Cardioactive C₁₉-Diterpenoid Alkaloids from the Lateral Roots of *Aconitum carmichaeli* “Fu Zi”

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Bioassay-guided fractionation of an *n*-BuOH extract of the lateral roots of *Aconitum carmichaeli* DEBX. led to the isolation of 5 cardioactive C₁₉-diterpenoid alkaloids: *N*-deethylaconine (1), beiwutinine (2), hypaconine (3), mesaconine (4), and 15 α -hydroxyneoline (5). *N*-Deethylaconine and beiwutinine are new aconitine-type C₁₉-diterpenoid alkaloids. Hypaconine was isolated from this species for the first time. Among them, mesaconine, hypaconine, and beiwutinine showed the strongest cardiac actions on the isolated perfused bullfrog heart. Furthermore, mesaconine has protective effects, including improved inotropic effect and left ventricular diastolic function, on myocardial ischemia-reperfusion injury in rat at a dose of 10⁻⁹ mol/L. However, mesaconine has almost no effect on heart rate.

Key words *Aconitum carmichaeli*; cardioactive constituent; Fu Zi; *N*-deethylaconine; beiwutinine

Fu Zi, a distinguished traditional Chinese medicine, is the lateral roots of *Aconitum carmichaeli* DEBX. (Ranunculaceae). The species, originally growing in the district of Jiangyou in Sichuan province of China, has been clinically used for more than two thousand years. *Aconitum carmichaeli* DEBX. has now been widely grown in Hanzhong of Shangxi Province, LiJiang of Yunnan Province, etc. in addition to the district of Jiangyou. Furthermore, other Eastern Asian countries, such as Japan and Korea, also grow *Aconitum carmichaeli* DEBX. for the clinical use as traditional medicine. Fu Zi, as an critical emergency drug in traditional Chinese medicines, is used to treat heart failure.¹⁾ The mother roots of the same plant (*A. carmichaeli* DEBX.), designated as “Chuan Wu,” is also used in traditional Chinese medicine for the clinical treatment of pains and rheumatics.²⁾

For several decades, researchers paid more attention to the cardioactive components of the lateral roots of *A. carmichaeli*. Chinese and Japanese scholars^{3–8)} have independently verified that Fu Zi has indeed cardiac effects. In 1976, Japanese scholar Kosuge *et al.*⁹⁾ isolated a trace amount of cardioactive component, DL-demethylcoclaurine, from the roots of Japanese *A. japonicum* THUM, which are of interest to a lot of scientists. Three years later, another Japanese scholar Konno and his co-workers¹⁰⁾ isolated a water-soluble component coryneine chloride, which can improve blood pressure, from Japanese “Bushu” (*A. carmichaeli*). Afterwards, Chen and Liang¹¹⁾ obtained a weak cardioactive component, salsoline, from Fu Zi. During the period of 1975–1980, extensive investigation of the synthesis^{12,13)} and clinical study¹⁴⁾ of DL-demethylcoclaurine were carried out by Chinese scientists. It was concluded that DL-demethylcoclaurine was suitable to the clinical treatment for chronic arrhythmia and low cardiac output syndrome,¹⁴⁾ and could be used as a diagnostic agent for coronary heart disease.¹³⁾ During the 10 years after this, there was almost no report on the investigation of the cardioactive components of Fu Zi until Han *et al.*^{15,16)} and Xu *et al.*^{17,18)} isolated the

cardioactive components, uracil and fuzinoside, from Fu Zi, respectively. In addition to the above-mentioned cardioactive compounds, numerous of the components including the diterpenoid alkaloids have also been isolated from the lateral roots of *Aconitum carmichaeli*^{19–46)}. However, no cardiac effect of these compounds has been reported. In this paper, we used an isolated bullfrog heart testing to guide the fractionation of water-soluble constituents of the lateral roots of *Aconitum carmichaeli*, leading to the isolation of five cardioactive C₁₉-diterpenoid alkaloids: *N*-deethylaconine (1), beiwutinine (2), hypaconine (3), mesaconine (4), and 15 α -hydroxyneoline (5). Of them, *N*-deethylaconine and beiwutinine are new aconitine-type C₁₉-diterpenoid alkaloids. We wish herein to report the isolation, structure elucidation, and cardiac effects of these alkaloids.

