**Separation and characterization of bufadienolides in toad skin using
two-dimensional normal-phase liquid
chromatography×reversed-phase liquid chromatography coupled
with mass spectrometry**

Yun Zhang^{1,a}, Hongli Jin^{1,a}, Xiaolong Li^a, Jianqiang Zhao^b, Xiujie Guo^a, Jixia Wang^a,
Zhimou Guo^a, Xiuli Zhang^a, Yanduo Tao^b, Yanfang Liu^{*a}, Deliang Chen^{**c}, Xinmiao
Liang^a

1: these authors contributed equally to this work

^a Dalian Institute of Chemical Physics, Chinese Academy of Sciences, Key Laboratory of
Separation Science for Analytical Chemistry, Dalian 116023, Peoples R China

^b Key Laboratory of Tibetan Medicine Research, Northwest Institute of Plateau Biology, Chinese
Academy of Sciences, Xining 810001, Peoples R China

^c Wuhan Univ, ZhongNan Hosp, Dept Internal Med, Div Cardiol, Wuhan 430071, Hubei Province,
Peoples R China

Fax: +86-411-84379539

Tel.: +86-411-84379541

Email: liuyanfang@dicp.ac.cn

Keywords: Bufadienolides, Normal-phase liquid chromatography, Two-dimensional
liquid chromatography, MS/MS, Toad skin

■ Abstract

Bufadienolides possess various bioactivities especially antitumor. Due to the high structural diversity, the separation of bufadienolides often suffers from coelution problem on conventional RP columns. In this work, an off-line two-dimensional normal-phase liquid chromatography×reversed-phase liquid chromatography (2D-NPLC×RPLC) method was developed to separate and characterize bufadienolides in toad skin. Several RP and NP columns were evaluated with five reference bufadienolides. The XUnion C18 and XAmide columns exhibited superior chromatographic performances for bufadienolide separation, and were selected in RPLC and NPLC, respectively. RPLC was used in the second-dimension for the good compatibility with MS, while NPLC was adopted in the first-dimension. The orthogonality of the 2D-NPLC×RPLC system was investigated by the geometric approach using fifteen bufadienolide mixtures. The result was 49.6%, demonstrating reasonable orthogonality of this 2D-LC system. By combining the 2D-LC system with MS, 64 bufadienolides including 33 minor ones and 11 pairs of isomers in toad skin were identified. This off-line 2D-NPLC×RPLC allowed to solve the coelution problem of bufadienolides in one-dimension RPLC, and thus facilitated the identification significantly.

1. Introduction

The skin of *Bufo bufo gargarizans* Cantor, also known as toad skin, is one of the most famous traditional Chinese Medicine (TCMs). It has been used to treat numerous diseases in China for a long time, such as antitumor [1-3], anti-arrhythmia [4], and prevention of heart disease [5]. In recent years, increasing evidences have demonstrated that bufadienolides, a kind of polyhydroxy C-24 steroids with **2H-pyrone-2-one** ring in the molecule, are main active components in toad skin. These compounds possess various bioactivities, including cardiotonic, renal sodium excretion, blood pressure stimulating [6, 7], immunoregulatory [8], and antimicrobial [9]. Most notably, bufadienolides exhibit remarkable anticancer activities and a broad spectrum to many cancer cell lines [10, 11]. The investigation of bufadienolides in toad skin has been a hotspot in the natural product research.

Up to now, about 60 bufadienolides in total have been reported in toad skin [12-16]. Due to the high structure diversity, the comprehensive separation and analysis of bufadienolides remains challenging. Reversed-phase liquid chromatography (RPLC) has been widely used for the separation of bufadienolides [17-19]. And coupled with mass spectrometry (MS) or tandem mass spectrometry (MS/MS), convenient identification of bufadienolides is achieved [20-24]. Hu et al. [21] have characterized bufadienolides in Chinese medicine-Venenum Bufonis by RPLC-ESI-MS/MS. A total of 30 bufadienolides were identified. Nonetheless, it was found that the separation of bufadienolides in toad skin was rather difficult, owing to the similar retention behaviors in RPLC. Besides, the characterization of minor

bufadienolides or isomers may suffer from co-elution problems in one-dimensional RPLC. Hence, improved chromatographic separation of bufadienolides is of great significance for the identification.

Two-dimensional liquid chromatography (2D-LC) has become a potent tool for the separation of complex mixtures [25-28]. Liu et al. [29] has developed a two-dimensional reversed-phase liquid chromatography/hydrophilic interaction chromatography (2D-RPLC/HILIC) method for the purification of bufadienolides from toad skin. In addition, RPLC coupled with positively charged C18 column was used to efficiently separate the bufadienolides in toad skin [30]. These works indicated that 2D-LC is capable of providing significantly improved separation for bufadienolides comparing to one-dimension LC, mainly ascribed to the increased peak capacity and selectivity.

In the case of constructing a successful 2D-LC system, Orthogonality is an important issue. Commonly, a pair of phases with different separation mechanisms would minimize the retention correlation between dimensions [31, 32], and thus good orthogonality could be obtained. Normal-phase liquid chromatography (NPLC) has been adopted as an alternative mode to RPLC for the distinct chromatographic mechanisms [33, 34]. And the combination of NPLC and RPLC offers a high degree of orthogonality in the analysis of various compounds [35, 36]. To our best knowledge, two-dimensional NPLC×RPLC (2D-NPLC×RPLC) method has not been applied in the bufadienolide separation.

In this work, we aimed to develop an off-line 2D-NPLC×RPLC method for the

separation and identification of bufadienolides in toad skin. RP and NP columns were assessed to establish the 2D-LC system. Its orthogonality was evaluated by the geometric approach. Using this 2D-LC method coupled with mass spectrum, bufadienolides in toad skin were characterized systematically.

2. Materials and Methods

2.1 Apparatus and reagents

Most HPLC separation was performed on an Alliance HPLC system consisting of a Waters 2695 HPLC pump and a Waters 2996 photodiode array detector. Data acquisition and processing were conducted by Waters Empower software. The second dimensional RPLC-MS/MS was performed on an Agilent 1290 Infinity LC instrument (Agilent, USA) coupled to Agilent 6540 series QTOF-MS (Agilent, USA), which equipped with electrospray ionization (ESI) source, diode-array detector (DAD), automatic sample injector and column thermostat.

