ORIGINAL ARTICLE

Structure, property, biogenesis, and activity of diterpenoid alkaloids containing a sulfonic acid group from Aconitum carmichaelii

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Abstract Three new C20-diterpenoid alkaloids with a sulfonic acid unit, named aconicarmisulfonines B and C (1 and 2) and chuanfusulfonine A (3), respectively, were isolated from the Aconitum carmichaelii lateral roots (“fu zi” in Chinese). Structures of 1–3 were determined by spectroscopic data analysis. Intriguing chemical properties and reactions were observed for the C20-diterpenoid alkaloids: (a) specific selective nucleophilic addition of the carbonyl (C-12) in 1 with CD3OD; (b) interconversion between 1 and 2 in D2O; (c) stereo- and/or regioselective deuterations of H-11α in 1–3 and both H-11α and H-11β in aconicarmisulfonine A (4); (d) TMSP-2,2,3,3-d4 promoted cleavage of the C-12–C-13 bond of 4 in D2O; (e) dehydrogenation of 4 in pyridine-d5, and (f) Na2SO3-assisted dehydrogenation and N-deethylation of songorine (5, a putative precursor of 1–4). Biogenetically, 1 and 2 are correlated with 4, for which the same novel carbon skeleton is proposed to be derived from semipinacol rearrangements via migrations of C-13–C-16 and C-15–C-16 bonds of the napelline-type skeleton, respectively. Meanwhile, 3 is a highly possible precursor or a concurrent product in the biosynthetic pathways of 1, 2, and 4. In the acetic acid-induced mice writhing assay, at 1.0 mg/kg (i.p.), compounds 1, 2, 5, 5a, and 5b exhibited analgesic effects against mice writhing.

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1. Introduction

C20-Diterpenoid alkaloids are a group of natural products with distinctive diverse carbon skeletons\textsuperscript{1,2}. Approximate 400 members have been reported and divided into four classes including at least 24 types, 34 subtypes, and 42 groups\textsuperscript{1–5}. Plants of two genera Aconitum and Delphinium in the Ranunculaceae family and of one genus Spiraea in the Rosaceae family are the richest source of the C20-diterpenoid alkaloids\textsuperscript{1,2,6}. Due to variable chemical properties, notable pharmacological profiles, and structurally sophisticated architectures, considerable interest from the community of medicinal and organic chemistry has been elicited over 130 years\textsuperscript{1,2,6}, leading to recent fruitful achievements in total synthesis of a subset of C20-diterpenoid alkaloids\textsuperscript{7–12}. The dried lateral root (“fu zi” in Chinese) of the poisonous plant Aconitum carmichaelii Debx., an important ingredient of many preparations and formulae\textsuperscript{13–15} in the oriental countries\textsuperscript{13–15}. To reduce fatal poisoning, “fu zi” is cautiously utilized by processing, decocting, or formulating\textsuperscript{16–19}. Previous studies chemically and pharmacologically revealed aconitine-type C19-diterpenoid alkaloids representing the predominant toxic and active constituents of this herbal medicine\textsuperscript{20}, while a majority of toxic C19-diterpenoid alkaloid diesters were hydrolyzed into less toxic monoester and/or alcohol forms during processing and decocting\textsuperscript{21–23}. Nevertheless, the currently known chemical constituents were mostly separated from organic solvent extracts of the drug materials. Meanwhile, HCl and/or NH\textsubscript{4}OH were used in extraction and/or isolation steps\textsuperscript{5,14,15,24–30}. Particularly the extraction methods differ from that classically decocts “fu zi” with water. Thus, as part of a program to detailly search for water-soluble active constituents from several popular traditional Chinese medicines and to test their pharmacological effects\textsuperscript{31–38}, we focused on an aqueous extract of the air-dried raw material of “fu zi”, which is closer to a practically prepared decoction. From the extract, 54 previously unknown constituents have been reported, including 12 unprecedented glycosidic C19-diterpenoid alkaloids and three sulfonated C19-diterpenoid alkaloids with two novel carbon architectures, as well as their notable analgesic effects\textsuperscript{39–47}. Further examination of a remaining fraction of the extract has resulted in characterization of three additional sulfonated C20-diterpenoid alkaloids (1–3, Fig. 1). Herein, we report details of their isolation, structural elucidation, distinctive chemical properties, plausible biogenesis, and analgesic effects.