Results and Discussion

The fractions A, B, C were obtained from the lateral roots of *Aconitum carmichaeli* (22 kg) by sequential reflux with hot water, precipitation with 75% ethanol, and extraction with diethyl ether and *n*-butanol after basifying. Among them, only *n*-butanol extract (fraction B) exhibited significant cardiac actions on the isolated bullfrog hearts. Consequently, fraction B was subjected to repeated column chromatography over silica gel H (CHCl₃–MeOH), leading to the isolation of *N*-deethylaconine (1), beiwutinine (2), hypaconine (3), mesaconine (4), and 15 α -hydroxyneoline (5).

N-Deethylaconine (1): white amorphous powder, $[\alpha]_D^{20} + 25.4$ ($c = 0.61$, MeOH). Its molecular formula was determined as C₂₃H₃₇NO₉ by the ¹³C-NMR spectrum and high resolution-electrospray ionization-mass spectra (HR-ESI-MS) data (Found: m/z 472.2549 [M+H]⁺, Calcd: m/z 472.2547 [M+H]⁺). The IR spectrum showed an absorbance band at 3357 cm⁻¹ for hydroxyl groups. The ¹H-NMR spectrum showed the presence of four methoxyl groups (δ 3.34, 3.39, 3.43, 3.64, each 3H, s). The ¹³C-NMR spectrum suggested the existence of nine oxygenated carbons (δ 91.1 d, 81.7 d, 80.2 d, 79.9 d, 78.2 d, 77.9 s, 76.8 t, 76.0 s, 69.1 d), indicating the presence of five hydroxyl groups in addition to four methoxyl groups. The

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Fig. 1. Key $^1H-^1H$ COSY, HMBC, and NOESY Correlations of Compound 1

above-mentioned evidence, in combination with the biogenetic consideration, suggested this compound to be a C_{19} -diterpenoid alkaloid.⁴⁷⁾ On the other hand, a doublet signal at δ_H 3.89 ($J=4.1$ Hz) was attributed to H-14 β .⁴⁷⁾ A one-proton doublet signal at δ_H 4.18 ($J=3.9$ Hz) was assigned to H-3 β based on the correlations between this signal and C-1 (δ 79.9 d) as well as C-18 (δ 76.8 t) in the heteronuclear multiple bond connectivity (HMBC) spectrum. The assignment of H-15 (δ_H 4.51, d, $J=6.0$ Hz) was based on the correlations of H-15 and C-8 (δ 77.9 s) and C-16 (δ 91.6 d) in the HMBC spectrum. Four methoxyl groups were located to C-1, C-6, C-16, and C-18, respectively, based on the correlations between δ_H 3.39/C-1 (δ_C 77.9 d), δ_H 3.43/C-6 (δ_C 81.7 d), δ_H 3.64/C-16 (δ_C 91.6 d), and δ_H 3.34/C-18 (δ_C 76.8 t) in the HMBC spectrum (Fig. 1). The α -orientation of H-3 was established according to the correlation between H-3 (δ 4.18) and H₂-18 (δ 3.51, 3.59) in its nuclear Overhauser effect spectroscopy (NOESY) spectrum (Fig. 1). Its Dreiding molecular modeling shows that the dihedral angles between H-16 α and H-15 β , and between H-16 α and H-15 α are around 45° and 75°, respectively (ring D possesses

boat conformation⁴⁷⁾). Theoretically, $J_{16\alpha-15\beta}$ and $J_{16\alpha-15\alpha}$ should be *ca.* 8 Hz and *ca.* 3 Hz. The observed value is 6.0 Hz, suggesting the α -orientation of 15-OH. All signals in the 1H (^{13}C) spectra of this compound (Table 1) were assigned based on its 2D-NMR spectra. Thus, the structure of *N*-deethylaconine was established as 1.