Water was prepared by a Milli-Q system (Billerica, MA, USA). Methanol, isopropanol and n-hexane were of chromatographic grade (Yuwang, China). Acetonitrile was purchased from Tedia (Fairfield, USA) and formic acid was obtained from Acros (Cambridge, USA). Fifteen reference bufadienolides were listed as follows: bufarenogin (1), desacetylcinobufotalin (2), desacetylbufotalin (3), argentinogenin (4), telocinobufagin (5), desacetylcinobufagin (6), bufadienolide (7), bufotalin (8), 3-Dehydrobufagin (9), marinobufagin (10), bufalin (11), 3-keto-Deacetylcinobufagin (12), bufadienolide (13) cinobufagin (14) and resibufogenin (15). Compounds 7 and 13 belonged to bufadienolides, according to

UV and MS data. The chemical structures required further identification. Other reference bufadienolides were isolated from toad skin in our previous work [30]. The structures (shown in Fig. 1) were identified by UV, MS and NMR.

All the columns used in this work were purchased from Acchrom (Beijing, China) and listed as follows: XAmide (150 mm × 4.6 mm, I. D., 5 μm), Unitary Diol (150 mm × 4.6 mm, I. D., 5 μm), Click XIon (150 mm × 4.6 mm, I. D., 5 μm) and XUnion C18 (150 mm × 4.6 mm, I. D., 5 μm).

2.2 Sample preparation

Toad skin was collected from Shandong Province and authenticated by the Institute of Medication, Xiyuan Hospital of China Academy of Traditional Chinese Medicine. 100 g of dried toad skin was cut up into pieces and decocted with 700 mL of methanol for three hours. The extraction procedure was repeated twice. The decoctions were combined and dried with rotary evaporation at 60 °C in vacuum. 3.5 g of the extract was redissolved in 50 mL of 70% methanol, and then extracted with n-heptane (3×50 mL each). The methanol fraction was dried in vacuum and yielded 2 g of residue. Subsequently, the residue was redissolved in 40 mL of dichloromethane and extracted with 40 mL of water (2×40 mL each). The dichloromethane fraction was dried in vacuum. 500 mg of the concentrate of dichloromethane layer was dissolved in 3 mL of 95% ethanol and loaded on Click XIon SPE cartridge (20 g, 60 mL, Acchrom, Beijing, China). The target fraction was collected by elution with 60 mL of 95% acetonitrile. And the column was washed with 60 mL of 50% acetonitrile (0.1% FA). The sample was concentrated to dryness in vacuum. 150 mg of the residue

was dissolved in 1 mL of methanol and filtered through 0.45 μm filter for NP chromatography separation. Twelve fractions were collected according to the UV absorption. All of these fractions were concentrated to dryness in vacuum. A proper quantity of each fraction was resolved in methanol and filtered through 0.22 μm filter prior to RPLC/MS analysis.

2.3 Chromatographic and mass spectrometric conditions

HPLC experiments were performed at the flow rate of 1.0 mL/min. Column temperature was maintained at 30 °C. Detection was carried out at 300 nm.

The development of the 2D-LC system: in RPLC mode, the mobile phase A was acetonitrile and B was water. Gradient elution steps were as follows: 0-10 min, 10%-30% A; 10-30 min, 30%-45% A; 30-40 min, 45%-60% A. In NPLC mode, the mobile phase A was isopropanol and B was n-hexane. Gradient elution steps were as follow: 0-10 min, 10%-30% A; 10-30 min, 30%-50% A.

The orthogonality evaluation: an XUnion C18 column (150 mm $\times$ 4.6 mm, I. D., 5 μm) was used in RPLC. The mobile phase A was acetonitrile and B was water. Gradient elution steps were as follows: 0-10 min, 10%-30% A; 10-30 min, 30%-45% A; 30-40 min, 45%-60% A. An XAmide column (150 mm $\times$ 4.6 mm, I. D., 5 μm) was used in NPLC. The mobile phase A was isopropanol and B was n-hexane. Gradient elution steps were as follows: 0-50 min, 10%-60% A.

The analysis of practical bufadienolides sample with NPLC $\times$ RPLC-QTOF-MS/MS: the first-dimension separation was performed on the XAmide column (150 mm $\times$ 4.6 mm, I. D., 5 μm) in NPLC. The mobile phase A was

isopropanol and B was n-hexane. Gradient elution steps were as follows: 0-50 min, 10%-60% A.

The second-dimension separation was performed on the XUnion C18 column (150 mm × 4.6 mm, I. D., 5 μm). The mobile phase A was acetonitrile and B was water. Gradient elution steps were as follow: 0-10 min, 10%-30% A; 10-30 min, 30%-50% A; 30-35 min, 80%-100% A. The elution from the second-dimension separation was split and then delivered into the mass spectrometer. The mass spectrometer conditions were as follows: Nebulizer (N₂) was set at 35 psi. The dry gas was set to 8 L/min. The gas temperature was set to 350 °C. The capillary voltage was set to 3500 V. The collision energy was 25 eV. MS scan ranged from 100 to 800 m/z, and the MS/MS scan ranged from 50 to 500 m/z, Spectra were collected in the positive ESI mode.

2.4 Data analysis

The orthogonality evaluation was described, according to the literatures [33]. The retention times of the 15 bufadienolides were converted to normalized $RT_{i(norm)}$ calculated by formula (1). where RT_{max} and RT_{min} represented the retention times of the most and least retained solute in the data set. **Then the normalized retention data were plotted into a 2D separation space which was divided into 4×4 bins.** The orthogonality O was calculated according to the equation (2). The $\sum bins$ was the number of the bins containing data points which was 9 and the P_{max} was the sum of all bins which was 16.

$$RT_{i(norm)} = \frac{RT_i - RT_{min}}{RT_{max} - RT_{min}} \quad (1)$$

$$O = \frac{\sum \text{bins} - \sqrt{P_{\max}}}{0.63 P_{\max}} \quad (2)$$

3. Results and discussion

3.1 The development of the 2D-LC system

In this section, five reference bufadienolides (Fig. 1) were used for the column evaluation, including bufarenogin, telocinobufagin, desacetylcinobufagin, bufotalin and 3-dehydrobufagin, RPLC is a common HPLC method for the separation of bufadienolides [18, 20]. Several conventional RP columns were tested with reference bufadienolides. The RP columns exhibited the similar chromatographic performances for the bufadienolides (results shown in supporting information). Hence, the widely used XUnion C18 column was selected in this work. As shown in Fig. 2a, satisfactory separation was obtained.

