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C19-Norditerpenoid Alkaloids from *Aconitum szechenyianum* and Their Effects on LPS-Activated NO Production

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Abstract: Three new C_{19}-norditerpenoid alkaloids (**1**–**3**), along with two known C_{19}-norditerpenoid alkaloids (**4**–**5**) have been isolated from *Aconitum szechenyianum*. Their structures were established by extensive spectroscopic techniques and chemical methods as *szechenyianine* A (**1**), *szechenyianine* B (**2**), *szechenyianine* C (**3**), *N*-deethyl-3-acetylaconitine (**4**), and *N*-deethyldeoxyaconitine (**5**). Additionally, compounds **1**–**5** were tested for the inhibition of NO production on LPS-activated RAW264.7 cells with IC_{50} values of 36.62 ± 6.86, 3.30 ± 0.11, 7.46 ± 0.89, 8.09 ± 1.31, and 11.73 ± 1.94 μM, respectively, while the positive control drug dexamethasone showed inhibitory activity with IC_{50} value of 8.32 ± 1.45 μM. The structure-activity relationship of aconitine alkaloids were discussed.

Keywords: *Aconitum szechenyianum*; C_{19}-norditerpenoid alkaloids; anti-inflammatory activity; NO production; structure-activity relationship

1. Introduction

The plant *Aconitum szechenyianum* Gay., a species in the *Aconitum* genus of Ranunculaceae, is widely distributed in the west of China and used as a folk medicine in Shaanxi province, known as "Tie-Bang-Chui" [1]. Phytochemical studies revealed that *A. szechenyianum* contained mainly C_{19} and C_{20} diterpenoid alkaloids [2–5], possessing aconitine-type, 7,17-secoaconitine-type, and napeline-type skeletons. Aconitine-type have no oxygen-containing functionality at C-7, and secoaconitine-type skeleton contains N, C-17, and C-7, C-8 double bonds. Pharmacological studies revealed that these C_{19} and C_{20} diterpenoid alkaloids had demonstrated various activities as anti-inflammatory, analgesic, anticancer, anti-epileptiform, antiparasite, and cardiovascular action [6,7]. As part of our research project to explore more bioactive lead compounds from the medicinal herbs in the Qinba mountains of China [8–16], the chemical constituents and pharmacological studies of *A. szechenyianum* were studied, and three new C_{19}-norditerpenoid alkaloids, *szechenyianine* A (**1**), *szechenyianine* B (**2**), and *szechenyianine* C (**3**), along with two known ones, *N*-deethyl-3-acetylaconitine (**4**) and *N*-deethyldeoxyaconitine (**5**) were isolated (Figure 1). Since the roots of *A. szechenyianum* were commonly used to treat rheumatism and fracture [17], the isolated compounds were evaluated for their effects on the inhibition of NO production on LPS-activated RAW264.7 cells (Table 2 and Figure 5), and the structure-activity relationship of these compounds were discussed.

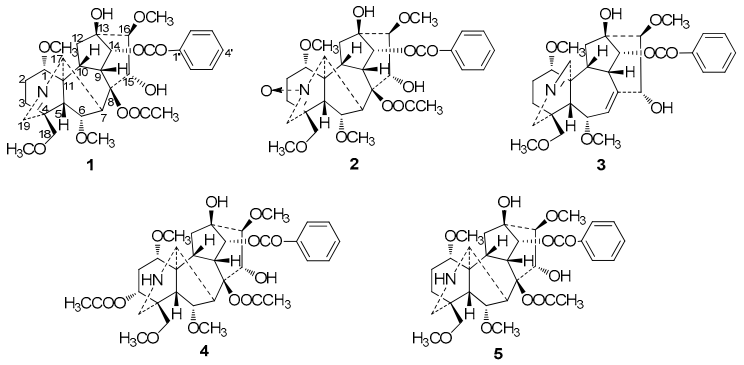

Figure 1. Structures of compounds **1**–**5**.