2. Results and discussion

2.1. Structure elucidation of 1–3

Compound 1 was obtained as a white amorphous powder with [\alpha\textsubscript{D}\textsuperscript{20} \text{L} = -38.1 (c 0.29, MeOH). The presence of hydroxyl and/or amino (3393 cm\textsuperscript{-1}) and ketone carbonyl (1738 and 1678 cm\textsuperscript{-1}) units in its molecule was deduced from the characteristic absorption bands in the IR spectrum of 1. Hydrogen adduct ion peaks of the molecule at m/z 438 [M+H]\textsuperscript{+} and 436 [M–H]\textsuperscript{−} were detectable by positive and negative modes of ESI-MS, respectively. Subsequent measurements of HR-ESI-MS data (see Experimental Section) determined the molecular formula as C\textsubscript{22}H\textsubscript{31}NO\textsubscript{6}S. Diagnostic signals for a C20-diterpenoid alkaloid\textsuperscript{40,42,43,45,47} were exhibited in the \textsuperscript{1}H NMR spectrum of 1 in D\textsubscript{2}O, including two heteroatom-bearing methines at \text{δ}\textsubscript{H} 4.12 (brdd, J = 9.0 and 8.4 Hz, H-1) and 4.00 (brs, H-20), a methyl group at \text{δ}\textsubscript{H} 0.91 (s, H\textsubscript{3}-18), and a characteristic N-ethyl unit at \text{δ}\textsubscript{H} 3.32 (m, H\textsubscript{2}-21) and 1.38 (t, J = 7.2 Hz, H-22), in addition to five aliphatic methines and seven aliphatic methylenes between \text{δ}\textsubscript{H} 3.61 and 1.46 (partially overlapping multiplets). Corresponding to the abovementioned structural units, 22 carbon resonances including two carbonyl carbons at \text{δ}_{\text{C}} 217.0 (C-12) and 216.3 (C-15) as well as three additional sp\textsuperscript{3} hybrid quaternary carbons at \text{δ}_{\text{C}} 35.2 (C-4), 53.5 (C-8), and 52.6 (C-10) (Table 1) were observable in the \textsuperscript{13}C NMR and DEPT spectra of 1. These spectroscopic data were similar to those reported for aconicarmisulfonine A (4, Scheme 1) from the same extract\textsuperscript{45}. Analyzing the 2D NMR spectroscopic data of 1, the same rare skeletal architecture and 1-hydroxy-12-one substitution pattern sharing by the two compounds were readily determined. However, a 15-ketone in 1 replacing the 16-one in 4 was revealed by the long-range heteronuclear correlation signals of C-15/H-9, H-2-14, H-2-16, and H-17 in the HMBC spectrum and the homonuclear vicinal coupling correlation peaks of H-16/H-17/H-13 in the \textsuperscript{1}H–\textsuperscript{1}H COSY spectrum (Fig. 2). A 17-sulfonate in 1 was assigned to satisfy the molecular formula and substitution of C-17, and its α-orientation was elucidated from the NOE cross-peak between H-9 and H-17 in the ROESY spectrum (Fig. 3), wherein the remaining NOE correlation signals proved identity of the relative configuration of other chiral centers in 1 and 4. Based on biogenetic consideration of the identical carbon skeleton deriving from the same precursor (Scheme 2), stereochemistry of 1 and 4 was predicted to be the same except for an additional 17R-configuration in 1, of which 4 was verified by a single crystal X-ray diffraction\textsuperscript{45}. Further supportive evidences for the prediction were obtained from explanation of a negative Cotton effect at 290 (\text{Δ}ε = -0.43) nm arising from \pi–\pi* transitions of the carbonyl chromophores in the CD spectrum (Supporting Information Fig. S11) by the octant rule for the cyclohexanones\textsuperscript{38,49} as well as the consistency between the measured CD and theoretically calculated ECD spectra of 1. Thus, compound 1 was determined to have the structure analogized to aconicarmisulfonine A and named aconicarmisulfonine B.

Compound 2, a white amorphous powder with [\alpha\textsubscript{D}\textsuperscript{20} = -37.4 (c 0.14, MeOH), had spectroscopic data very similar to those of 1. However, comparing the NMR spectroscopic data of the two compounds in the same solvent D\textsubscript{2}O (Table 1), coupling constant and chemical shift changes of H-13 and H-17 from the broad singlet (\text{δ}\textsubscript{H} 3.27, J\textsubscript{13,16} \sim 0 Hz) and broad doublet (\text{δ}\textsubscript{H} 2.74,
$J_{16,17b} = 10.2$ Hz) in 1 into a doublet ($\delta_{H} = 3.29, J_{13,16} = 8.4$ Hz) and a double doublet ($\delta_{H} = 3.11, J_{13,16} = 8.4$ Hz and $J_{16,17b} = 9.0$ Hz) in 2 were distinguishable. In addition, upfield shifts by $\Delta\delta_{C} = -1.0$, $-1.5$, and $-2.3$ were observed for C-6, C-8, and C-16 in 2, respectively, whereas downfield shifts by $\Delta\delta_{C} = +1.2$, $+2.4$, $+1.1$, and $+1.8$, and $+3.1$ were observed for C-7, C-9, C-11, C-14, and C-17. From these data, 2 was deduced to be an epimer of 1 having a $\beta$-orientation of 17-sulfonate. The deduction was verified by 2D NMR spectroscopic data analysis (Figs. 2 and 3) and by the NOE correlation signals between H-11a with H-11b and H-20 as well as between H-14b and H-17 (Fig. 3) in the ROESY spectrum of 2. Based on similarity of the CD spectra between 1 and 2 as well as the consistency between the measured CD and calculated ECD spectra of 2, the configurational change at C-17 does not affect significantly on the measured CD and calculated ECD spectra of the two compounds (Fig. 4). Therefore, compound 2 was determined as the 17-epimer of 1 and named acconicarmisulfonine C.

Compound 3, a white amorphous powder with $[\alpha]^{20}_D = -7.3$ (c 0.15, MeOH), is another C_{20}-diterpenoid alkaloid having the molecular formula C_{22}H_{33}NO_{7}S as deduced from HR-ESI-MS and spectroscopic data. This compound displayed the NMR spectroscopic data (previously isolated from the same plant) $50$. However, significant and a double doublet ($\delta_{H} = 1.57$, $J_{13,16} = 1.0$, and $C-17 = 2.3$) as well as the consistency between the measured CD and calculated ECD spectra of 3, the configurational change at C-17 does not affect significantly on the measured CD and calculated ECD spectra of the two compounds (Fig. 4).

The elucidation was sup-

2.2. Chemical properties of 1–4

During measurements of the NMR spectra, distinctive stability in D_{2}O or CD_{3}OD was observable for 1 and 2, respectively. Compound 2 was partially converted into 1 in D_{2}O accompanying by deuteration of H-11a (Fig. 5a and b), whereas 1 was partially transformed into a new product 1a in CD_{3}OD (Fig. 6a and b). After storage in a refrigerator at 4 °C around 14 months, the