Three commercially available NP columns with the identical length and diameter were evaluated to separate five bufadienolides (Fig. 2b-d). As can be seen in Fig. 2, the elution orders of the bufadienolides on the NP columns were quite different from those on the RP column. This result revealed the orthogonal combination of RPLC and NPLC for the separation of bufadienolides.

Furthermore, it was found that three NP columns exhibited distinct chromatographic performances (Fig. 2b-d). The Diol column contains diol groups bonded to the surface. The Click XIon column possesses zwitterionic groups grafted to the silica through polar groups. And the XAmide column contains amide groups attached to the silica surface. Compound 1, 2 and 4 almost co-eluted on the Diol

column (Fig. 2b). Peak broadening and tailing were observed on the Click XIon stationary phase (Fig. 2c). Contrastively, satisfactory separation quality was obtained on the XAmide column (Fig. 2d). Thus, this stationary phase was used in NPLC.

Given that RPLC has high separation efficiency and good compatibility with MS detection, this chromatographic mode was chosen as the second-dimension separation. And NPLC was adopted in the first-dimension. Accordingly, a 2D-NPLC×RPLC system was developed to separate bufadienolides, based on the XAmide and the XUnion C18 columns.

3.2 The orthogonality evaluation

In order to evaluate the orthogonality of the established 2D-LC system, fifteen bufadienolides (Fig. 1) purified from toad skin were used as a test mixture. As shown in Fig. 3, the XAmide and XUnion C18 columns displayed very different chromatographic retention for bufadienolides, indicating the good orthogonality of this 2D-LC system. Interestingly, it was noticed that 2D-LC system allowed to separate co-eluted bufadienolides in one-dimension HPLC. For example, compounds 10 and 11, co-eluted in RPLC, were well separated in NPLC. Compounds 1 and 4 had close retention time in NPLC, but acquired satisfactory resolution in RPLC.

In addition, the orthogonality was also assessed using a geometric approach [34]. The details for data analysis were expounded in the section 2.3. Reportedly, the surface coverage was average 63% for a 2D separation space with the same peak capacity [37]. As a result, the orthogonality of the 2D-LC system was 49.6% (Fig. 4). This data confirmed the above result.

3.3 Analysis of bufadienolides in toad skin

The 2D-NPLC×RPLC system had been tested with reference bufadienolides. Nonetheless, the application of this 2D-LC system to the separation of complex sample needed further validation.

The amino acid conjugated bufadienolides in toad skin, such as bufatoxin, telocinobufatoxin and hellebritoxin, might cause irreversible adsorption in NPLC separation. Therefore it is necessary to separate this kind of bufadienolide from the sample. The sample pretreatment, including liquid-liquid extraction and SPE [38], was used to prepare toad skin sample. Subsequently, the first-dimension separation was performed on the XAmide column in NPLC (shown in the supporting information). Twelve fractions were collected according to the UV absorbance. The second-dimension separation was conducted on the XUnion C18 column.

The 2D-LC separation of the toad skin sample was shown in a three-dimensional projection chromatogram (Fig. 5). It resulted from each HPLC/UV chromatogram (300 nm) on the XUnion C18 column stacked side by side and viewed from a side perspective. The fractions on the XAmide column could be read from the x-axis from 1 to 12 (0 was the total sample used as reference), while their separation on the XUnion C18 was given on the y-axis. The heights of the peaks were determined by the UV absorbance. As can be seen in Fig. 5, much more peaks were observed in 2D-LC compared with that in the unidimensional HPLC.

Above all, the 2D-NPLC×RPLC system performed considerable orthogonality in the separation of bufadienolides, which would be beneficial to their analysis.

The established 2D-LC system was combined with MS to characterize bufadienolides in toad skin, with NPLC as the first-dimension and RPLC-MS/MS as the second-dimension. In total, 64 bufadienolides were identified by comparing the retention time and the MS fragments with the references and the literature [11, 17, 23, 37]. All of the data were listed in Table 1.

Fractions 2, 6, 8, 9, 12 were taken as example to illustrate the identification. Their chromatograms on the XAmide and XUnion C18 columns were presented in Fig. 6 and 7, respectively (other results shown in the supporting information).

The comparison results between Fig. 6 and 7 showed that the elution order in these chromatograms was different from each other, and much more peaks were observed on the XUnion C18 column compared to that on the XAmide column. The use of this 2D-LC method could facilitate the characterization of minor bufadienolides in the toad skin. Taking fraction 9 as an example, minor compounds 17, 21, 22, 24 in fraction 9 coeluted with major compound 36 on the XAmide column (Fig. 6D). This increased the difficulty of the identification. In the second-dimension separation, these minor compounds were separated from the main compound on the XUnion C18 column (Fig. 7b), benefiting from the good orthogonality of the 2D-LC system. As shown in Table 1, the molecular ion of peak 17 was at m/z 461.2506 ($[M + H]^+$). And in the MS^2 analysis, its molecular ion was fragmented to many product ions, such as m/z 365 and m/z 347, corresponding to $[M+H-HOAc-2H_2O]^+$ and $[M+H-HOAc-3H_2O]^+$, respectively. Comparing these results with the reference [37, 39], peak 17 was identified as monohydroxybufotalin. The molecular ion of peak 21

at m/z 401.2264 ($[M + H]^+$) was fragmented to m/z 383 ($[M+H-H_2O]^+$), 365 ($[M+H-2H_2O]^+$), 347 ($[M+H-3H_2O]^+$), 337 ($[M+H-2H_2O-CO]^+$), 319 ($[M+H-2H_2O-2CO]^+$), and 301 ($[M+H-3H_2O-2CO]^+$). Comparing above data with the reference [37], peak 21 was identified as resibufaginol. Using the similar method, peak 22 was identified as desacetylbufotalin. In comparison with the reference bufadienolide used in this work by retention time and MS data, peak 21 was confirmed to be resibufaginol and the peak 24 was identified as bufotalinin stereoisomer. Above phenomenon was also observed in many other fractions, and finally 33 minor bufadienolides were identified.

More importantly, this 2D-LC system solved the coelution problem of bufadienolide isomers in RPLC. For instance, peak 1 in fraction 12 and peak 2 in fraction 8 had very close retention time on the XUnion C18 column (Fig. 7c and e). And as can be seen in Table 1, these two peaks possessed the same molecular ions at m/z 417 ($[M+H]^+$) and fragment ion at m/z 399 ($[M+H-H_2O]^+$). If solely analyzed these two compounds with RPLC-MS, only one compound could be detected. However, after the first-dimension separation on the XAmide column (Fig. 6c and e), peaks 1 and 2 were separated into two fractions. And they were detected by RPLC-MS. According to the reference [40], peaks 1 and 2 were tentatively identified as hellebrigenol-9,11-ene and its isomer. Peak 57 and 58 also suffered from the coelution problem in RPLC (Fig. 7a and b). Based on this 2D-LC method (Fig. 6a and b), these two peaks were differentiated and characterized as a pair of cinobufagin isomers. By comparing the data in Table 1, it was found that 11 pairs of

bufadienolide isomers, almost coeluted in RPLC, were well separated by means of the 2D-LC method.