2. Results and Discussion

Szechenyianine A (**1**) was isolated as a white amorphous powder and showed a positive reaction with Dragendorff's reagent. Its molecular formula $C_{32}H_{41}NO_{10}$ was derived from a protonated molecular ion peak at *m/z* 600.2842 $[M + H]^+$ (calcd. 600.2809) of the HR-ESI-MS spectrum. Comparison of the NMR data of **1** and **5**, indicated almost similar NMR spectroscopic features, except for the number of C-4, C-17, C-19, this deduction was also confirmed by the chemical shift (Table 1) of C-4 (δ_C 39.0), C-19 (δ_C 49.0), and C-17 (δ_C 56.7) to upfield in ^{13}C-NMR spectra of **5** compared with C-4 (δ_C 46.8), C-19 (δ_C 165.9) and C-17 (δ_C 60.6) of **1,** we predicted the existence of *N*=CH group in compound **1**. The ^{1}H-NMR spectrum (Table 1) of **1** showed the presence of five aromatic proton signals due to a monosubstituted benzene at δ_H 8.02 (2H, d, *J* = 7.6 Hz), 7.55 (1H, t, *J* = 7.6 Hz), and 7.43 (2H, t, *J* = 7.6 Hz); a methine proton of an *N* = CH group at δ_H 7.31 (1H, s), four OMe protons at δ_H 3.75 (3H, s), 3.29 (3H, s), 3.18 (3H, s), and 3.03 (3H, s); and a strongly shielded proton of an acetoxyl group at δ_H 1.32 (3H, s). The ^{13}C-NMR spectrum (Table 1) displayed 32 carbon resonances. Among them, resonances at δ_C 166.2, 133.6, 130.0, 129.8 (C × 2), and 128.9 (C × 2) were attributed to a benzoyloxy group; δ_C 61.3, 59.3, 57.4 and 56.3 were attributed to four OMe groups, δ_C 172.6 and 21.5 were attributed to an acetoxyl group, and the NMR features of the remained 19 resonances were characteristic to an aconitine-type alkaloid, in which δ_C 165.9 was attributed to a *N*=CH group and δ_C 74.3 and 78.9 were attributed to two oxygenated carbons associated with hydroxyl groups. The assignments of the NMR signals associated with **1** were derived from HSQC, HMBC, and ROESY experiments. In the HMBC spectrum (Figure 2), correlations of H-5 (δ_H 2.23) and H-17 (δ_H 3.97) to C-19 (δ_C 165.9) suggested that C-19 was involved in the *N*=CH group; correlation of H-14 (δ_H 4.90) to the carbonyl carbon signal of benzoyl group (δ_C 166.2) suggested that the benzoyl group was located at C-14; correlation of the proton signal of the acetoxyl group (δ_H 1.32) to C-8 (δ_C 90.6) suggested the acetoxyl group was located at C-8; correlations of OCH_3 (δ_H 3.18) to C-1 (δ_C 82.3), OCH_3 (δ_H 3.03) to C-6 (δ_C 84.1), OCH_3 (δ_H 3.75) to C-16 (δ_C 89.9), and OCH_3 (δ_H 3.29) to C-18 (δ_C 78.2) suggested four methoxyl groups were linked at C-1, C-6, C-16, and C-18, respectively; correlations of H-12 (δ_H 2.20, 2.21), H-14 (δ_H 4.90), and H-16 (δ_H 3.42) to C-13 (δ_C 74.3), H-9 (δ_H 2.70) and H-16 (δ_H 3.42) to C-15 (δ_C 78.9), suggested two hydroxyl groups were linked at C-13 and C-15, respectively. Thus, the planar structure of **1** was deduced as 14-benzoyloxy-8-acetoxyl-13,15-dihydroxy-1,6,16,18-tetramethoxy-19-en-aconitane. Meanwhile, in the ROSEY spectrum (Figure 2) of **1**, the NOE correlations of H-1/H-10, H-10/H-14, H-14/H-9, and H-9/H-6 indicated β-orientation of H-1, H-6, H-9, H-10, and H-14, and α-axial configurations of 1-OCH_3, 6-OCH_3 and 14-benzoyloxy; NOE correlations of H-6/H-5 and H-5/H-18 revealed β-orientation of H-18 and 18-OCH_3, and α-axial of H-19; NOE correlations of H-17/H-7, H-16 and 15-OH, revealed α-axial of H-16, H-17 and 15-OH, and β-orientation of 16-OCH_3, 13-OH and 8-acetoxyl. Moreover, the NOE correlations of H-1/H-3 and H-5 while no correlation between H-2 and H-5 indicated **1** had ring A (C-1, C-2, C-3, C-4, C-5, and C-11) in the

chair conformation. Thus, according to the literature [18], compound **1** was assigned the name as (A-*c*)-14α-benzoyloxy-8β-acetoxyl-13β,15α-dihydroxy-1α,6α,16β,18β-tetramethoxy-19-en-aconitane.