| No. | $\delta_{H}$ | $\delta_{C}$ | $\delta_{H}$ | $\delta_{C}$ | $\delta_{H}$ | $\delta_{C}$ |
|-----|-------------|-------------|-------------|-------------|-------------|-------------|
| 1   | 4.12 brdd (9.0, 8.4) | 67.3        | 4.08 brt (9.0) | 67.3        | 4.12 dd (9.6, 8.4) | 68.0        |
| 2   | 2.14 m      | 30.9        | 2.11 m      | 30.9        | 2.13 m      | 31.0        |
| 3   | 1.57 m      | 1.56 m      | 1.53 m      |             |             |             |
| 4   | 1.68 brd (13.8) | 35.5        | 1.68 m      | 35.6        | 1.67 m      | 35.5        |
| 5   | 1.46 dt (3.6, 13.8) | 35.2        | 1.45 dt (3.0, 13.8) | 35.2        | 1.47 brt (13.8) |             |
| 6   | 1.72 brd (7.8) | 47.3        | 1.73 brd (7.8) | 47.6        | 1.72 d (8.4) | 47.1        |
| 7   | 2.91 dd (15.0, 7.8) | 22.1        | 3.31 dd (15.0, 7.8) | 21.1        | 2.83 dd (14.4, 8.4) | 22.4        |
| 8   | 1.77 dd (15.0, 4.8) | 40.8        | 1.66 dd (15.0, 4.8) | 42.0        | 1.61 dd (14.4, 4.2) | 44.2        |
| 9   | 2.85 d (4.8) | 53.5        | 2.98 d (4.8) | 52.0        | 2.72 brd (4.2) | 49.2        |
| 10  | 2.19 dd (11.4, 7.2) | 42.9        | 2.03 dd (11.4, 7.2) | 45.3        | 2.20 dd (10.2, 7.2) | 36.6        |
| 11  | 3.61 dd (16.8, 11.4) | 37.9        | 3.52 dd (16.8, 11.4) | 39.0        | 3.29 dd (12.0, 10.2) | 38.1        |
| 12  | 2.60 dd (16.8, 7.2) | 52.6        | 2.49 dd (16.8, 7.2) | 52.8        | 2.39 dd (12.0, 7.2) | 38.6        |
| 13  | 3.27 brs     | 47.4        | 3.29 d (8.4) | 47.0        | 3.05 d (3.6) | 56.2        |
| 14  | 2.52 brd (13.2) | 29.8        | 2.65 brd (12.6) | 31.6        | 2.06 d (13.2) | 29.5        |
| 15  | 2.12 brd (13.2) | 21.6        | 2.14 brd (12.6) | 21.7        | 1.69 dd (13.2, 3.6) |             |
| 16  | 3.23 brd (14.4) | 50.8        | 3.11 brd (14.4) | 48.5        | 4.29 s       | 76.7        |
| 17  | 3.01 dd (14.4, 10.2) | 48.5        | 2.83 dd (14.4, 9.0) | 48.5        | 1.69 dd (13.2, 3.6) |             |
| 18  | 2.74 brd (10.2) | 47.6        | 3.11 dd (9.0, 8.4) | 50.7        | 3.26 d (14.4) | 59.3        |
| 19  | 0.91 s       | 24.5        | 0.91 s       | 24.5        | 0.91 s       | 24.7        |
| 20  | 3.40 d (13.8) | 57.2        | 3.39 d (13.2) | 57.3        | 3.36 d (13.8) | 57.7        |
| 21  | 2.96 d (13.8) | 57.2        | 2.97 d (13.2) | 57.2        | 2.96 d (13.8) |             |
| 22  | 4.00 brs     | 65.1        | 4.03 brs     | 65.6        | 3.96 brs     | 65.4        |
| 23  | 3.32 m       | 54.9        | 3.32 m       | 54.9        | 3.30 q (7.2) | 54.7        |
| 24  | 1.38 t (7.2) | 10.1        | 1.39 t (6.6) | 10.1        | 1.37 t (7.2) | 10.1        |

*aChemical shift values (J) were measured at 600 MHz for $^1$H NMR and at 150 MHz for $^{13}$C NMR, respectively. Proton coupling constants (J) in Hz are given in parentheses. The assignments were based on DEPT, $^1$H–$^1$H COSY, HSQC, and HMBC experiments.*
samples of 1 and 2 in D$_2$O became almost identical, containing two components (1/1-d and 2-1-d) in an approximate 4:1 ratio (Fig. 5c–f). The gradual generation of 1a-d from 1-d was also demonstrated by measuring the $^1$H NMR spectra of the mixture (Fig. 6c and d) as well as of a purified sample of 1-d in CD$_3$OD (Fig. 7). The structures of 1-d and 2-d were readily deduced as the H-11a deuterated 1 and 2, respectively, by comparing the NMR spectroscopic data (Supporting Information Tables S1 and S2) which were unambiguously assignable by analysis of the 2D NMR spectra of the mixture. Particularly disappearance of the H-11a signals and variation of the splitting patterns of H-9 and H-11b in the $^1$H NMR spectra (Figs. 5 and 6) were the determinative proofs for the deduction. The $\alpha$-orientation of H-11a was deducible from comparing the NMR spectroscopic data between 1-d and 1 as well as between 2-d and 2, along with the NOE correlation H-1/H-11b in the ROESY spectrum of the mixture. The structures of 1a and 1a-d were elucidated to be the semi-acetals deriving from nucleophilic addition of the solvent CD$_3$OD to 12-ketone in 1 and 1-d, respectively. The elucidation was based on distinguishing the signals between 1a and 1 as well as between 1a-d and 1-d (Table S1) in the NMR spectra of the mixtures, especially on a characteristic resonance for a dioxygen-bearing quaternary carbon (dC 104.0) of 1a-d or 1a replacing the carbonyl carbon resonance (dC 213.9) or 1 (dC 213.6), in combination with the HMBC correlations of the dioxygen-bearing quaternary carbon with H-11b, H-13, and H-14a. After evaporation of the CD$_3$OD solutions, the signals of 1a or 1a-d completely disappeared in the NMR spectra of the samples in D$_2$O, whereas those of 1 and 1-d were retained, indicating that 1a and 1a-d survived only in CD$_3$OD. Therefore, accompanying the selective deuteration of H-11a (H-11a), 1 and 2 are able to interconvert to reach an equilibrium in the approximate 4:1 ratio in D$_2$O, wherein 1 is a stable species with the higher abundance while 2 is less stable and relatively easier to isomerize into 1 (Scheme 3). Whereas, the 12-ketone in 1 has a more active chemical character to be selectively attacked by a nucleophile (CD$_3$OD) to produce a returnable ketone.
semiacetal. An explanation for the different properties between 1 and 2 is from the varied effects of the sulfonate group.