4. Conclusion

In this work, an off-line 2D-NPLC×RPLC method was successfully developed for the separation and characterization of bufadienolides in toad skin. After column evaluation, XUnion C18 and XAmide columns were selected to separate bufadienolide in RPLC and NPLC, respectively. The geometric approach showed that the orthogonality of the 2D-NPLC×RPLC system was 49.5%. Combining this 2D-LC method with QTOF-MS/MS, 64 bufadienolides, including 33 minor ones and 11 pairs of isomers, were identified in toad skin. The 2D-NPLC×RPLC method not only contributed to the characterization of minor bufadienolides, but also solved the co-elution problem of bufadienolide isomers in RPLC. All of these results indicated that the off-line 2D-NPLC×RPLC method was available to realize the comprehensive analysis of bufadienolides in toad skin. And it appeared to be a potent candidate for the purification of bufadienolides from natural products.

References

- [1] K.Q. Han, G. Huang, W. Gu, Y.H. Su, X.Q. Huang, C.Q. Ling, Anti-tumor activities and apoptosis-regulated mechanisms of bufalin on the orthotopic transplantation tumor model of human hepatocellular carcinoma in nude mice, *World Journal of Gastroenterology*, 13 (2007) 3374-3379.
- [2] T. Huang, W.H. Gong, X.C. Li, C.P. Zou, G.J. Jiang, X.H. Li, H. Qian, Efficient killing effect of osteosarcoma cells by cinobufacini and cisplatin in combination, *Asian Pacific Journal of Cancer Prevention*, 13 (2012) 2847-2851.
- [3] Y. Kamano, A. Yamashita, T. Nogawa, H. Morita, K. Takeya, H. Itokawa, T. Segawa, A. Yukita, K. Saito, M. Katsuyama, G.R. Pettit, QSAR evaluation of the Ch'an Su and related bufadienolides against the colchicine-resistant primary liver carcinoma cell line PLC/PRF/51, *Journal of Medicinal Chemistry*, 45 (2002) 5440-5447.
- [4] W. Schoner, G. Scheiner-Bobis, Endogenous and exogenous cardiac glycosides: their roles in hypertension, salt metabolism, and cell growth, *American Journal of Physiology-Cell Physiology*, 293 (2007) C509-C536.
- [5] I. Prassas, E.P. Diamandis, Novel therapeutic applications of cardiac glycosides, *Nature Reviews Drug Discovery*, 7 (2008) 926-935.
- [6] D.L. Lichtstein, M. Steinitz, I. Gati, S. Samuelov, J. Deutsch, J. Orly, Biosynthesis of digitalis-like compounds in rat adrenal cells: Hydroxycholesterol as possible precursor, *Life Sciences*, 62 (1998) 2109-2126.
- [7] J.M. Hamlyn, Biosynthesis of endogenous cardiac glycosides by mammalian adrenocortical cells: Three steps forward, *Clinical Chemistry*, 50 (2004) 469-470.
- [8] P. Terness, D. Navolan, C. Dufter, B. Kopp, G. Opelz, The T-cell suppressive effect of bufadienolides: structural requirements for their immunoregulatory activity, *International Immunopharmacology*, 1 (2001) 119-134.
- [9] G.A. Cunha Filho, C.A. Schwartz, I.S. Resck, M.M. Murta, S.S. Lemos, M.S. Castro, C. Kyaw, O.R. Pires, Jr., J.R.S. Leite, C. Bloch, Jr., E.F. Schwartz, Antimicrobial activity of the bufadienolides marinobufagin and telocinobufagin isolated as major components from skin secretion of the toad *Bufo rubescens*, *Toxicon : official journal of the International Society on*

Toxinology, 45 (2005) 777-782.

[10] L.M.Y. Banuls, E. Urban, M. Gelbcke, F. Dufrasne, B. Kopp, R. Kiss, M. Zehl, Structure-Activity Relationship Analysis of Bufadienolide-Induced in Vitro Growth Inhibitory Effects on Mouse and Human Cancer Cells, *Journal of Natural Products*, 76 (2013) 1078-1084.

[11] K. Alevizopoulos, T. Calogeropoulou, F. Lang, C. Stournaras, Na⁺/K⁺ ATPase Inhibitors in Cancer, *Current Drug Targets*, 15 (2014) 988-1000.

[12] K. Shimada, Y. Fujii, E. Yamashita, Y. Niizaki, Y. Sato, T. Nambara, Studies on steroids. 119. Studies on cardiotoxic steroids from skin of Japanese toad *Chemical & Pharmaceutical Bulletin*, 25 (1977) 714-730.

[13] K. Shimada, N. Ishii, T. Nambara, Studies on steroids. 222. Occurrence of bufadienolides in the skin of *Bufo viridis* *Chemical & Pharmaceutical Bulletin*, 34 (1986) 3454-3457.

[14] K. Shimada, J.S. Ro, C. Kanno, T. Nambara, Studies on steroids . 235. Occurrence of bufogenin conjugates in the skin of Korean toad *Chemical & Pharmaceutical Bulletin*, 35 (1987) 4996-4999.

[15] Y. Kamano, T. Nogawa, A. Yamashita, M. Hayashi, M. Inoue, P. Drasar, G.R. Pettit, Isolation and structure of a 20,21-epoxybufenolide series from "Ch'an Su", *Journal of Natural Products*, 65 (2002) 1001-1005.

[16] T. Nogawa, Y. Kamano, A. Yamashita, G.R. Pettit, Isolation and structure of five new cancer cell growth inhibitory bufadienolides from the Chinese traditional drug Ch'an Su, *Journal of Natural Products*, 64 (2001) 1148-1152.

[17] L. Qiao, D.W. Juan, Y. Yao, C. Huan, L. Tao, P.Y. Hu, Isolation and identification of bufadienolides from traditional Chinese drug *Venenum Bufonis*, *Journal of Shenyang Pharmaceutical University*, 24 (2007) 611-614.