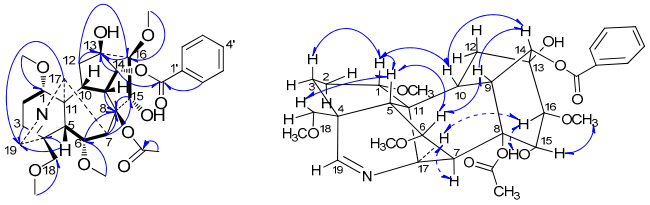

Figure 2. Key ^{1}H-^{1}H COSY (H▬H), HMBC (H→C) and ROESY (H↔H) correlations of compound **1**.

Table 1. ^{1}H-NMR and ^{13}C-NMR spectral data of compounds **1**–**5**.

NO.	1		2		3		4	5
	δ_C	δ_H (*J* in Hz)	δ_C	δ_H (*J* in Hz)	δ_C	δ_H (*J* in Hz)	δ_C	δ_C
1	82.3	3.20 (d, 4.1)	80.5	3.32 (m)	89.6	2.99 (dd, 4.4, 11.3)	81.0	82.3
2	22.9	1.66 (m, H-2a) 1.57 (m, H-2b)	22.0	1.45 (m, H-2a) 1.81 (m, H-2b)	24.7	1.10 (m) 1.86 (m)	31.8	23.5
3	28.2	1.63 (m, H-3a) 1.64 (m, H-3b)	29.5	1.70 (m, H-3a) 1.79 (m, H-3b)	37.4	1.55 (m) 1.69 (m)	72.3	29.0
4	46.8		42.1		39.7		43.2	39.0
5	45.8	2.23 (d, 7.1)	44.7	2.35 (d, 7.0)	46.2	2.32 (d, 8.9)	51.2	48.7
6	84.1	3.92 (d, 7.1)	82.7	4.00 (d, 7.0)	80.1	4.45 (m)	83.8	83.2
7	49.6	2.87 (s)	49.7	3.30 (s)	132.1	5.62 (d, 5.5)	45.2	43.6
8	90.6		89.3		137.5		91.7	91.5
9	42.6	2.70 (t, 6.1)	41.9	2.74 (m)	43.0	3.18 (s)	43.9	43.2
10	40.6	2.17 (m)	39.1	2.26 (m)	41.7	2.43 (s)	40.9	40.3
11	51.4		51.9		48.5		49.4	49.7
12	36.4	2.20 (m, H-12a) 2.21 (m, H-12b)	36.5	2.27 (m, H-12a) 1.98 (m, H-12b)	38.9	2.45 (m)	35.2	35.4
13	74.3		74.1		75.6		74.4	74.0
14	79.3	4.90 (d, 4.9)	78.8	4.89 (d, 4.8)	79.4	5.08 (d, 4.2)	79.1	78.9
15	78.9	4.48 (dd, 2.9, 5.3)	78.7	4.48 (dd, 3.0, 4.9)	74.1	4.80 (dd, 3.0, 5.8)	79.2	79.0
16	89.9	3.42 (d, 5.3)	89.6	3.45 (d, 5.0)	92.2	3.30 (d, 6.0)	90.0	89.5
17	60.6	3.97 (s)	72.9	4.02 (s)	166.4	7.86 (br s)	55.8	56.7
18	78.2	3.78 (d, 8.5, H-18a) 3.42 (d, 8.5, H-18b)	77.9	3.79 (d, 8.5, H-18a) 3.33 (d, 8.5, H-18b)	80.6	3.16 (d, 8.4) 3.86 (d, 8.4)	73.8	79.8
19	165.9	7.31 (s)	138.9	6.70 (d, 1.2)	58.3	3.53 (m) 3.45 (m)	41.6	49.0
8-OAc	172.6		172.1				172.3	172.0
	21.5	1.32 (s)	21.4	1.32 (s)			21.5	21.3
1-OCH_3	56.3	3.18 (s)	56.6	3.21 (s)	58.2	3.20 (s)	56.0	55.4
6-OCH_3	57.4	3.03 (s)	57.3	3.05 (s)	56.9	3.19 (s)	58.3	57.9
16-OCH_3	61.3	3.75 (s)	61.4	3.77 (s)	61.8	3.75 (s)	61.4	61.1
18-OCH_3	59.3	3.29 (s)	59.3	3.27 (s)	59.1	3.27 (s)	59.1	59.1
ArC=O	166.2		166.2		166.4		166.2	165.9
ArC-1′	130.0		129.9		130.0		130.0	130.7
3′, 5′	128.9	7.43 (t, 7.6)	129.0	7.44 (t, 7.3)	128.7	7.42 (t, 7.5)	128.9	128.6
2′, 6′	129.8	8.02 (d, 7.6)	129.9	8.01 (d, 7.3)	130.1	8.03 (d, 7.5)	129.8	129.6
4′	133.6	7.55 (t, 7.6)	133.8	7.57 (t, 7.3)	133.5	7.53 (t, 7.5)	133.5	133.3

