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Croomine- and tuberostemonine-type alkaloids from roots of *Stemona tuberosa* and their antitussive activity

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ABSTRACT

Three new croomine-type *Stemona* alkaloids, tuberocrooline (**1**), 10-hydroxycroomine (**2**), and dehydrocroomine (**3**), and four new tuberostemonine-type alkaloids, tuberostemoline (**4**), tridehydrotuberostemonine (**5**), 9α-bisdehydrotuberostemonine (**6**), and 9α-bisdehydrotuberostemonine A (**7**), along with ten known constituents, were isolated from the roots of *Stemona tuberosa* collected from Yunnan province. The structures of the new compounds were established on the basis of one- and two-dimensional NMR spectra and other spectroscopic studies. The antitussive activity of the major alkaloids was tested using the citric acid-induced guinea pig cough model. Croomine (**8**) exhibited a dose-dependent inhibition of coughing with an ID_{50} value of 0.18 mmol/kg.

1. Introduction

The herb Radix Stemonae ('Baibu' in Chinese) has been used as an antitussive and insecticide remedy for thousands of years.[1,2] The species *Stemona tuberosa* (Stemonaceae), distributed widely in southern China, is documented in China Pharmacopoeia as one of three authentic plant resources for Baibu.[1] Many investigations on *S. tuberosa* of different localities have led to the isolation of more than 60 alkaloids,[3] which are classified into tuberostemonine-type,[4–8] stemoninine-type,[9,10] and croomine-type.[11,12] Some major alkaloids in Baibu, such as neotuberostemonine and neostenine, have been reported to exhibit antitussive potency comparable to codeine but without interactions with opioid receptors.[7] Bisdehydrostemoninine and stemoninine were reported to show significant antitussive activity in the guinea pig after cough induction by citric acid aerosol stimulation.[9,10] However, the major alkaloids of *S. tuberosa* vary greatly in samples collected from different locations,[4] which causes problems when attempting to determine the relationship between the specific medicinal use and the herbal growing locations. Thus, further investigation on this species appears warranted.

Herein, we describe the isolation and structural elucidation of alkaloids from the roots of *S. tuberosa* collected from Wenshan County, Yunnan province. Seven new alkaloids, including three croomine-type alkaloids, tuberocrooline (**1**), 10-hydroxycroomine (**2**), and dehydrocroomine (**3**), and four tuberostemonine-type ones, tuberostemoline (**4**), tridehydrotuberostemonine (**5**), 9α-bisdehydrotuberostemonine (**6**), and 9α-bisdehydrotuberostemonine A (**7**), were isolated and identified (Fig. 1). In addition to the new compounds, ten known alkaloids, croomine (**8**),[12] tuberospironine (**9**),[4] bisdehydrotuberostemonine,[6] tuberostemospironine,[8] 6-hydroxycroomine (**10**),[11,12] stemotinine,[13] isostemotinine,[13] tuberostemoenone,[14] sessilifoliamide F,[15] and tuberostemoninoamide[16] were also found. The structures of new compounds were elucidated by one- and two-dimensional NMR and other spectroscopic studies. The major alkaloids **2**, **3**, **8**, **9**, and **10** were tested for antitussive activity in the citric acid-induced guinea pig cough model. The results revealed that croomine-type alkaloids, especially croomine (**8**), might be responsible for the antitussive activity of *S. tuberosa* collected from Yunnan province.

2. Results and discussion

Tuberocrooline (**1**) was obtained as colorless oil. The HR-EIMS afforded the molecular formula $C_{18}H_{29}NO_5$ (*m*/*z* 339.2042 $[M]^+$) with five unsaturation degrees. The IR absorption band at

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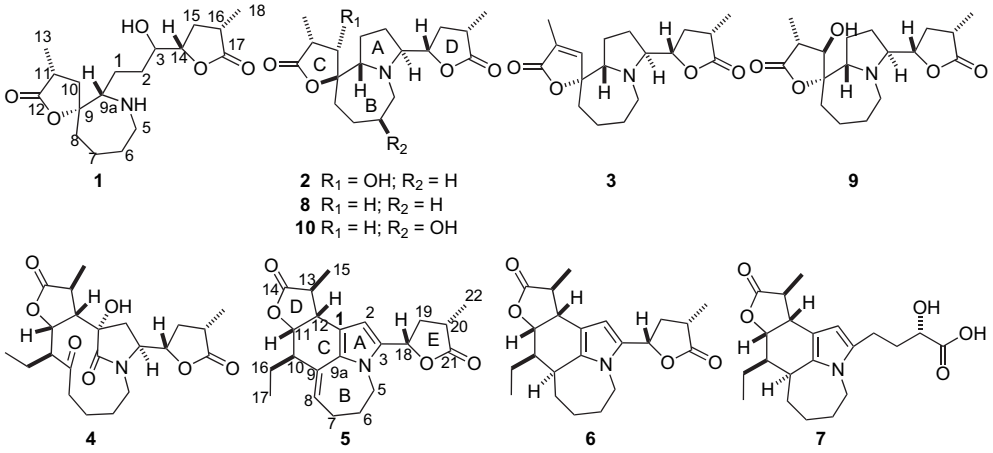

Figure 1. Alkaloids from *S. tuberosa*.