Differing from 1 and 2, compound 3 was almost insoluble in CD3OD. The selective deuteration of H-11α was also observed in the NMR spectra of 3 in D2O. However, both H-11α and H-11β in 4 were readily deuterated in D2O45. Because deuteration of the carbon-bearing protons was not observed in other diterpenoid alkaloids42, the intriguing property of 1−4 must be due to the characteristic structural sulfonation. Particularly in the measurements of the NMR spectra of 4 in D2O with and without using sodium 3-trimethylsilylpropionate (TMSP-2,2,3,3-d4) as an internal reference, a product 4a was gradually formed only in the solution containing TMSP. This suggested a TMSP promoted reaction of 4 to yield 4a. HPLC separation of the sample afforded 4a with recovering 4. HR-ESI-MS indicated an isomeric relationship between 4a and 4. However, the NMR spectroscopic data of 4a differed significantly from those of 4 (Supporting Information Table S3), showing replacement of the ketone carbonyl (C-12) and methine (CH-13) units in 4 by carboxylic carbon (Δδ 176.5) and methylene [δH 2.76 (dd, J = 13.2, 12.0 Hz) and 2.65 (brd, J = 12.0 Hz)] in 4a, respectively, along with the remarkable downfield shifts of H-1 and C-1. These spectroscopic data revealed a skeletal change from 4 to 4a. The structure of 4a was subsequently determined by analyzing the 2D NMR spectroscopic data. Briefly, a cleavage of the C-12/C-13 bond in 4 to give a 12-carboxyl in 4a was deduced from the vicinal coupling cross-peaks H-9/H2-11 and H2-13/H2-14 in the 1H−1H COSY spectrum as well as the long-range heteronuclear correlations H-1/C-2, C-9, and C-20; H-9/C-5, C-8, C-10, C-11, C-12, and C-14; H2-11/C-8, C-9, C-10, and C-12; H2-13/C-8, C-14, and C-16; H2-14/C-8, C-15, and C-16; and H-15/C-8, C-9, C-14, C-16, and C-17 in the HMBC spectrum (Supporting Information Fig. S8). A lactone formation was constructed by the requirements of the molecular formula and chemical shift values of H-1 and C-1 and C-12. The NOE correlations between H-1 with H-9 and H-11a (Supporting Information Fig. S9) in the ROESY spectrum were supportive for the lactone and configuration of 4a, though the expected correlation from H-1 to C-12 did not appear in the HMBC spectrum (possibly due to limitation of the sample amount). Hence, compound 4a was assigned to have the structure deriving from an unprecedented intermolecular cleavage of the C-
Figure 5  The overlaid $^1$H NMR spectra of 2 (a)–(c) and 1 (d)–(f) in D$_2$O showing selective deuteration of H-11a and equilibration between 1-d and 2-d. (a) initially obtained for 2; (b) reacquired with the same sample of (a) after evaporated under reduced pressure to dry then re-dissolved in D$_2$O; (c) reacquired with the same sample of (b) after stored at 4 °C for 14 months (the splitting of H-9 and appearing of H-11a and H-11b may be explained by the presence of a relative larger amount of H$_2$O, which was partially introduced during storage of the sample, see the full spectra in Fig. S93–S95); (d) reacquired with the same sample of (c) after stored at 4 °C for 14 months; (e) reacquired with the same sample of (f) after evaporated under reduced pressure to dry and re-dissolved in D$_2$O; (f) initially obtained for 1.

Figure 6  The overlaid $^1$H NMR spectra of 1 in CD$_3$OD showing production of 1a or 1a-d (a)–(d) and the $^1$H NMR of 2 in CD$_3$OD showing no product formation (e). (a) initially obtained for 1; (b) reacquired with the same sample of (a) after measurements of the $^{13}$C NMR and DEPT spectra; (c) reacquired with the same sample of (b) after stored in D$_2$O at 4 °C for 14 months then evaporated under reduce pressure and re-dissolved in CD$_3$OD; (d) reobtained with the same sample of (c) after stored at 4 °C for two weeks; (e) initially obtained for 2 in CD$_3$OD.
12—C-13 bond in 4. Although transformation mechanisms from 4 to 4a could temporarily be proposed via a ketene intermediate with migration of one proton from C-11 to C-13 or a retro-Claisen reaction (Scheme 1), the two plausible mechanisms should result in deuteration of one proton at C-11 (via the ketene intermediate to give 4a-11d) or C-13 (via the retro-Claisen reaction to give 4a-13d). However, this was contrary to the observable H2-11 and H2-13 signals in the NMR spectra of 4a (Supporting Information Fig. S108—S119). Therefore, the transformation mechanisms from 4 to 4a seemed highly abnormal.