δ in $CDCl_3$, in ppm from TMS; coupling constants (*J*) in Hz; ^{1}H-NMR at 400 MHz and ^{13}C-NMR at 100 MHz.

Szechenyianine B (**2**) was isolated as a white amorphous powder. The NMR spectroscopic data indicated that **2** was an analogu of **1** with similar skeleton and substituent groups. However, the molecular formula of **2** was deduced as $C_{32}H_{41}NO_{11}$ from the protonated molecular ion peak at *m/z* 616.2783 $[M + H]^+$ (calcd. 616.2758), suggesting that an *N*-oxidation group was included in compound **2**. This deduction was also confirmed by the chemical shift (Table 1) of C-4 (δ_C 42.1) and C-19 (δ_C 138.9) to upfield, and C-17 (δ_C 72.9) to downfield in ^{13}C-NMR spectra of **2** compared with C-4 (δ_C 46.8), C-19 (δ_C 165.9) and C-17 (δ_C 60.6) of **1**. Thus, compound **2** was identified by HSQC, ^{1}H-^{1}H COSY, HMBC, and ROESY experiments (Table 1 and Figure 3) as

(A-*c*)-14α-benzoyloxy-8β-acetoxyl-13β,15α-dihydroxy-1α,6α,16β,18β-tetra-methoxy-19-en-aconitane-*N*-oxide.

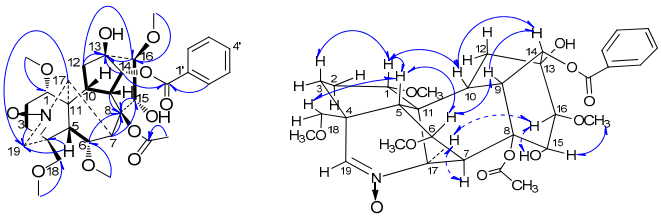

Figure 3. Key ^{1}H-^{1}H COSY (H▬H), HMBC (H→C) and ROESY (H↔H) correlations of compound **2**.