Beiwutinine (2): White amorphous powders, $[\alpha]_D^{20} +22.0$ ($c=0.51$, MeOH). Its molecular formula was established as $C_{24}H_{39}NO_{10}$ based on its ^{13}C -NMR spectrum and HR-MS data (Found m/z 502.2656 $[M+H]^+$, Calcd $C_{24}H_{40}NO_{10}$, 502.2652). Its structure was initially suggested to be 2 based on the comparison of its TLC behavior and electrospray ionization-mass spectra (ESI-MS) data with those of the authentic sample,^{48,49)} and was finally confirmed by the 2D-NMR spectra. All of the signals (Table 1) in its 1H - (^{13}C -) NMR spectra were assigned by the correlations in the 2D-NMR spectra.

Bioassay results exhibited that 4 C_{19} -diterpenoid alkaloids (1–4) have cardioactive effect on the isolated bullfrog hearts,⁵⁰⁾ while 15 α -ishydroxyneoline (5) only showed very short cardiac action (Table 2). Of them, hypaconine, mes-

Table 1. NMR Data of *N*-Deethylaconine (**1**) and Beiwutinine (**2**) [400 MHz for ^1H , δ_{H} mult. ($J=\text{Hz}$), 100 MHz for ^{13}C]

Position	1^{a)}		2^{b)}	
	δ_{H}	δ_{C}	δ_{H}	δ_{C}
1	3.54 m	79.9 d	4.16 d (6.4)	84.4 d
2	2.28 m 1.57 ddd (16.0, 3.9, 3.9)	29.0 t	2.32 m (α) 2.40 m (α)	42.8 t
3	4.18 d (3.9)	69.1 d	3.71 dd (12.0, 4.8)	70.6 d
4	—	43.2 s	—	44.4 s
5	2.42 m	42.3 d	2.35 (hidden)	42.4 d
6	4.31 d (8.0)	81.7 d	3.68 d (7.6)	84.4 d
7	2.94 s	40.5 d	2.02 m	35.0 d
8	—	77.9 s	—	78.1 s
9	2.28 (hidden)	40.4 d	2.09 d (5.2)	59.4 d
10	2.94 m	40.5 d	—	79.4 s
11	—	50.3 s	—	56.8 s
12	1.69 m 2.14 m	36.4 t	1.79 d (10.8) 2.97 (hidden)	42.9 t
13	—	76.0 s	—	77.7 s
14	3.89 d (4.1)	78.2 d	4.27 d (5.2)	78.4 d
15	4.51 d (6.0)	80.2 d	4.45 d (6.0)	83.2 d
16	3.08 d (6.0)	91.6 d	2.96 d (6.0)	92.6 d
17	3.26 s	66.9 d	2.97 s	64.6 d
18	3.51 m (hidden) 3.59 m (hidden)	76.8 t	3.82 ABq (8.4) 3.43 ABq (8.4)	75.8 t
19	3.40 m (hidden) 3.50 m (hidden)	51.5 t	2.52 ABq (10.8) 2.70 ABq (10.8)	51.1 t
NCH ₃	—	—	2.35 s	47.8 q
1-OCH ₃	3.39 s	54.1 q	3.33 s	58.3 q
6-OCH ₃	3.43 s	57.2 q	3.27 s	55.8 q
16-OCH ₃	3.64 s	60.4 q	3.57 s	61.5 q
18-OCH ₃	3.34 s	58.0 q	3.28 s	59.1 q
NH	hidden	—	—	—

^{a)} CD₃OD, ^{b)} CDCl₃.Table 2. Cardiac Effect of Compounds **1**—**5** and β -Strophatin-K on the Isolated Bullfrog Hearts

Compound	Amplitude (cm)		Amplitude increase (cm)	Rate of amplitude increase (%)	Average increase (%)	Result
	Before	After				
Control ^{a)}	1.6	1.6	0	0	0	
<i>N</i> -Deethylaconine (1) ^{b)}	1.6	2.1	0.5	31.0	28.0	+
	1.7	2.1	0.4	24.0		
Beiwutinine (2) ^{c)}	1.3	2.6	1.3	100.0	71.5	+++
	1.4	2.0	0.6	42.9		
Hypaconine (3) ^{c)}	1.8	3.8	2.0	111.0	118.0	+++
	1.7	3.8	2.1	124.0		
Mesaconine (4) ^{c)}	1.7	2.3	1.6	94.0	82.0	+++
	1.7	2.9	1.2	70.0		
15 α -Hydroxyneoline (5) ^{c,d)}	1.3	1.8	0.5	38.5	38.5	+ ^{e)}
	1.3	1.8	0.5	38.5		
β -Strophathin-K ^{e)}	1.3	5.4	4.1	315.4	284.6	++++
	1.3	4.6	3.3	253.8		