Because TMSP is a water-soluble salt with basicity, to inspect a possible promotion of the reaction by basicity (a suitable condition of the retro-Claisen reaction), 4 was treated with pyridine-d5 in the NMR tube for a convenient detection of the reaction. After the pyridine-d5 solution of 4 was stored at room temperature for 12 h, a new product 4b was detectable by the NMR spectra. Analyzing the 1D and 2D NMR spectroscopic data of the reaction mixture (Supporting Information Table S3 and Figs. S8 and S9), 4b was preliminarily elucidated to have an aza acetal structure deriving from dehydrogenation of 4. Follow-up HPLC isolation of the reaction mixture afforded the product with recovering 4. However, an iminium structure 4c instead of the aza acetal 4b was deducted from HR-ESI-MS as well as 1D and 2D NMR spectroscopic data of the product in D2O (Table S4 and Figs. S8 and S9). Based on our previous investigation on transformation between the alcohol iminium and aza acetal forms of several C20-diterpenoid alkaloids from the same extract42, 4b in pyridine-d5 was speculated to be transformed into 4c in D2O (Scheme 1). The speculation was proved by the reacquired 1H NMR spectrum of the sample in pyridine-d5. Therefore, under the basic condition of pyridine-d5, the C-12—C-13 bond in 4 was not cleaved to give 4a, whereas 4b was formed existing only as the alcohol iminium 4c in D2O. In addition, 4 was individually treated with equimolar sodium propionate (replacing TMSP) in H2O or TMS in CD3OD under room temperature for 48 h and then heating at 50 ºC, TLC and HPLC analysis indicated that no product was produced in the solutions. Hence, TMSP must play an important role in transforming 4 into 4a, which is unprecedented with currently unknown mechanism.

Additionally, in order to investigate possible artificial formation of 1—4, the putative biosynthetic precursor songorine (5), obtained from the same extract in this study, was refluxed with sodium bisulfite (Na2SO3) in CH3OH/H2O (1:1) for 8 h. TLC and HPLC analysis of the reaction mixture showed production of two products 5a and 5b (Scheme 4). Following HPLC isolation of 5a and 5b from the reaction mixture, their structures were deduced from the HR-ESI-MS and NMR experimental data (Supporting Information Table S4 and Figs. S8 and S9) as deethylsongorine (5a)43 and aconicarmichinium B (5b)44, respectively. Although the former was isolated from Aconitum monticola in 1965 and recently studied by a single crystal X-ray diffraction, the full NMR spectroscopic data (Table S4) were absent in literature51. The later was previously obtained from the extract and proved to exist as the aza acetal and alcohol iminium forms in D2O and pyridine-d545, respectively. The Na2SO3-induced deethylation and dehydrogenation of diterpenoid alkaloids have never been reported. Furthermore, transformation from 3 to 1, 2, and/or 4 was not observed by refluxing 3 in water or HPLC mobile phase for 8 h. Therefore, artificial production of 1—4 from 5 as well as 1, 2, and/or 4 from 3 could preliminarily be excluded.

2.3 Biogenetic relationships of 1—4

According to the structural architectures of 1—3, the co-occurring napelvine-type C20-diterpenoid alkaloid songorine (5)45 is proposed to be their biosynthetic precursor (Scheme 2). The precursor undergoes sequentially enzyme-assisted double bond oxidation and epoxide ring opening through 6 to generate a key enzyme-adducting intermediate 7. Semipinacol rearrangement52 via 1,2-migration of the C-13—C-16 bond in 7 affords 8. Subsequent

Figure 7 The overlaid 1H NMR spectra of 1-d in CD3OD showing gradual formation 1a-d. (a)—(g) successively acquired with the same sample via a two-day interval.
enzyme-catalyzed selective hydrogenation of the newly formed carbonyl group in 8, followed by concurrent or sequential dehydratation via 9, produces an enol intermediate 10. The enol intermediate undergoes either oxidative hydrolysis and isomerization via 11 or a reverse sequence via 12 to afford 1 and/or 2. Anyway, an equilibration between 1 and 2 would be achieved by interconversion in the aqueous biosystems since the equilibration was proved by the aforementioned experiments. Compound 3 is generated by oxidative hydrolysis of the intermediate 7. As predicted in the biosynthetic pathway of 4 (red arrow part in Scheme 2)\textsuperscript{95}, 3 may be produced by a direct sulfonation of 5 and/or 6. Alternatively, 1 and 2 would be generated via semipinacol rearrangement of the C-13–C-16 bond in 3, followed by sequential or spontaneous reduction, dehydration, and isomerization through 13, 14, and 12. Although 1 and 2 possess the same carbon skeleton as that of 4\textsuperscript{58}, biogenetically 1 and 2 are proposed to be generated from the C-13–C-16 bond migration of the precursors while 4 from the C-15–C-16 bond migration. In particular, the coexistence of 1–4 in the extract not only supports the proposed biogenetic pathway (Scheme 2) but also indicates the biogenetic unselective migration of the C-13–C-16 and C-15–C-16 bonds in the key intermediates 7 or 3. In order to keep the consistency with that of the precursor, the numbering for C-16 and C-17 in 4 is exchanged in 1 and 2. In addition, the proposed biosynthetic pathways fully support assignment of the absolute configurations of 1–3 since the absolute configurations of 4\textsuperscript{15} and a derivative of 5\textsuperscript{45} were proved by single crystal X-ray diffraction.

2.4. Analgesic activities of 1–3, 4a, 4c, 5a, and 5b

Because “fu zi” is clinically used as an analgesic ingredient for the treatment of various pains and molecular targets of the unusual alkaloids are unpredictable, analgesic effects of the compounds on an available animal model of acetic acid-induced writhing assay\textsuperscript{95} were preliminarily tested in this study (approved by the Animal Care & Welfare Committee Institute of Materia Medica, CAMS & PUMC). In spite of toxicity, flaconitine (3-acetylaconitine) was used as the positive control since it is a well identified analgesic constituent from the plants of the genus Aconitum. The assay result is given in Table 2 and Supporting Information Fig. S7. Comparing with the vehicle group, at 1.0 mg/kg (i.p.), compounds 1, 2, 5, 5a, and 5b exhibited analgesic effects against mice writhing with inhibition rates >26%. Especially, at 1.0, 0.3, and 0.1 mg/kg (i.p.), dose-dependent inhibitions were exhibited by 5 and 5a. Although the positive control gave a better inhibition (71.2%) at 0.3 mg/kg (i.p.), its toxicity was shown at 1.0 mg/kg (i.p.), however, toxicity was not observed for the other tested compounds. Due to interconversion between 1 and 2 is inevitable, their pharmacological effects must be contributed by equilibrations of the two compounds in the test samples. The two samples in this study were much less active than 4\textsuperscript{15}, demonstrating that the absence of the conjugated ketene moiety in 1 and 2 significantly decreased the activity. In addition, ineffectiveness of 4a and 4c reveals that the carbon skeleton shared by 1, 2, and 4 was essential for the mice writhing inhibition. Comparison of the analgesic effects of 3, 5, 5a, and 5b sharing the napelline skeleton indicates that the exocyclic double bond is likely the most important pharmacophore since 3 was inactive, meanwhile, the activity was decreased by dislodging N-ethyl (5a) or leading-in iminium double bond (5b) in the structure.