[18] X.C. Ma, B.J. Zhang, X.L. Xin, S.S. Huang, S. Deng, H.L. Zhang, X.H. Shu, Y.P. Diao, J. Cui, Simultaneous quantification of seven major bufadienolides in three Traditional Chinese Medicinal Preparations of ChanSu by HPLC-DAD, *Natural Product Communications*, 4 (2009) 179-184.

[19] F. Zhang, J.S. Wang, Y.C. Gu, L.Y. Kong, Cytotoxic and Anti-inflammatory Triterpenoids from *Toona ciliata*, *Journal of Natural Products*, 75 (2012) 538-546.

[20] J.X. Chen, J.W. Deng, H. Chen, H.Y. Tian, Y.Y. Yang, W.C. Ye, Identification of

Bufadienolides and Its Metabolites in Rat Plasma by High Performance Liquid Chromatography Coupled with Linear Trap Quadrupole-Orbitrap Mass Spectrometry, *Chinese Journal of Analytical Chemistry*, 39 (2011) 1858-1863.

[21] Y.M. Hu, Z.L. Yu, Z.J. Yang, G.Y. Zhu, W.F. Fong, Comprehensive chemical analysis of Venenum Bufonis by using liquid chromatography/electrospray ionization tandem mass spectrometry, *Journal of Pharmaceutical and Biomedical Analysis*, 56 (2011) 210-220.

[22] H.M. Gao, M. Zehl, A. Leitner, X.Y. Wu, Z.M. Wang, B. Kopp, Comparison of toad venoms from different Bufo species by HPLC and LC-DAD-MS/MS, *Journal of Ethnopharmacology*, 131 (2010) 368-376.

[23] M. Ye, D.A. Guo, Analysis of bufadienolides in the Chinese drug ChanSu by high-performance liquid chromatography with atmospheric pressure chemical ionization tandem mass spectrometry, *Rapid Communications in Mass Spectrometry*, 19 (2005) 1881-1892.

[24] Y.F. Liu, Y.S. Xiao, X.Y. Xue, X.L. Zhang, X.M. Liang, Systematic screening and characterization of novel bufadienolides from toad skin using ultra-performance liquid chromatography/electrospray ionization quadrupole time-of-flight mass spectrometry, *Rapid Communications in Mass Spectrometry*, 24 (2010) 667-678.

[25] M. Vollmer, P. Horth, E. Nagele, Optimization of two-dimensional off-line LC/MS separations to improve resolution of complex proteomic samples, *Analytical Chemistry*, 76 (2004) 5180-5185.

[26] X. Guo, X. Zhang, J. Feng, Z. Guo, Y. Xiao, X. Liang, Purification of saponins from leaves of *Panax notoginseng* using preparative two-dimensional reversed-phase liquid chromatography/hydrophilic interaction chromatography, *Analytical and Bioanalytical Chemistry*, 405 (2013) 3413-3421.

[27] H. Jin, Y. Liu, J. Feng, Z. Guo, C. Wang, Z. Zhong, X. Peng, J. Dang, Y. Tao, X. Liang, Efficient purification of high-purity compounds from the stem of *Lonicera japonica* Thunb using two-dimensional preparative chromatography, *Journal of Separation Science*, 36 (2013) 2414-2420.

[28] X. Li, R. Huang, Y. Liu, H. Jin, H. Wan, J. Zhao, W. Zhao, X. Liang, Efficient purification of low molecular weight nitrogen polar compounds from the skin of *Bufo bufo* gargarizans Cantor by

reversed-phase high performance liquid chromatography with a polar-copolymerized C18 stationary phase, *Analytical Methods*, 6 (2014) 5183-5190.

[29] Y.F. Liu, J.T. Feng, Y.S. Xiao, Z.M. Guo, J. Zhang, X.Y. Xue, J. Ding, X.L. Zhang, X.M. Liang, Purification of active bufadienolides from toad skin by preparative reversed-phase liquid chromatography coupled with hydrophilic interaction chromatography, *Journal of Separation Science*, 33 (2010) 1487-1494.

[30] X.L. Li, Z.M. Guo, C.R. Wang, A.J. Shen, Y.F. Liu, X.L. Zhang, W.J. Zhao, X.M. Liang, Purification of bufadienolides from the skin of *Bufo bufo gargarizans* Cantor with positively charged C18 column, *Journal of Pharmaceutical and Biomedical Analysis*, 92 (2014) 105-113.

[31] D.R. Stoll, X. Li, X. Wang, P.W. Carr, S.E.G. Porter, S.C. Rutan, Fast, comprehensive two-dimensional liquid chromatography, *Journal of Chromatography A*, 1168 (2007) 3-43.

[32] P. Jandera, Column selectivity for two-dimensional liquid chromatography, *Journal of Separation Science*, 29 (2006) 1763-1783.

[33] K.Y. Li, W.Y. Zhu, Q. Fu, Y.X. Ke, Y. Jin, X.M. Liang, Purification of amide alkaloids from *Piper longum* L. using preparative two-dimensional normal-phase liquid chromatography x reversed-phase liquid chromatography, *Analyst*, 138 (2013) 3313-3320.

[34] M. Gilar, P. Olivova, A.E. Daly, J.C. Gebler, Orthogonality of separation in two-dimensional liquid chromatography, *Analytical Chemistry*, 77 (2005) 6426-6434.

[35] P. Dugo, V. Skerikova, T. Kumm, A. Trozzi, P. Jandera, L. Mondello, Elucidation of carotenoid patterns in citrus products by means of comprehensive normal-phase x reversed-phase liquid chromatography, *Analytical Chemistry*, 78 (2006) 7743-7750.

[36] P. Jandera, J. Fischer, H. Lahovska, K. Novotna, P. Cesla, L. Kolarova, Two-dimensional liquid chromatography normal-phase and reversed-phase separation of (co)oligomers, *Journal of Chromatography A*, 1119 (2006) 3-10.

[37] J.M. Davis, Statistical-overlap theory for elliptical zones of high aspect ratio in comprehensive two-dimensional separations, *Journal of Separation Science*, 28 (2005) 347-359.

[38] X.L. Li, Y.F. Liu, A.J. Shen, C.R. Wang, J.Y. Yan, W.J. Zhao, X.M. Liang, Efficient purification of active bufadienolides by a class separation method based on hydrophilic solid-phase extraction and reversed-phase high performance liquid chromatography, *Journal of*

Pharmaceutical and Biomedical Analysis, 97 (2014) 54-64.