1768 cm^{-1} and the characteristic EIMS cleavage fragment *m*/*z* 240 $[M-C_5H_7O_2]^+$ indicated the presence of a typical α-methyl-γ-lactone moiety.[9] The strong IR absorption at 3396 cm^{-1}, as well as the EIMS fragments *m*/*z* 321 $[M-H_2O]^+$ and *m*/*z* 222 $[M-C_5H_7O_2-H_2O]^+$, revealed a hydroxyl group in the structure. The ^{13}C NMR and DEPT spectra displayed 18 carbon signals, involving 2 carbonyl carbons, 1 quaternary carbon, 5 methine carbons, 8 methylene carbons, and 2 methyl carbons (Table 1). Thus, two active protons were revealed in the molecule. The ^1H NMR spectrum (Table 2) showed 2 secondary methyls at δ 1.20 (3H, d, *J*=4.5 Hz, H_3-18) and 1.24 (3H, d, *J*=4.0 Hz, H_3-13), 2 oxymethines at δ 4.51 (1H, m, H-3) and 4.77 (1H, m, H-14), and 1 methylene and 1 methine bearing a nitrogen functionality at δ 3.53 (1H, m, H-5), 3.58 (1H, m, H-5), and 3.65 (1H, dd, *J*=5.7, 7.8 Hz, H-9a). Three spin systems (bold lines in Fig. 2a) were constructed by the ^1H–^1H COSY spectrum and further connected by the HMBC correlations (arrows in Fig. 2a). Since two α-methyl-γ-lactone rings and one azepine ring accounted for all the unsaturation degrees, no other ring existed in the molecule. The obvious down-fielded chemical shift of H-3 (δ 4.51), in comparison with the corresponding resonance (δ 3.25) in croomine,[4] indicated that a hydroxyl group instead of a nitrogen functionality was attached to C-3. Therefore, **1** was deduced to be a croomine-type alkaloid with an opened ring A.

The relative configuration was revealed by ROESY spectrum (broken arrows in Fig. 2b) and its biogenetic consideration. Since CH_3-18 is α-oriented in croomine-type alkaloids, the ROESY correlation of H-14/H-16 indicated that H-14 is β-oriented. The correlations of H-9a/H-11 and H-9a/H-10β suggested that H-9a, H-10β, and H-11 were in the same face and β-oriented, and C-9 was therefore in rel-(*S*) configuration. These results allowed assignment of the relative configurations of chiral centers as rel-(9*S*, 9a*S*, 11*R*, 14*S*, and 16*S*), but the stereochemistry of C-3 remained unknown. Compound **1** is the first example of 3,4-*seco* croomine-type alkaloid.

10-Hydroxycroomine (**2**) was obtained as a yellow amorphous powder. The HR-EIMS of **2** indicated the molecular formula as $C_{18}H_{27}NO_5$ (*m*/*z* 337.1892 $[M]^+$) with six degrees of unsaturation. The IR absorption at 1768 cm^{-1} and the characteristic EIMS cleavage fragment *m*/*z* 238 $[M-C_5H_7O_2]^+$ indicated the existence of an α-methyl-γ-lactone ring annexed at C-3. The strong and broad IR absorption band at 3431 cm^{-1} suggested the presence of a hydroxyl group. The ^1H and ^{13}C NMR data suggested that

Table 1
^{13}C NMR data of alkaloids **1**–**7** (100 MHz; δ in ppm)