Szechenyianine C (**3**) was isolated as a white amorphous powder. Its molecular formula $C_{30}H_{39}NO_8$ was derived from a protonated molecular ion peak at *m/z* 542.2783 $[M + H]^+$ (calcd. 542.2754) of the HR-ESI-MS spectrum. The ^{1}H-NMR spectrum (Table 1) of **3** showed the presence of five aromatic protons signals due to a monosubstituted benzene at δ_H 8.03 (2H, d, *J* = 7.5 Hz), 7.53 (1H, t, *J* = 7.5 Hz), and 7.42 (2H, t, *J* = 7.5 Hz); two olefinic protons signals at δ_H 7.86 (1H, brs) due to *N*=CH and δ_H 5.62 (1H, d, *J* = 5.5 Hz) due to C=CH, respectively; and four OMe protons at δ_H 3.75 (3H, s), 3.27 (3H, s), 3.20 (3H, s) and 3.19 (3H, s). The ^{13}C-NMR spectrum (Table 1) displayed 30 carbon resonances. Among them, resonances at δ_C 166.4, 133.5, 130.0, 130.1 (C × 2) and 128.7 (C × 2) were attributed to a benzoyl group; δ_C 61.8, 59.1, 58.2 and 56.9 were attributed to four OMe groups; and the NMR features of the remained 19 resonances were characteristic to a 7, 17-secoaconitine alkaloid, in which δ_C 166.4 was attributed to a *N*=CH group, and δ_C 132.1 and 137.5 were attributed to an olefinic bond. In the HMBC spectrum (Figure 4), correlations of H-1 (δ_H 2.99), H-5 (δ_H 2.32), H-10 (δ_H 2.43), and H-19 (δ_H 3.53) to C-17 (δ_C 166.4) suggested that C-17 was involved in the *N*=CH group, and correlations of H-5 (δ_H 2.32), H-6 (δ_H 4.45) to C-7 (δ_C 132.1), H-6 (δ_H 4.45), H-14 (δ_H 5.08), and H-15 (δ_H 4.80) to C-8 (δ_C 137.5) suggested the olefinic bond was located at C-7 and C-8, which supported the presence of skeleton of the 7,17-secoaconitine alkaloid. Moreover, HMBC correlation of H-14 (δ_H 5.08) to the carbonyl carbon signal of benzoyl group (δ_C 166.4) suggested that the benzoyl group was located at C-14; correlations of OCH_3 (δ_H 3.20) to C-1 (δ_C 89.6), OCH_3 (δ_H 3.19) to C-6 (δ_C 80.1), OCH_3 (δ_H 3.75) to C-16 (δ_C 92.2), and OCH_3 (δ_H 3.27) to C-18 (δ_C 80.6) suggested four methoxyl groups were linked at C-1, C-6, C-16 and C-18, respectively; correlations of H-10 (δ_H 2.43) and H-14 (δ_H 5.08) to C-13 (δ_C 75.6), H-7 (δ_H 5.62) and H-16 (δ_H 3.30) to C-15 (δ_C 74.1) suggested two hydroxyl group were linked at C-13 and C-15, respectively. Thus, the planar structure of **3** was deduced as 14-benzoyloxy-13,15-dihydroxy-1,6,16,18-tetramethoxy-7(8),17-dien-7,17-secoaconitane. Meanwhile, in the ROSEY spectrum (Figure 4) of **3**, the NOE correlations of H-1/H-10, H-10/H-14 and H-14/H-9 indicated *β*-orientation of H-1, H-9, H-10 and H-14, and *α*-axial configurations of 1-OCH_3 and 14-benzoyloxy; the NOE correlations of H-6/H-5 and H-5/H-18 revealed β-orientation of H-18 and 18-OCH_3, and *α*-axial of 6-OCH_3; NOE correlations of H-17/H-16 and 15-OH, H-15/16-OCH_3 revealed *α*-axial of H-16 and 15-OH, and β-orientation of 16-OCH_3 and 13-OH. Moreover, the NOE correlations of H-1/H-3 and H-5 while no correlation between H-2 and H-5 indicated **3** had ring A (C-1, C-2, C-3, C-4, C-5, and C-11) in the chair conformation. Thus, according to the literature [18], compound **3** was assigned the name as (A-*c*)-14α-benzoyloxy-13β, 15α-dihydroxy-1α,6α,16β,18β-tetramethoxy-7(8),17-dien-7,17-secoaconitane.

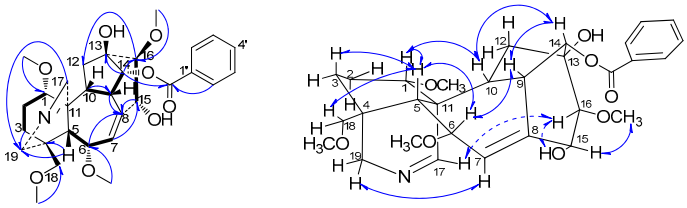

Figure 4. Key ^{1}H-^{1}H COSY (H▬H), HMBC (H→C) and ROESY (H↔H) correlations of compound **3**.