[39] H. Linde, P. Hofer, K. Meyer, Cinobufaginol - Uber krotengifte. 32, Helvetica Chimica Acta, 49 (1966) 1243-&.

[40] J.Q. Liu, N. Si, J. Yang, H.Y. Zhao, B.L. Bian, H.J. Wang, Identification of bufadienolides profiling in cinobufacino by HPLC-DAD-FT-ICR-MS method, Acta Pharmaceutica Sinica, 49 (2014) 244-248.

[41] Y. Kamano, P. Drasar, G.R. Pettit, M. Tozawa, Bufadienolides and steroids and related natural-products. 39. Synthesis of 5- α -cinobufagin, Collection of Czechoslovak Chemical Communications, 52 (1987) 1325-1330.

[42] Y. Kamano, A. Kotake, H. Hashima, M. Inoue, H. Morita, K. Takeya, H. Itokawa, N. Nandachi, T. Segawa, A. Yukita, K. Saitou, M. Katsuyama, G.R. Pettit, Structure-cytotoxic activity relationship for the toad poison bufadienolides, Bioorganic & Medicinal Chemistry, 6 (1998) 1103-1115.

Acknowledgement

This work was supported by “Project of National Science Foundation of China (21305138)” and the External Cooperation Program of BIC, Chinese Academy of Science (No. 121421KYSB20130013). We also appreciate the supporting from Key Projects in the National Science & Technology Pillar Program in the twelfth Five-year Plan (2012BAI29B08)

Figure captions

Fig. 1 The chemical structures of the reference bufadienolides. * used for the development of 2D-LC system.

Fig. 2 The chromatograms of five bufadienolides on an XUnion C18 (a) and Unitary Diol (b), Click XIon (c), and XAmide (d). 1: bufarenogin; 2: telocinobufagin; 3: desacetylcinobufagin; 4: bufotalin; 5: 3-dehydrobufagin. **Conditions on the XUnion C18 columns: mobile phase A: acetonitrile and B: water; Gradient elution steps were as follow: 0-10 min, 10%-30% A; 10-30 min, 30%-45% A; 30-40 min, 45%-60% A; Conditions on the NP columns: the mobile phase A: isopropanol and B: n-hexane; Gradient elution steps were as follow: 0-10 min, 10%-30% A; 10-30 min, 30%-50% A. Flow rate: 1.0 mL/min; Column temperature: 30 °C; Detection: 300 nm.**

Fig. 3 The chromatograms of reference bufadienolides on the XUnion C18 (a) and XAmide (b) columns. **Conditions on the XUnion C18 column (150 mm × 4.6 mm, I. D., 5 μm): the mobile phases A: acetonitrile and B: water; Gradient elution steps were as follow: 0-10 min, 10%-30% A; 10-30 min, 30%-45% A; 30-40 min, 45%-60% A; Conditions on the XAmide (150 mm × 4.6 mm, I. D., 5 μm); The mobile phase A: isopropanol and B: n-hexane; Gradient elution steps were as follow: 0-50 min, 10%-60% A. Other conditions were same as those in Fig. 2.**

Fig. 4 Normalized retention time plots for the 2D-NPLC×RPLC system.

Fig. 5 Analysis of fractions 1-12 on the XUnion C18 column (150 mm × 4.6 mm, I. D., 5 μm). **Conditions: the mobile phase A: acetonitrile and B: water; Gradient elution steps were as follow: 0-10 min, 10%-30% A; 10-30 min, 30%-50% A; 30-35 min, 80%-100% A. Other conditions were same as those in Fig.2.**

Fig. 6 The chromatograms of fractions 2 (a), 6 (b), 8 (c), 9 (d), and 12 (e) on the XAmide column (150 mm × 4.6 mm, I. D., 5 μm). **Conditions: the mobile phase A:**

isopropanol and B: n-hexane; Gradient elution steps were as follow: 0-50 min, 10%-60% A. Other conditions were same as those in Fig. 2.

Fig. 7 The chromatograms of fractions 2 (a), 6 (b), 8 (c), 9 (d), and 12 (e) on the XUnion C18 column (150 mm $\times$ 4.6 mm, I. D., 5 μ m). Conditions: the mobile phase A: acetonitrile and B: water; Gradient elution steps were as follow: 0-10 min, 10%-30% A; 10-30 min, 30%-50% A; 30-35 min, 80%-100% A. Other conditions were same as those in Fig.2.

- * 1. Bufarenogin: $R_1 = H$ $R_2 = O$ $R_3 = H$ $R_4 = \beta-OH$
- 3. Desacetylbufotalin: $R_1 = H$ $R_2 = H$ $R_3 = \beta-OH$ $R_4 = H$
- * 5. Telocinobufagin: $R_1 = OH$ $R_2 = H$ $R_3 = H$ $R_4 = H$
- * 8. Bufotalin: $R_1 = H$ $R_2 = H$ $R_3 = \beta-OAc$ $R_4 = H$
- 11. Bufalin: $R_1 = H$ $R_2 = H$ $R_3 = H$ $R_4 = H$

- 2. Desacetylcinobufotalin: $R_1 = OH$ $R_2 = OH$ $R_3 = \beta-OH$
- * 6. Desacetylcinobufagin: $R_1 = OH$ $R_2 = H$ $R_3 = \beta-OH$
- 10. Marinobufagin: $R_1 = OH$ $R_2 = OH$ $R_3 = H$
- 12. 3-keto-Desacetylcinobufagin: $R_1 = O$ $R_2 = H$ $R_3 = \beta-OH$
- 14. Cinobufagin: $R_1 = OH$ $R_2 = H$ $R_3 = \beta-OAc$
- 15. Resibufogenin: $R_1 = OH$ $R_2 = H$ $R_3 = H$