^{a)} Distillated water; ^{b)} 2.5 mg/mL; ^{c)} 5 mg/mL; ^{d)} the cardioactive duration of **5** is 10', while the cardioactive durations of other alkaloids can reach 30'; ^{e)} 0.25 mg/mL.

aconine, and beiwutinine have optimal cardiac actions. To observe the effects of these alkaloids on heart failure, hypaconine and mesaconine were selected for further study on myocardial ischemia-reperfusion injury in isolated rat hearts. In this study, the cardiac parameters of maximal rise rate of coronary flow, left ventricular pressure, left ventricular end-diastolic pressure, and heart rate were observed. It can be concluded from the observed results: 1) hypaconine and mes-

aconine has almost no effect on coronary flow and heart rate; 2) mesaconine exhibited significant effects on left ventricular pressure in 10—40 min relative to the control group (Table 3); 3) mesaconine showed significant effects on left ventricular end-diastolic pressure in 20—60 min relative to the control group, while hypaconine did not show significant effects (Table 4). The above-mentioned evidence indicated that mesaconine has protective effects, including improved inotropic

Table 3. Effect of Hypaconine (3) and Mesaconine (4) on Maximal Rise Rate of Left Ventricular Pressure ($dP/dt_{\max}$) (mmHg/s, $n=3$)

Group	Time						
	Before	10 min	20 min	30 min	40 min	50 min	60 min
Control	1011±75	728±49	891±139	890±56	947±93	920±88	920±111
Hypaconine (3)	985±79	856±93	958±138	953±227	1025±142	943±159	927±168
Mesaconine (4)	991±36	1142±88**	1214±98*	1217±86**	1258±61**	1157±124	1150±134
Deslanoside	982±79	992±62**	1237±128*	1286±297	1240±301	1127±239	1177±177

Relative to control group, * $p<0.05$, ** $p<0.01$.

Table 4. Effect of Hypaconine (3) and Mesaconine (4) on Left Ventricular-End Diastolic Pressure (LVEDP) (mmHg, $n=3$)

Group	Time						
	Before	10 min	20 min	30 min	40 min	50 min	60 min
Control	-5.5±1.6	-8.9±0.8	-10.6±1.5	-12.0±1.4	-13.2±1.7	-14.4±1.8	-15.7±2.7
Hypaconine (3)	-6.4±3.3	-10.2±3.1	-12.8±3.4	-15.3±3.4	-17.1±3.5	-18.6±3.0	-20.1±2.6
Mesaconine (4)	-5.5±0.4	-9.3±4.1	-14.2±1.5*	-16.8±1.3*	-19.2±0.7*	-21.1±0.7**	-22.8±1.6*
Deslanoside	-6.2±1.0	-10.3±1.6	-13.0±1.1	-16.0±0.6*	-18.3±0.6*	-20.3±0.4*	-22.5±0.8*

Relative to control group, * $p<0.05$, ** $p<0.01$.

effect and left ventricular diastolic function, on myocardial ischemia-reperfusion injury in rat at a dose of 10^{-9} mol/L.

Conclusion

It is known that numerous of diterpenoid alkaloids have already been isolated from the lateral roots of *Aconitum Carmichaeli*. However, none of them showed cardiac effects. In this paper, we firstly reported 5 cardioactive C_{19} -diterpenoid alkaloids. Among them, *N*-deethylaconine and beiwutinine are new diterpenoid alkaloids; while hypaconine was isolated for the first time from this species. All these compounds are soluble in water. *N*-Deethylaconine is not stable under acidic and basic conditions.