3. Conclusions

Three sulfonated C\textsubscript{20}-diterpenoid alkaloids (1–3) were discovered from A. carmichaelii lateral roots (fu zi). These compounds, along with aconicamisulfonfaine A (4)\textsuperscript{45} and aconicitamsulfonfaines A and B\textsuperscript{47} from the same extract, are the only sulfonated diterpenoid alkaloids from nature so far, architecturally covering three carbon skeletons. This unravels the general occurrence of sulfonation of the different types of diterpenoid alkaloids in this plant or the genus. Differential deuteration of H\textsubscript{2}–11 in 1–4 in D\textsubscript{2}O reveals that these compounds may actively interact with bioenvironments and biomolecules or targets to exhibit biological functions under physiological conditions. Chemical activity of H\textsubscript{2}–11 in these compounds is highly peculiar since deuteration of H-13 locating between the two carbonyl groups (1, 2, 4, 4b, and 4c) or attaching to one carbonyl group (3, 4a, 5, 5a, and 5b) was not observable. Mutual transformation between 1 and 2 in D\textsubscript{2}O indicates that the equilibration possibly exists in the aqueous biosystems to regulate their specific biological activities. Especially abundance in the equilibration and selectivity of the nucleophilic addition show that 1 is more stable but chemically more active than 2. These properties would play some roles in the pharmacological and biological functions of 1 and/or 2. In addition, all the reactions, including the TMSP-2,2,3,3-d\textsubscript{4} promoted C-12–C-13 bond cleavage and dehydrogenation of 4 as well as the Na\textsubscript{2}SO\textsubscript{3} assisted

\begin{equation}
\text{Scheme 3} \quad \text{Deuteration and conversion of 1 and 2 in D}_{2}\text{O and ketoacetal formation of 1 in CD}_{3}\text{OD.}
\end{equation}
spontaneous dehydrogenation and deethylolation of 5, are unprecedented and useful for structural modification of the diterpenoid alkaloids under a relatively mild condition. The transformation from the azal acetal (4b) in pyridine-d$_5$ into alcohol iminium (4c) in D$_2$O is fully consistent with the behavior of several napelline-type diterpenoid alkaloids, indicating that the structural variation exists and possibly has biological/pharmacological significances under neutral, acidic, and alkali microenvironments of the biosystems$^{32}$. Moreover, simultaneous isolation of 1–4 demonstrates a rational path for biogenetic and chemical synthesis of these C$_{20}$-diterpenoid alkaloids. Although 1 and 2 share the same carbon skeleton with 4, the co-occurrence of 1–4 strongly supports the novel carbon skeleton deriving from the different carbon-bond migrations of the napelline-type precursor(s) via the semipinacol rearrangements in enzyme-involved biosynthesis. Especially 3 represents the most possible intermediate or the concurrent product in the biosynthetic pathway of 1, 2, and 4 though our attempts of chemical transformation from 3 to 1, 2, and 4 failed. Like amino acids having both the alkali (amine/iminium) and acidic (sulfonic acid) functionalities, the sulfonated diterpenoid alkaloids have specific zwitterionic properties and good bio-compatibilities to play biological and/or pharmacological functions in the biosystems. The analgesic effects of the different types of C$_{20}$-diterpenoids testify that the multiple components work together in the clinic effects of this herbal medicine. The observed preliminary structure–activity relationships are helpful for new drug development based on the C$_{20}$-diterpenoid leads. In summary, this study is opening a new window for the diterpenoid alkaloids in candidate discovery and new drug development as well as for the discovery of unknown functional molecules in the herbal medicine to support its clinic utilization. The protocols focusing on diverse constituents of the water extract, especially on minor components, should be validated for many of the Chinese herbal medicines.

4. Experimental

4.1. General experimental procedures

See Supporting Information.

4.2. Plant material

See Ref. 39.

4.3. Extraction and isolation

For preliminary extraction and isolation, see Refs. 39–46 and Supporting Information. Fraction C2-1-C-5 (13 g) was chromatographed over Sephadex LH-20 (H$_2$O) to yield C2-1-C-5-1–C2-1-C-5-6, of which C2-1-C-5-1 (0.8 g) was further fractionated by column chromatography (CC) over HW-40C, eluting with H$_2$O, to afford C2-1-C-5-1–C2-1-C-5-6. Purification of C2-1-C-5-1-4 (28 mg) by reversed phase HPLC (Ultimate XB-phenyl semi-preparative column, 20% CH$_3$OH in H$_2$O, containing 0.2% TFA, flow rate 2 mL/min) yielded 1 (5 mg, t$_R$ = 56 min) and 2 (2 mg, t$_R$ = 51 min). Fraction C2-1-C-5-4 (4 g) was separated by CC over Sephadex LH-20 (H$_2$O) to give C2-1-C-5-4–C2-1-C-5-4-7, of which C2-1-C-5-4-6 (1.1 g) was isolated by CC over reversed phase C-18 silica gel, eluted by a gradient mobile phase increasing MeOH (0–50%) in H$_2$O, to yield C2-1-C-5-4-6-1–C2-1-C-5-4-6-5. Separation of C2-1-C-5-4-6-5 (16 mg) by reversed phase HPLC using the same column and the mobile phase of 20% CH$_3$OH in H$_2$O containing 0.5% TFA (flow rate 2 mL/min) obtained 3 (5.1 mg, t$_R$ = 44 min).