4. Argentinogenin

* 9. 3-Dehydrobufagin

Fig. 1

Fig. 2

Fig. 3

Fig. 4

Fig. 5

Fig. 6

Fig. 7

Table 1 Characterization of bufadienolides in toad skin by NPLC/RPLC-MS/MS

No.	Frac. in NPLC	Rt in RPLC (min)	[M+H] ⁺	Formula	MS/MS										Identification	
1	F-12	8.700	417.2262	C ₂₄ H ₃₂ O ₆	417(100)	399(20)										Hellebrigenol-9,11-ene [40]
2	F-8	8.730	417.2238	C ₂₄ H ₃₂ O ₆	417(100)	399(24)										Hellebrigenol-9,11-enestereoisomer
3	F-11	9.199	403.2465	C ₂₄ H ₃₄ O ₅	403(32)	385(14)	367(6)	349(9)		253(11)		145(48)		105(100)		Gamabufotalin [23]
4	F-11	9.673	417.2252	C ₂₄ H ₃₂ O ₆	417(100)	399(29)										Ψ Bufarenogin [23]
5	F-4	10.266	415.2086	C ₂₄ H ₃₀ O ₆	415(100)	397(7)	381(6)	361(10)	351(7)	333(12)	253(7)					19- <i>oxo</i> -Desacetylcinobufagin [40]
6	F-4	10.363	403.2442	C ₂₄ H ₃₄ O ₅	403(47)	385(15)	367(7)	349(19)			253(29)		145(62)		105(100)	12β-Hydroxyl-Bufalin [40]
7	F-10	10.436	419.2388	C ₂₄ H ₃₄ O ₆	419(25)	401(7)		371(6)	365(7)	353(14)	337(7)	305(6)	251(12)		91(100)	Hellebrigenol [40]
8	F-11	10.855	417.2256	C ₂₄ H ₃₂ O ₆	417(100)	399(30)		363(8)	353(5)	335(7)						Bufarenogin stereoisomer
9	F-7	11.226	417.2253	C ₂₄ H ₃₂ O ₆	417(100)	399(34)		371(6)	353(5)	335(8)	251(5)					Bufarenogin stereoisomer
10	F-4	11.415	401.2330	C ₂₄ H ₃₂ O ₅	401(54)	383(26)	365(18)	347(8)	319(7)	269(6)	251(12)					Resibufaginol [23]
11	F-8	11.426	417.2247	C ₂₄ H ₃₂ O ₆	417(100)	399(47)				335(6)						Bufarenogin
12	F-11	11.562	403.2457	C ₂₄ H ₃₄ O ₅	403(11)	385(7)	367(5)	349(11)			253(10)		145(46)	105(67)	91(100)	12β/1β Hydroxy Bufalin [23]
13	F-10	11.572	417.2234	C ₂₄ H ₃₂ O ₆	417(30)	399(9)		363(14)	353(6)	335(21)	317(8)	289(5)			91(100)	Arenobufagin [23]
14	F-5	11.830	417.2280	C ₂₄ H ₃₂ O ₆	417(55)		381(5)	363(12)			255(8)				81(100)	Hellebrigenin [23]
15	F-4	11.850	415.2042	C ₂₄ H ₃₀ O ₆	415(100)	397(29)			351(9)	333(8)	255(14)					19- <i>oxo</i> -Desacetylcinobufagin [23]
16	F-12	11.873	417.2267	C ₂₄ H ₃₂ O ₆	417(53)		381(6)	363(7)		335(7)	255(10)	253(8)			81(100)	Desacetylcinobufotalin
17	F-9	12.340	461.2506	C ₂₆ H ₃₆ O ₇	461(15)			365(42)	347(63)	323(11)	267(13)	251(14)			81(100)	Monohydroxybufotalin [23]
18	F-7	12.516	389.2472	C ₂₃ H ₃₃ O ₅	389(29)	371(10)	353(12)	335(23)		319(7)	239(29)	213(24)			105(100)	*
19	F-10	12.603	459.2315	C ₂₆ H ₃₄ O ₇	459(8)		363(10)	345(7)	335(5)	333(5)	321(6)	317(7)	237(11)		91(100)	Cinobufaginol [39]
20	F-12	12.788	415.2081	C ₂₄ H ₃₀ O ₆	415(100)	397(5)	387(8)				323(6)	221(6)	215(8)	161(38)	91(59)	3- <i>keto</i> -Arenobufagin [20]
21	F-9	12.806	401.2264	C ₂₄ H ₃₂ O ₅	401(8)	383(6)	365(8)	347(14)	337(10)	319(5)	301(9)	253(10)	251(12)	213(9)	91(100)	Resibufaginol [23]

Table 1 Continued

No.	Frac. in NPLC	Rt in RPLC (min)	[M+H] ⁺	Formula	MS/MS										Identification	
22	F-9	13.039	403.2486	C ₂₄ H ₃₄ O ₅	403(0)	385(6)	367(10)	349(11)		253(11)	215(35)	151(73)		105(100)	Desacetylbufotalin	
23	F-6	13.195	403.2462	C ₂₄ H ₃₄ O ₅	403(26)	385(20)	367(10)	349(15)		253(19)	213(15)			105(100)	1β-Hydroxylbufalin [23]	
24	F-9	13.314	415.2090	C ₂₄ H ₃₀ O ₆	415(16)			361(9)	351(10)	333(7)	273(7)	237(23)		91(100)	Bufotalinin stereoisomer	
25	F-7	13.478	415.2095	C ₂₄ H ₃₀ O ₆	415(98)	397(36)	379(10)	361(10)	351(15)	333(8)	323(5)	315(7)		131(100)	Bufotalinin stereoisomer	
26	F-10	13.549	459.2322	C ₂₆ H ₃₄ O ₇	459(18)	424(9)	417(15)	381(23)	363(52)		345(16)	253(29)			Cinobufotalin stereoisomer [24]	
27	F-1	13.621	415.2083	C ₂₄ H ₃₀ O ₆	415(100)	397(45)	379(11)		351(19)	319(11)			187(15)	131(74)	91(55)	Bufotalinin stereoisomer
28	F-11	13.809	415.2021	C ₂₄ H ₃₀ O ₆	415(100)	397(16)		369(11)	351(8)	319(12)		237(29)				Bufotalinin [23]
29	F-8	14.066	401.2294	C ₂₄ H ₃₂ O ₅	401(23)		365(7)	347(15)	337(14)	253(6)						19-oxo-Bufalin [23]
30	F-10	14.074	473.2146	C ₂₆ H ₃₂ O ₈	473(31)	431(6)	395(13)	377(11)	367(6)	359(8)	349(18)		199(70)	143(81)	91(100)	19-oxo-Cinobufotalin [24]
31	F-6	14.159	415.2127	C ₂₄ H ₃₀ O ₆	415(95)	397(76)	379(8)	361(6)	351(11)	333(18)	323(10)	305(11)	271(17)	253(19)	105(100)	Argentinogenin
32	F-12	14.363	447.2350	C ₂₆ H ₃₈ O ₆	447(100)	429(15)	415(77)	411(10)	397(5)	387(20)	383(6)	379(9)	361(6)	333(10)	275(42)	BN ₄ [24]
33	F-6	14.791	403.2459	C ₂₄ H ₃₄ O ₅	403(33)	385(15)	367(17)	349(25)	271(7)		253(16)	213(16)			105(100)	Telocinobufagin
34	F-12	15.428	397.1950	C ₂₄ H ₂₈ O ₅	397(29)		361(6)	333(6)			261(5)	257(6)			97(100)	3-keto-resibufagin
35	F-10	15.499	403.2463	C ₂₄ H ₃₄ O ₅	403(8)		367(14)	349(20)			253(15)	215(37)	151(92)		105(100)	Telocinobufagin stereoisomer
36	F-9	15.589	401.2314	C ₂₄ H ₃₂ O ₅	401(30)		365(6)								105(100)	Desacetylcinobufagin
37	F-11	15.659	401.2314	C ₂₄ H ₃₂ O ₅	401(25)		365(7)			319(5)	253(9)					11-Hydroxyl resibufogenin [21]
38	F-2	15.688	401.2303	C ₂₄ H ₃₂ O ₅	401(31)		365(6)				213(6)				105(100)	Monohydroxyl resibufogenin
39	F-6	15.822	445.2519	C ₂₆ H ₃₆ O ₆	445(8)		385(8)	367(20)	349(36)		255(7)	253(8)			105(100)	Bufotalin stereoisomer [24]
40	F-5	16.633	403.2468	C ₂₄ H ₃₄ O ₅	403(40)	385(33)	367(22)	349(16)	321(9)	271(20)	253(14)				91(100)	Telocinobufagin stereoisomer
41	F-6	16.906	399.2112	C ₂₄ H ₃₀ O ₅	399(9)		363(6)	353(5)	335(7)	267(7)	257(12)	249(15)			91(100)	9-19 Resibufagin [23]
42	F-7	17.281	457.2149	C ₂₆ H ₃₂ O ₇	457(17)	439(5)	415(6)	397(9)	379(6)	361(8)	351(13)	319(14)	315(11)	267(18)	91(100)	19-oxo-Cinobufagin [23]
43	F-4	17.329	401.2398	C ₂₄ H ₃₂ O ₅	401(12)	383	365(12)	347(8)	339(6)	337(5)	319(5)	269(5)	253(15)			22,23-Epoxyresibufogenin [21]