Since the roots of *A. szechenyianum* are commonly used to treat rheumatism and fracture [17], in which inflammation is involved in the pathophysiological process and inhibitors of NO release are considered as potential anti-inflammatory agents for the treatment of these diseases [18–20], the isolated compounds from *A. szechenyianum* were evaluated using the Griess assay [21] for their effects on the inhibition of NO production in LPS-activated RAW264.7 cells. Dexamethasone (DEX) was selected as a positive control. As shown in Table 2 and Figure 5, all compounds with aconitine or 7,17-secoaconitine skeleton exhibited anti-inflammatory activities in a dose-dependent manner. Compared the activity with the substituent groups of **1**, **2**, **4**, and **5**, the structure-activity relationship may be due to the chemical environment of *N* atom. The compound **1** could hinder the inhibition of NO production with IC_{50} value of 36.62 ± 6.86 μM. The compound **2** exhibited excellent active performance with IC_{50} value of 3.30 ± 0.11 μM, indicated that the presence of $N\rightarrow O$ might increase anti-inflammatory activities. Moreover, compound **4** exhibited effective inhibitory activity with IC_{50} value of 8.09 ± 1.31 μM and compound **5** showed inhibitory activity with IC_{50} value of 11.73 ± 1.94 μM. In addition, compound **3** as a 7,17-secoaconitine type alkaloid also exhibited potent inhibitory activity on NO production with IC_{50} value of 7.46 ± 0.89 μM.