C	**1**[a]	**2**[b]	**3**[b]	**4**[b]	**5**[b]	**6**[c]	**7**[b]
1	26.7 (t)	26.8 (t)	26.4 (t)	74.3 (s)	116.6 (s)	114.0 (s)	112.9 (s)
2	26.7 (t)	26.5 (t)	26.0 (t)	34.4 (t)	106.0 (d)	105.9 (d)	103.1 (d)
3	60.3 (d)	68.0 (d)	67.1 (d)	61.4 (d)	128.6 (s)	136.2 (s)	131.5 (s)
5	63.2 (t)	48.2 (t)	49.3 (t)	43.1 (t)	46.4 (t)	44.7 (t)	44.2 (t)
6	34.6 (t)	22.1 (t)	22.6 (t)	27.1 (t)	31.3 (t)	27.0 (t)	29.3 (t)
7	21.4 (t)	29.7 (t)	38.6 (t)	19.7 (t)	25.5 (t)	29.7 (t)	30.3 (t)
8	41.1 (t)	27.9 (t)	27.9 (t)	43.0 (t)	125.3 (d)	35.6 (t)	27.0 (t)
9	73.5 (s)	89.5 (s)	91.1 (s)	213.3 (s)	124.1 (s)	36.3 (d)	34.9 (d)
9a	65.4 (d)	68.7 (d)	66.6 (d)	175.7 (s)	129.7 (s)	128.7 (s)	132.7 (s)
10	41.9 (t)	72.4 (d)	150.6 (d)	51.0 (d)	48.2 (d)	35.1 (d)	42.2 (d)
11	35.4 (d)	40.1 (d)	130.5 (s)	74.8 (d)	80.6 (d)	79.6 (d)	79.9 (d)
12	176.7 (s)	171.7 (s)	173.2 (s)	53.8 (d)	39.8 (d)	41.2 (d)	42.6 (d)
13	18.7 (q)	10.0 (q)	10.7 (q)	34.7 (d)	36.0 (d)	42.3 (d)	41.4 (d)
14	81.7 (d)	79.9 (d)	79.9 (d)	179.0 (s)	179.8 (s)	178.9 (s)	180.1 (s)
15	34.4 (t)	34.7 (t)	35.0 (t)	16.8 (q)	15.6 (q)	14.7 (q)	14.0 (q)
16	37.2 (d)	34.9 (d)	34.9 (d)	22.5 (t)	23.2 (t)	21.3 (t)	20.9 (t)
17	182.2 (s)	179.3 (s)	179.3 (s)	8.2 (q)	12.1 (q)	14.0 (q)	11.2 (q)
18	15.6 (q)	14.8 (q)	14.8 (q)	77.5 (d)	71.6 (d)	71.8 (d)	38.6 (d)
19				34.3 (t)	35.2 (t)	30.3 (t)	33.3 (t)
20				34.4 (d)	45.0 (d)	42.7 (d)	24.1 (d)
21				179.0 (s)	178.8 (s)	179.1 (s)	179.9 (s)
22				14.6 (q)	15.0 (q)	11.1 (q)	17.0 (q)

[a] In CD_3OD.
[b] In $CDCl_3$.
[c] In CD_3COCD_3.

Table 2
^{1}H NMR spectral data of alkaloids **1**–**3** (400 MHz; δ in ppm, J in Hz)

H	**1**[a]	**2**[b]	**3**[b]
1	2.14, 2.03 each m	1.84, 1.50 each m	1.86, 1.46 each m
2	2.04, 1.75 each m	1.98, 1.74 each m	1.90, 1.80 each m
3	4.51 m	3.46 (dd, 7.5, 13.9)	3.15 m
5	3.53, 3.58 each m	3.30, 3.11 each m	3.18 m, 2H
6	1.54 m, 2H	1.78, 1.64 each m	1.62 m, 2H
7	1.46 m, 2H	1.80, 1.56 each m	1.80, 1.60 each m
8	1.57, 1.50 each m	2.02, 1.74 each m	1.92, 1.78 each m
9a	3.65 (dd, 5.7, 7.8)	3.60 (t, 7.8)	3.54 (dd, 3.9, 8.2)
10	2.18 (dd, 9.0, 14.3), 1.54 m	4.32 (d, 7.8)	7.10 br s
11	2.48 m	2.80 m	
13	1.24 (d, 4.0)	1.27 (d, 2.8)	1.89 (d, 1.5)
14	4.77 m	4.38 m	4.31 m
15	2.35, 1.68 each m	2.39, 1.51 each m	2.38, 1.45 each m
16	2.74 m	2.63 m	2.59 m
18	1.20 (d, 4.5)	1.25 (d, 5.9)	1.23 (d, 6.8)

[a] In CD_3OD.
[b] In $CDCl_3$.