Bioassay results exhibited that 5 C_{19} -diterpenoid alkaloids (1–5) have cardioactive effect on the isolated bullfrog hearts and that hypaconine, mesaconine, and beiwutinine have optimal cardiac actions. 15 α -Hydroxyneoliine has a significant short duration of action relative to other alkaloids. We also observed that mesaconine has protective effects, including improved inotropic effect and left ventricular diastolic function, on myocardial ischemia-reperfusion injury in rat at a dose of 10^{-9} mol/L. This result is inconsistent with the results reported by Hirohisa,⁵¹⁾ in which mesaconine was reported to have no cardiac effect on both frog and toad hearts. This inconsistency might be caused by the different animal models and concentrations of the samples. In literature⁵¹⁾ the test concentration of mesaconine is 1 mg/mL, while a concentration of 5 mg/mL was employed in our testing.

In conclusion, the results from this study might be helpful to elucidate the mechanism of action of the cardiac effect of Fu Zi, and we have indeed found a lead compound with cardiac effect.

Further structure–activity relationship study on the cardiac effect of mesaconine and its derivatives is in progress, which will be reported in due course.

Experimental

General Experimental Procedures Melting points were determined on a Kofler block (uncorrected). Optical rota-

tions were measured in a 1.0-dm cell on a Perkin-Elmer 341 polarimeter at $20\pm1^\circ\text{C}$. IR spectra were obtained on a Nicolet FT-IR 200 SXV spectrophotometer. ^1H - and ^{13}C -NMR spectra were taken on a Varian Unity INOVA 400/54 NMR spectrometer in CDCl_3 with TMS as the internal standard. The ESI-MS and HR-ESI-MS were recorded on a VG Auto Spec 3000 or a Finnigan-MAT 90 instrument. Silica gel H (Qingdao Marine Chemical Factory, Qingdao, China) was used for column chromatography. Development system: S_1 (CHCl_3 –MeOH–conc. NH_4OH = 85:14:1), S_2 (EtOAc–MeOH–conc. NH_4OH = 83:16:1), S_3 (hexane–EtOAc–EtOH = 1:3:1). TLC developing chambers were saturated with conc. NH_4OH . Zones on TLC (silica gel G) plates were detected with the modified Dragendorff's reagent.

Plant Material The lateral roots of *Aconitum Carmichaeli* were collected in the district of Jiangyou, Sichuan province, China, in June 2004. The plant was identified by Professor Wang Tian-Zi, West China College of Pharmacy, Sichuan University, and a voucher specimen (No. 04601) has been deposited in the West China College of Pharmacy at Sichuan University.

Extraction and Isolation The powdered lateral roots of *Aconitum Carmichaeli* (22 kg) were macerated with water (88 L) overnight, and then refluxed for 1 h. After filtration, the powders were refluxed twice with water (66 L each time) for 1 h. The combined filtrates were concentrated to about 20 L, to which was added 95% ethanol (75 L) to adjust the mixture in 75% ethanol. After standing at room temperature overnight, the insoluble material was removed by filtration and the solvents were evaporated to furnish a residue (175 g). The residue was dissolved in water (1000 mL) and basified with concentrated ammonium hydroxide to pH 10. The subsequent mixture was extracted with diethyl ether (1000 mL $\times$ 3, fraction A, 15 g), the aqueous layer was extracted with *n*-butanol (saturated with water, 1000 mL $\times$ 3). The combined *n*-butanol extracts were concentrated to give fraction B (92 g), and the aqueous fraction was concentrated to give fraction C (70 g). Fraction B (80 g) was subjected to column chromatography (CC) (silica gel H, 400 g; CHCl_3 –MeOH = 95:5 $\rightarrow$ 85:15) to

give the fractions 1–6. Frs. 4–5 were applied repeatedly to CC (silica gel H, 260, 50, 15g, respectively) eluting with CHCl_3 –MeOH=90:10→80:20) to furnish *N*-deethylaconine (**1**, 60mg), beiwutinine (**2**, 56mg), hyaconine (**3**, 125mg), mesaconine (**4**, 105mg), and 15 α -hydroxyneoline (**5**, 150mg). The known alkaloids **3**–**5** were identified based on the comparison of TLC behavior (S_1 , S_3) and ^1H - (^{13}C)-NMR spectra with those of authentic samples, which were provided by our laboratory.