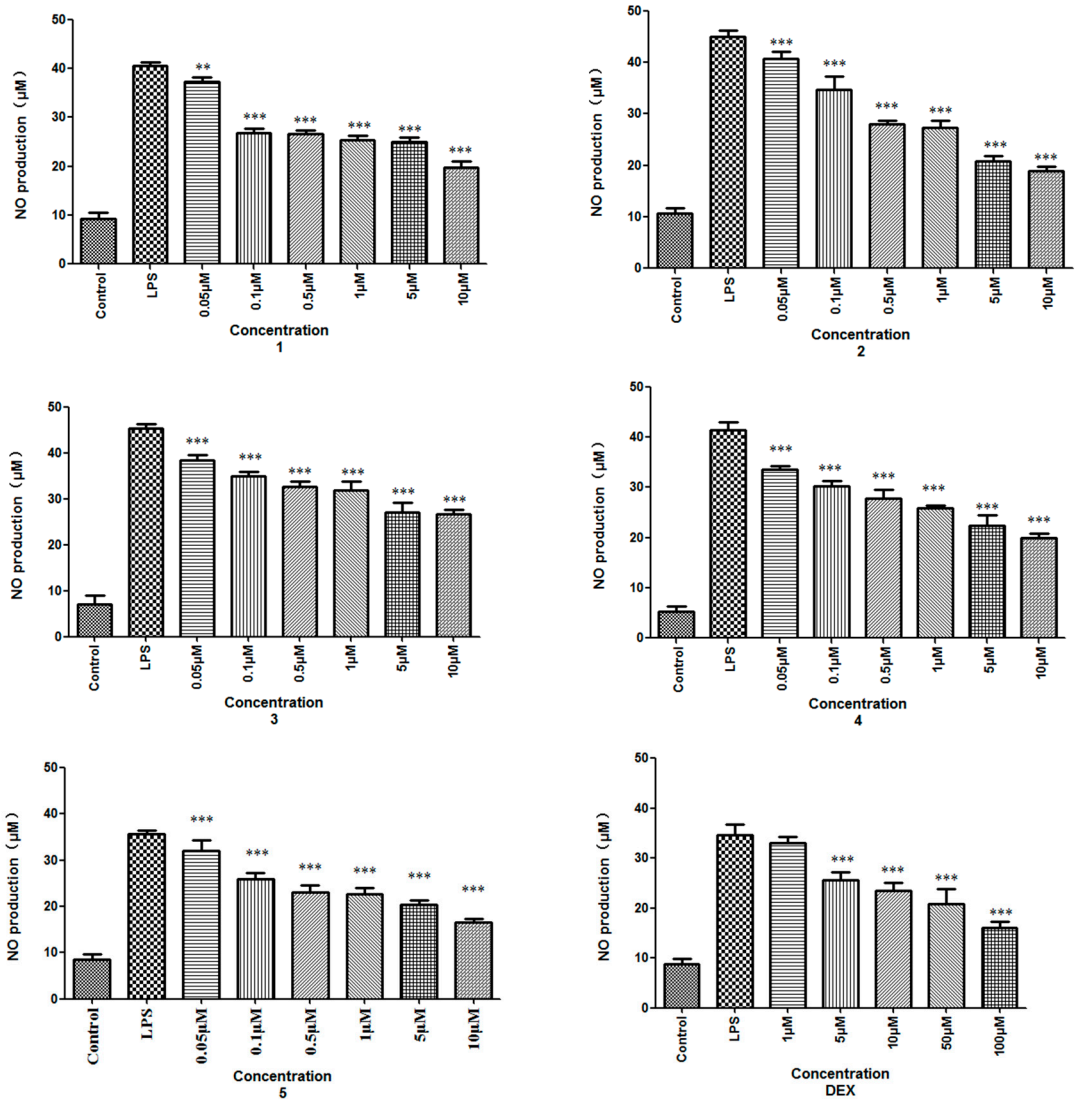

Figure 5. NO inhibitory effects of compounds from *A. szechenyianum* on LPS-activated RAW264.7 cells. Results represent the mean ± SD of three independent experiments; results differ significantly from the LPS-treated, ** $p < 0.01$, *** $p < 0.001$; dexamethasone (DEX) was used as a positive control.

Table 2. IC_{50} values of the compounds from *A. szechenyianum* on NO production in LPS-activated RAW264.7 cells.

Compound	1	2	3	4	5	Dexamethasone
IC_{50} (μM)	36.62 ± 6.86	3.30 ± 0.11	7.46 ± 0.89	8.09 ± 1.31	11.73 ± 1.94	8.32 ± 1.45

Results are expressed as IC_{50} values in μM and the values are means ± SD; n = 3; dexamethasone was used as a positive control.

3. Experimental Section

3.1. General Information

ESI-MS was performed on a Quattoro Premier instrument (Waters, Milford, MA, USA). The HR-ESI-MS spectra were recorded on an Agilent Technologies 6550 Q-TOF (Santa Clara, CA, USA). 1D and 2D-NMR spectra were recorded on Bruker-AVANCE 400 instrument (Bruker, Rheinstetten, Germany) with TMS as an internal standard. The analytical HPLC was performed on a Waters e2695 Separations Module coupled with a 2998 Photodiode Array Detector and a Accurasil C-18 column (4.6 mm × 250 mm, 5 μm particles, Ameritech, Chicago, IL, USA). Semipreparative HPLC was performed on a system comprising an LC-6AD pump equipped with an SPD-20A UV detector (Shimadzu, Kyoto, Japan) and an Ultimate XB-C18 (10 mm × 250 mm, 5 μm particles) or YMS-Pack-ODS-A (10 mm × 250 mm, 5 μm particles). Silica gel was purchased Qingdao Haiyang Chemical Group Corporation (Qingdao, China).

3.2. Plant Material

The roots of *Aconitum szechenyianum* Gay. were collected from the Xi Mountains of Gansu Province of China in July 2014, and identified by senior experimentalist Jitao Wang. A voucher specimen (herbarium No. 20140728) has been deposited in the Medicinal Plants Herbarium (MPH), Shaanxi University of Chinese Medicine, Xianyang, China.

3.3. Extraction and Isolation

The air-dried and powdered underground parts of *A. szechenyianum* (5.0 kg) were extracted with 80% EtOH at 80 °C for three times (each time 40 L for 1.5 h). After removal of EtOH solvent under reduced pressure, the extract (2 L) was dispersed in water (1.5 L), adjusted with 9% HCl solution to pH 0.8, and extracted with petroleum ether (PE). The acidic water solution was alkalized to pH 10.26 with 25% ammonia solution, extracted with $CHCl_3$ three times, and evaporated under pressure to give crude alkaloids (50 g). The crude alkaloids (47 g) were chromatographed on silica gel column, eluting with gradient solvent system (PE/acetone/diethylamine, 50:1:0.1–1:1:0.1) to give 12 fractions (Fr.1–Fr.12). Fr.6 (3.2 g) was purified by HPLC (YMC-Pack-ODS-A, 10 mm × 250 mm, 5 μm particles, flow rate: 1.0 mL·min^{-1}) with CH_3OH/H_2O (70:30) as mobile phase to obtained Fr.6-1 (30 mg; t_R = 5 min), Fr.6-2 (120 mg; t_R = 30 min), Fr.6-3 (130 mg; t_R = 42 min), Fr.6-4 (120 mg; t_R = 63 min), Fr.6-5 (140 mg; t_R = 77 min), and Fr.6-6 (200 mg; t_R = 63 min). Fr.6-3 (130mg) was purified by HPLC with CH_3OH/H_2O (60:40) as mobile phase to afford **1** (13 mg; t_R = 50 min) and **2** (12 mg; t_R = 65 min). Fr.6-4 (120 mg) was purified by HPLC with CH_3OH/H_2O (65:35) as mobile phase to afford **3** (10 mg; t_R = 45 min), **4** (20 mg; t_R = 65 min), and **5** (25 mg; t_R = 75 min). See more detailed spectrums in the supplementary materials.