4.3.1. Aconicarmisulfonine B (1)

White amorphous powder (MeOH); [α]$_D^{20}$ = 38.1 (c 0.29, CH$_3$OH); UV (CH$_3$OH) $\lambda_{\text{max}}$ (log e) 202 (2.24), 223 (1.62), 267 (0.89) nm; CD (CH$_3$OH) $\lambda_{\text{max}}$ ($\Delta\varepsilon$) 200 (−1.18), 290 (−0.43), 329 (+0.04); IR $\nu_{\text{max}}$ 3393, 2970, 2922, 2850, 1738, 1768, 1485, 1466, 1420, 1321, 1202, 1140, 1049, 957, 905, 879, 838, 801, 722 cm$^{-1}$; $^1$H NMR (D$_2$O, 600 MHz) spectroscopic data, see Table 1; $^{13}$C NMR (D$_2$O, 150 MHz) spectroscopic data (Table S1); $^1$H NMR (CD$_3$OD, 600 MHz) spectroscopic data (Table S1); $^{13}$C NMR (CD$_3$OD, 150 MHz) spectroscopic data (Table S1); (+)-ESI-MS m/z 438 [M+H]$^+$, 460 [M+Na]$^+$, (+)-ESI-MS m/z 436 [M–H]$^-$; (+)-HR-ESI-MS m/z 438.1954 [M+H]$^+$ (Calcd. for C$_{22}$H$_{30}$NO$_6$S, 438.1945); (+)-HR-ESI-MS m/z 436.1813 [M–H]$^-$ (Calcd. for C$_{22}$H$_{29}$NO$_6$S, 436.1799).

4.3.2. Aconicarmisulfonine C (2)

White amorphous powder (CH$_3$OH); [α]$_D^{20}$ = −37.4 (c 0.14, CH$_3$OH); UV (CH$_3$OH) $\lambda_{\text{max}}$ (log e) 202 (2.46), 219 (1.65) nm; CD (CH$_3$OH) $\lambda_{\text{max}}$ (Δε) 200 (−0.54), 296 (−0.65); IR $\nu_{\text{max}}$ 3394, 2920, 2850, 1736 (sh), 1680, 1543, 1448, 1358, 1203, 1140, 1053, 983, 956, 907, 880, 842, 802, 724 cm$^{-1}$; $^1$H NMR (D$_2$O, 600 MHz) spectroscopic data, see Table 1; $^{13}$C NMR (D$_2$O, 150 MHz) spectroscopic data (Table S1); $^1$H NMR (CD$_3$OD, 600 MHz) spectroscopic data (Table S1); $^{13}$C NMR (CD$_3$OD, 150 MHz) spectroscopic data (Table S1); (+)-ESI-MS m/z 438 [M+H]$^+$, 460 [M+Na]$^+$, 476 [M+K]$^+$, (+)-ESI-MS m/z 436 [M–H]$^-$; (+)-HR-ESI-MS m/z 438.1945 [M+H]$^+$ (Calcd. for C$_{22}$H$_{32}$NO$_6$S, 438.1945); (+)-HR-ESI-MS m/z 436.1813 [M–H]$^-$ (Calcd. for C$_{22}$H$_{31}$NO$_6$S, 436.1799).

4.3.3. Chuanfusulfonine A (3)

White amorphous powder (CH$_3$OH); [α]$_D^{20}$ = −7.3 (c 0.15, CH$_3$OH); UV (CH$_3$OH) $\lambda_{\text{max}}$ (log e) 202 (2.48), 222 (1.48) nm; CD (CH$_3$OH) $\lambda_{\text{max}}$ (Δε) 220 (+0.05), 250 (−0.04), 296 (−0.09); IR
The samples of 1 (3.0 mg) and 2 (1.8 mg) in D$_2$O, which were stored in a refrigerator at 4 °C for 14 months and detected by the NMR spectral measurements to be identical, were combined and evaporated under reduced pressure to give a mixture. Isolation of the residue by HPLC (YMC-Pack Ph column, 15% CH$_3$OH in H$_2$O containing 0.1% TFA, flow rate 2 mL/min) yielded 2 (0.8 mg, $t_R = 28$ min) and 4 (3.1 mg, $t_R = 31$ min). Recovery of 4 was proved by complete identity of the $^1$H NMR spectrum with that of the original sample. 4a: white amorphous powder; $^1$H NMR (D$_2$O, 600 MHz) spectrpspic data (Table S3); $^{13}$C NMR (D$_2$O, 150 MHz) spectroscopic data (Table S3); (+)-HR-ESI-MS $m/z$ 436.1790 (Calcd. for C$_{22}$H$_{30}$NO$_6$S, 436.1788), 434.16345 [M+Na$^+$] (Calcd. for C$_{21}$H$_{28}$NO$_6$SNa, 478.1870), (−)-HR-ESI-MS $m/z$ 454.1916 [M−H]$^-$ (Calcd. for C$_{22}$H$_{29}$NO$_7$S, 454.1905).

4.3.4. ECD calculations of 1–3

See Supporting Information.

4.3.5. Isolation and structural characterization of 1-d, 2-d, 4a, 4b, and 4c

The samples of 1 (3.0 mg) and 2 (1.8 mg) in D$_2$O, which were stored in a refrigerator at 4 °C for 14 months and detected by the NMR spectral measurements to be identical, were combined and evaporated under reduced pressure to give a mixture. The mixture was separated by HPLC using the YMC-Pack Ph column and the mobile phase of 20% CH$_3$OH in H$_2$O containing 0.5% TFA (flow rate 2 mL/min) to afford two compounds. The $^1$H NMR spectrum of the major compound (3.8 mg, $t_R = 37$ min) in D$_2$O indicated it was 1-d. However, the $^1$H NMR spectrum of the minor one (0.4 mg, $t_R = 31$ min) in D$_2$O was almost identical to that of the mixture prior to HPLC separation. Because the two peaks in the HPLC chromatogram were completely separated, the minor compound must be 2-d and was partially transformed again into 1-d during evaporation of the HPLC mobile phase. Although further isolation of 2-d failed due to limitation of the sample amount, the structure was able to be determined by 2D NMR spectroscopic data analysis of the mixtures acquired prior to HPLC separation, while the $^1$H and $^{13}$C NMR (D$_2$O or CD$_3$OD, 600 MHz) spectroscopic data of 1-d and 2-d (Tables S1 and S2) were unambiguously assigned.