compound **2** contained a basic croomine-type skeleton (Tables 1 and 2). An oxymethine instead of a methylene in croomine was observed at δ 4.32, which showed HMBC correlations to C-9, C-9a, C-12, and C-13. It was evident that a hydroxyl group was attached to C-10. Thus, **2** was deduced as a 10-hydroxyl derivative of croomine. Such a structure has been previously constructed for tuberospironine.[4] These two compounds appeared to have different configurations. A NOE different experiment was applied to determine the relative configuration of **2** (broken arrows in Fig. 3). When the triplet methine at δ 3.60 (H-9a) was irradiated, enhancements of the signals of H-5β, H-8β, H-10, and H-11 suggested that H-9a, H-10, and H-11 are in β-orientation. When the oxymethine at δ 4.32 (H-10) was irradiated, enhancements of the signals of H-1β, H-9a, and H-11 indicated that CH_3-13 is α-oriented and C-9 is in rel-(*S*) configuration. The relative configurations of all chiral centers were assigned as rel-(3*S*, 9*R*, 9a*S*, 10*S*, 11*R*, 14*S*, and 16*S*).

Dehydrocroomine (**3**) was obtained as colorless needles. The molecular formula was determined by the HR-ESIMS to be $C_{18}H_{25}NO_4$ (m/z 342.1692 $[M+Na]^+$) with seven degrees of unsaturation. The IR spectrum showed the presence of a γ-lactone at 1767 cm^{-1}. The ^{1}H NMR spectrum of **3** (Table 2) exhibited the signals of a secondary methyl at δ 1.23 (3H, d, J=6.8 Hz, H_3-18), an allylic methyl at δ 1.89 (3H, d, J=1.5 Hz, H_3-13), and an olefinic proton at δ 7.10 (1H, br s, H-10). A detailed analysis of the NMR spectra revealed that compound **3** shared also the same basic skeleton of croomine (Tables 1 and 2).[4] The HMBC correlations from the olefinic proton to C-8, C-9, C-9a, C-11, C-12, and C-13 designated it as H-10. The configuration of C-9 was revealed by the

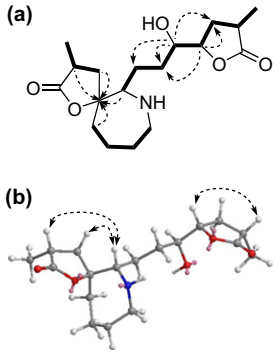

Figure 2. (a) ^{1}H–^{1}H correlations (bold lines) and key HMBC correlations (arrows) of **1**; (b) key NOE correlations and possible conformation of **1**.

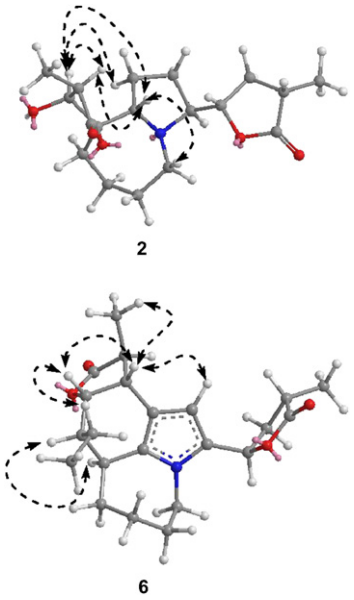

Figure 3. Key NOE correlations and possible conformations of **2** and **6**.

NOE difference experiment. When H-10 was irritated, the signals of H_3-13 and H-5β were enhanced. The result indicated H-5β, H-10, and H_3-13 were spatially adjacent and C-9 was in rel-(*R*) configuration.

Tuberostemoline (**4**) was obtained as a yellowish amorphous powder. The HR-EIMS data at m/z 421.2096 ($[M]^+$) established the molecular formula as $C_{22}H_{31}NO_7$ with eight degrees of unsaturation. The strong and sharp IR absorptions at 1776 and 1703 cm^{-1}, as well as the carbonyl signals at δ_C 179.0 and 213.3, suggested the presence of a saturated γ-lactone and a ketone group. The characteristic cleavage fragment m/z 322 $[M-C_5H_7O_2]^+$ in EIMS indicated the presence of a typical α-methyl-γ-lactone annexed at C-3.[9] The strong IR absorption at 3435 cm^{-1} and the EIMS fragments m/z 403 $[M-H_2O]^+$ and m/z 304 $[M-C_5H_7O_2-H_2O]^+$ revealed that a hydroxyl group was in the structure. The ^{13}C NMR and DEPT spectra showed 22 signals, including 4 carbonyl carbons, 1 quaternary carbon, 7 methine carbons, 7 methylene carbons, and 3 methyl carbons (Table 1). The ^{1}H NMR spectrum displayed signals for three methyls at δ 0.73 (3H, t, J=7.3 Hz, H_3-17), 1.29 (3H, d, J=7.3 Hz, H_3-22), and 1.45 (3H, d, J=6.9 Hz, H_3-15), and two oxymethines at δ 4.85 (1H, m, H-18) and 5.01 (1H, dd, J=6.5, 10.4 Hz, H-11) (Table 3). These data suggested that **4** was a tuberostemonine-type alkaloid.[3] The ^{1}H–^{1}H COSY spectrum constructed three spin systems (bold lines in Fig. 4a), which were further connected by HMBC experiment. Fragments **b** and **c** were connected by the HMBC correlations from H-7, H-10, and H-11 to the ketone carbon (δ 213.3), which was assigned at C-9. In addition, the HMBC cross-peaks from H-2, H-5, and H-12 to the carbonyl carbon (δ 175.7) suggested that this carbonyl was located at C-9a to form a lactam moiety. The hydroxyl group was attached to the quaternary carbon C-1 by the HMBC correlations from H-2 and H-12 to this carbon. Thus, **4** was constructed as a structural skeleton with an 11-membered macrocyclic ring, resulting from opening the C-9–C-9a bond in the perhydroazaazulene ring. Such a structural moiety has been observed in sessilifoliamide F.[14]

