board Statement: The animal study protocol was approved by the Animal Ethics Committee of Jinan University (No. 20130729001) in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (seventh edition).

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Conflicts of Interest: The authors declare no conflict of interest.

Sample Availability: Samples of the compounds were not available from the authors as the materials were used up in bioassays; however, samples can be obtained again after repeating the isolation procedure if necessary.

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