(A-c)-14α-Benzoyloxy-8β-acetoxyl-13β,15α-dihydroxy-1α,6α,16β,18β-tetramethoxy-19-en-aconitane (*szechenyianine* A): A white amorphous powder, $[\alpha]_D^{20}$ +60.2 (*c* 0.38, MeOH), IR (KBr) ν_{max}: 3508, 2936, 1718, 1637 and 714 cm^{-1}; ^{1}H-NMR (400 MHz, $CDCl_3$) and ^{13}C-NMR (100 MHz, $CDCl_3$) spectral data, see Table 1; *m/z* 600.2842 $[M + H]^+$ (calcd. for $C_{32}H_{41}NO_{10}$, 600.2809).

(A-c)-14α-Benzoyloxy-8β-acetoxyl-13β,15α-dihydroxy-1α,6α,16β,18β-tetramethoxy-19-en-aconitane-N-oxide (szechenyianine B): A white amorphous powder, $[\alpha]_D^{20}$ +10.5 (*c* 0.44, MeOH), IR (KBr) ν_{max}: 3510, 2938, 1719, 1603 and 716 cm^{-1}; ^{1}H-NMR (400 MHz, $CDCl_3$) and ^{13}C-NMR (100 MHz, $CDCl_3$) spectral data, see Table 1; *m/z* 616.2783 $[M + H]^+$ (calcd. for C_{32} $H_{41}NO_{11}$, 616.2758).

(A-c)-14α-Benzoyloxy-13β,15α-dihydroxy-1α,6α,16β,18β-tetramethoxy-7(8),17-dien-7,17-secoaconitane (szechenyianine C): A white amorphous powder, $[\alpha]_D^{20}$ +21.6 (*c* 0.64, MeOH), IR (KBr) ν_{max}: 3513, 2930, 2824, 1716, 1645, 1453, 1367, 1275, 1102 and 715 cm^{-1}; ^{1}H-NMR (400 MHz, $CDCl_3$) and ^{13}C-NMR (100 MHz, $CDCl_3$) spectral data, see Table 1; *m/z* 542.2783 $[M + H]^+$ (calcd. for C_{30} $H_{39}NO_8$, 542.2754).

3.4. Inhibitory Assay of NO Production

Assays for NO production were carried out according to the Griess reaction, using dexamethasone as positive control. Briefly, RAW264.7 cells were seeded into 96-well microplates at a density of 2×10^5 mL^{-1} and allowed to adhere for 4 h. RPMI1640 (100 μL) containing test samples (final concentration of 10, 5, 1, 0.5, 0.1, and 0.05 μM) dissolved in DMSO (final concentration less than 0.2%) and LPS (final concentration of 1 $\mu g \cdot mL^{-1}$) were added. After incubation at 37 °C for 18 h, 50 μL of cell-free supernatant was mixed with 50 μL of Griess Reagent I and 50 μL of Griess Reagent II to determine NO production. Absorbance was measured at 550 nm against a calibration curve with $NaNO_2$ standard. The NO productions of the isolated compounds were tested (Figure 5), the inhibitory rate on NO production induced by LPS was calculated by the NO_2^- levels as follows: Inhibitory rate (%) = $100 \times ([NO_2^-]_{LPS} - [NO_2^-]_{LPS+sample})/([NO_2^-]_{LPS} - [NO_2^-]_{untreated})$, the IC_{50} values were calculated (Table 2). Values are mean ± SD, $n = 3$, ** $p < 0.01$, *** $p < 0.001$ vs. LPS treated.

Supplementary Materials: IR, HR-ESI-MS, ^{1}H-NMR, ^{13}C-NMR and 2D NMR spectra for compounds **1**–**3** can be found, in the online version, at http://www.mdpi.com/1420-3049/21/9/1175/s1.

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Sample Availability: Samples of the compounds **4**–**5** are available from the authors.