Table 1 Continued

No.	Frac. in NPLC	Rt in RPLC (min)	[M+H] ⁺	Formula	MS/MS										Identification
44	F-5	17.604	459.2357	C ₂₆ H ₃₄ O ₇	459(17)	417(8)	381(15)	363(30)	345(8)	339(7)	335(10)				Cinobufotalin [23]
45	F-7	18.088	445.2487	C ₂₆ H ₃₆ O ₆	445(7)		385(6)	367(13)	349(61)	267(18)			131(100)		Bufotalin
46	F-4	18.792	385.2367	C ₂₄ H ₃₂ O ₄	385(0)	367(5)	349(9)				253(20)				3- <i>keto</i> -Bufalin stereoisomer
47	F-4	19.214	383.2250	C ₂₄ H ₃₀ O ₄	383(9)	365(9)	347(5)				269(6)	213(5)	97(100)		3-Dehydrobufagin
48	F-10	19.418	401.2305	C ₂₄ H ₃₂ O ₅	401(33)		365(6)	347(7)	337(6)			253(7)			Marinobufagin
49	F-1	19.563	443.2332	C ₂₆ H ₃₄ O ₆	443(8)		383(10)	365(26)	347(33)	337(7)	319(22)	305(10)	233(20)	131(100)	3- <i>keto</i> -Bufotalin [20]
50	F-3	19.793	387.2517	C ₂₄ H ₃₄ O ₄	387(21)	369(15)	351(11)				255(17)		105(100)		Bufalin
51	F-4	19.961	387.2514	C ₂₄ H ₃₄ O ₄	387(19)	369(16)	351(12)	333(6)			255(28)		105(100)		3- <i>epi</i> Bufalin [40]
52	F-10	20.165	399.2149	C ₂₄ H ₃₀ O ₅	399(64)		363(10)		335(7)	293(10)	253(12)		105(100)		3- <i>keto</i> -Deacetylcinobufagin
53	F-6	21.271	385.2375	C ₂₄ H ₃₂ O ₄	385(0)	367(9)	349(8)	321(5)			253(25)	213(8)			3- <i>keto</i> -Bufalin [20]
54	F-2	22.534	385.2343	C ₂₄ H ₃₂ O ₄	385(37)	367(29)	349(13)	321(6)					105(100)		15-21
55	F-5	23.454	439.2156	C ₂₆ H ₃₀ O ₆	439(5)	397(16)	379(7)	361(7)		319(5)	271(6)		159(100)		3-dehydrocinobufagin [41]
56	F-3	23.514	381.2067	C ₂₄ H ₂₈ O ₄	381(13)	363(6)				269(5)	251(5)		97(100)		Δ-3-oxoresibufogenin [42]
57	F-6	24.117	443.2381	C ₂₆ H ₃₄ O ₆	443(10)	401(12)	383(6)	365(21)	347(9)	319(5)	251(6)	215(35)	105(100)		Cinobufagin isomer [24]
58	F-2	24.187	443.2388	C ₂₆ H ₃₄ O ₆	443(5)	401(5)		365(9)	347(6)			215(21)	213(6)	105(100)	Cinobufagin
59	F-1	24.336	385.2336	C ₂₄ H ₃₂ O ₄	385(7)	367(8)	349(6)				253(8)		105(100)		Resibufogenin
60	F-2	26.176	385.2351	C ₂₄ H ₃₂ O ₄	385(6)	367(8)	349(6)				253(21)	213(5)	105(100)		3- <i>epi</i> -Resibufogenin [40]
61	F-3	27.387	443.2405	C ₂₆ H ₃₄ O ₆	443(5)	401(7)		365(9)	347(6)		251(6)	215(35)	105(100)		3- <i>epi</i> -Cinobufagin [40]
62	F-8	28.255	387.1919	C ₂₄ H ₃₄ O ₄	387(0)			331(10)	275(9)	231(5)			175(100)		Bufalin stereoisomer
63	F-2	28.331	387.1938	C ₂₄ H ₃₄ O ₄	387(0)			331(10)	275(6)	231(6)			175(100)		Bufalin stereoisomer
64	F-3	29.592	441.2265	C ₂₆ H ₃₂ O ₆	441(11)	399(22)	381(13)	363(12)	335(8)	319(5)	305(9)	253(6)	107(100)		3- <i>keto</i> -Cinobufagin [40]

* Could not be identified