The sample of 4 (4.2 mg) in D$_2$O with sodium 3-trimethylsilylpropionate (TMSP-2,2,3,3-trimethyl-1,1-d$_2$) as the internal standard, was further carried out in the 2D NMR experiments. The structure of 4b was determined by analysis of the 2D NMR spectroscopic data, for which the $^1$H NMR (D$_2$O, 600 MHz) spectrpspic data (Table S3); $^{13}$C NMR (D$_2$O, 150 MHz) spectroscopic data (Table S3); (+)-HR-ESI-MS $m/z$ 436.17902 [M+H]$^+$ (Calcd. for C$_{22}$H$_{30}$NO$_6$SNa, 478.1870), (−)-HR-ESI-MS $m/z$ 454.1916 [M−H]$^-$ (Calcd. for C$_{22}$H$_{29}$NO$_7$S, 454.1905).

| Table 2 | Experimental data for the analgesic effect of compounds 1–3, 4a, 4c, 5a, and 5b. |
|---|---|---|---|---|---|
| Group | Reagent | Dose (mg/kg) | Number of mice | Number of withering | Percent inhibition (%) |
| Vehicle group | Normal saline | 0.0 | 10 | 36.22 ± 2.50 | 0.0 |
| Positive group | Flaconitine | 0.3 | 10 | 10.4 ± 2.62*** | 71.28 |
| Test group | 1 | 1.0 | 10 | 24.9 ± 4.61* | 31.26 |
| | 0.3 | 10 | 35.3 ± 1.60 | 2.55 |
| | 0.1 | 10 | 34.9 ± 2.41 | 3.65 |
| | 0.3 | 10 | 26.5 ± 3.53* | 26.84 |
| | 0.1 | 10 | 35.5 ± 1.69 | 1.99 |
| | 3 | 1.0 | 10 | 31.8 ± 4.13 | 12.20 |
| | 0.3 | 10 | 32.9 ± 4.35 | 9.17 |
| | 0.1 | 10 | 33.1 ± 4.42 | 8.62 |
| | 4a | 1.0 | 10 | 29.9 ± 4.21 | 17.45 |
| | 0.3 | 10 | 30.1 ± 4.03 | 16.07 |
| | 0.1 | 10 | 38.2 ± 3.17 | 0.0 |
| | 4c | 1.0 | 10 | 31.4 ± 6.66 | 13.31 |
| | 0.3 | 10 | 30.9 ± 4.13 | 14.69 |
| | 0.1 | 10 | 35.8 ± 5.94 | 1.16 |
| | 5 | 1.0 | 10 | 17.7 ± 2.45*** | 51.13 |
| | 0.3 | 10 | 25.9 ± 2.98* | 28.49 |
| | 0.1 | 10 | 35.6 ± 2.32 | 1.72 |
| | 5a | 1.0 | 10 | 18.5 ± 2.29** | 48.92 |
| | 0.3 | 10 | 26.2 ± 4.61 | 27.66 |
| | 0.1 | 10 | 30.3 ± 4.82 | 16.35 |
| | 5b | 1.0 | 10 | 23.8 ± 3.10* | 34.29 |
| | 0.3 | 10 | 25.6 ± 3.11 | 29.32 |
| | 0.1 | 10 | 36.5 ± 3.64 | 0.0 |

Note: data are expressed as mean ± SEM, *P < 0.05, **P < 0.01, ***P < 0.001 compared to the model group.
4.3.6. Reaction of 5 with sodium bisulfite

Compound 5 (10.2 mg) and sodium bisulfite (Na₂SO₃, 20.0 mg) were dissolved in CH₃OH/H₂O (1:1; 10.0 mL) and kept at room temperature for 24 h. With TLC detection, no product was formed in the reaction mixture. Then the solution was refluxed for 8 h and two compounds were produced as detected by TLC. After removal of the solvent, the residue was separated by HPLC (YMC-Pack PH column, 25% CH₃OH in H₂O containing 0.1% TFA, flow rate 2 mL/min) to yield 5a (1.6 mg, tR = 26 min) and 5b (3.8 mg, tR = 28 min). 5a: white amorphous powder; 1H NMR (D₂O, 600 MHz) spectroscopic data (Table S4); 13C NMR (D₂O, 150 MHz) spectroscopic data (Table S4); (--)HR-ESI-MS m/z 330.2070 [M-H]⁻ (Calcd. for C₂₀H₂₈NO₃, 330.2064). The spectroscopic data 5b were completely indentical with the reported data of aconicarmichinium B [37].

4.5. Acetic acid-induced writhing test

See Supporting Information.

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Author contributions

Jiangong Shi designed and guided all the chemical experiments, analyzed the data, and rewrote and revised the manuscript. Qinglan Guo and Huan Xia conducted the chemical experiments, doublechecked the data, and wrote the preliminary manuscript. Tiancai Zhang designed the pharmacological test and analyzed the corresponding data. Shuai Shao conducted the pharmacological experiments. Yuzhuo Wu and Chengbo Xu assisted the chemical experiments. All authors read and approved the final manuscript.

Conflicts of interest

The authors have no conflicts of interest to declare.

Appendix A. Supporting information

Supporting data to this article can be found online at https://doi.org/10.1016/j.apsb.2020.01.013.

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