Physical and Spectroscopic Data of the Compounds *N*-Deethylaconine (**1**): white amorphous powder, R_f 0.40 (S_1). $[\alpha]_D^{20} +25.4$ ($c=0.61$, MeOH). IR (KBr) $\lambda_{\max}$ 3357 cm^{-1} . ^1H -NMR (400MHz, CD_3OD) and ^{13}C -NMR (100MHz, CD_3OD), see Table 1. ESI-MS m/z 472 $[\text{M}+\text{H}]^+$. HR-ESI-MS m/z 472.2549 $[\text{M}+\text{H}]^+$ (Calcd for $\text{C}_{23}\text{H}_{38}\text{NO}_9$, 472.2547).

Beiwutinine (**2**): White amorphous powder, R_f 0.30 (S_1), 0.21 (S_2) 0.22 (S_3). $[\alpha]_D^{20} +22.0$ ($c=0.5$, MeOH). IR (KBr) $\lambda_{\max}$ 3380, 2937, 2825, 1645, 1454, 1097 cm^{-1} . ^1H -NMR (400MHz, CDCl_3) and ^{13}C -NMR (100MHz, CDCl_3), see Table 1. ESI-MS m/z 502 $[\text{M}+\text{H}]^+$. HR-ESI-MS m/z 502.2656 $[\text{M}+\text{H}]^+$ [Calcd for $\text{C}_{24}\text{H}_{39}\text{NO}_{10}$ $[\text{M}+\text{H}]^+$, 502.2652].

Animal Experimental Experimental Animals: Sprague-Dawley (SD) male rats used in the experiments were purchased from the laboratory animal center at the Chinese people's liberation army military academy of medical sciences with Qualified number: SCXK 2007-0004.

Samples: Compounds **1**–**5** and the extracts (A–C) were dissolved in Riger's solution to make the test concentrations; hyaconine (molecular w8: 485, soluble in water) was dissolved in Krebs–Henseleit (KH) to prepare the test concentrations; mesaconine (molecular w8: 469, soluble in water) was dissolved in KH to make the test concentrations.

Positive Control: Deslanoside Injection, manufactured by Shanghai Xudong Haipu Pharmaceutical Co., Ltd. with batch number: 090409, was dissolved in KH to corresponding concentration.

Krebs–Henseleit (KH) Buffer: Contained (g/2L) NaCl 13.7918, KCl 0.7008, CaCl_2 0.56605, MgSO_4 0.2841, KH_2PO_4 0.3212, NaHCO_3 4.1803, glucose: 4.3994.

Experimental Apparatus: Langendorff heart perfusion device (BIOPAC in U.S.A.), DC100A more physical recorder (BIOPAC in U.S.A.)

Experimental Methods: The experiment was carried out according to the procedure described in the literature.⁵⁰ Specifically, rats were anesthetized by injecting 50mg/kg sodium pentobarbital intraperitoneally. Hearts were isolated and kept in ice-cold KH buffer. Isolated hearts were gently squeezed and then perfused for 1min with KH buffer to remove the blood. Then each heart was perfused retrogradely using the Langendorff method with KH buffer at $(38\pm0.5)^\circ\text{C}$ and a head pressure of 70mmHg. A water-filled latex balloon was passed into the left ventricle through the mitral orifice and was connected to a pressure transducer. The balloon and the tube were filled with aqueous solution to achieve a stable left ventricular pressure for 30min. After the stabilization, hearts were perfused for 10min and then normothermic no-flow global ischemia for 20min, followed by reperfusion for 60min. The coronary effluent was measured with volumetric cylinder. Each heart was perfused in the presence or absence of hyaconine (10^{-9}mol/L), mesaconine (10^{-9}mol/L) or deslanoside (10^{-12}mol/L). Hypoconine, mesaconine or deslanoside

was treated 10min before the introduction of ischemia and continued to the end of the reperfusion.

LVEDP, the peak positive ($dP/dt_{\max}$) and HR were recorded from 10min before the introduction of ischemia to the end of reperfusion. A polygraph recorder (DC100A, BIOPAC, U.S.A.) and Acq373 software were used for recording and analysis. *t*-Test was applied to calculate the mean value and the standard error.

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