The ROESY correlations of H-11/H-12, H-11/H_3-15, H-11/H_3-17, and H-12/H_3-15 indicated that H-11, H-12, H_3-15, and H_3-17 were cis-related and all β-oriented (broken arrows in Fig. 4b). The cross-peaks of H-18/H-20 and H-18/H-6β suggested that H-18 and H-20 were also β-oriented and the 11-membered ring is in a stable chair conformation, and H-6β is axial as well. The ROESY cross-peaks of

Table 3
^{1}H NMR spectral data of alkaloids **4**–**6** (in $CDCl_3$, 400 MHz; δ in ppm, J in Hz)

H	4	5	6	7
2	2.02 m, 1.96 (dd, 8.8, 13.1)	6.00 s	6.02 s	5.73 s
3	3.58 m			
5	3.62 m	4.35 (dd, 7.5, 13.5)	4.23 (dd, 4.3, 11.4)	4.05 (dd, 4.8, 14.1)
5	3.32 m	3.76 (dd, 9.1, 13.5)	3.78 (dd, 12.0, 25.8)	3.68 (dd, 11.4, 14.1)
6	2.07, 1.81 each m	2.14, 1.86 each m	1.95, 1.48 each m	1.94, 1.28 each m
7	1.82 m, 2H	2.72, 2.25 each m	1.90, 1.42 each m	2.08 m, 1.51 (t, 12.1)
8	2.78 m, 2.29 (dd, 7.9, 16.2)	5.44 (t, 4.4)	1.98, 1.00 each m	1.92, 0.99 each m
9			2.88 m	2.85 (dd, 5.0, 11.2)
10	3.45 m	2.12 m	2.16 m	1.95 m
11	5.01 (dd, 6.5, 10.4)	4.37 (dd, 3.9, 5.4)	4.40 (dt, 2.0, 9.3)	4.40 (dd, 7.5, 11.2)
12	2.67 m	3.17 (d, 5.4)	3.17 (dd, 7.5, 11.3)	3.12 (dd, 7.5, 11.5)
13	3.48 m	2.70 m	2.45 m	2.45 m
15	1.45 (d, 6.9)	1.42 (d, 7.7)	1.40 (d, 7.4)	1.36 (d, 7.0)
16	1.91, 1.66 each m	1.40, 1.27 each m	1.92, 1.48 each m	1.95, 1.38 each m
17	0.73 (t, 7.3)	0.92 (t, 7.4)	0.95 (t, 6.7)	0.95 (t, 7.3)
18	4.85 m	5.36 (dd, 5.2, 11.2)	5.38 (dd, 5.7, 11.4)	2.57, 1.27 each m
19	2.59, 1.47 each m	2.16 m, 2H	2.79, 2.21 each m	2.00, 1.72 each m
20	2.74 m	2.66 m	2.71 m	2.59 m
22	1.29 (d, 7.3)	1.36 (d, 6.9)	1.36 (d, 7.2)	1.28 (d, 7.0)

H-2β/H-11 and H-2β/H-12 indicated that H-2β is also axial, which allowed only an *S*-configuration for C-1. Therefore the relative configurations of all chiral centers were assigned as rel-(1*S*, 3*S*, 10*S*, 11*R*, 12*S*, 13*S*, 18*S*, and 20*S*).

Tridehydrotuberostemonine (**5**) was isolated as a yellow amorphous powder. The molecular formula of **5** was established as $C_{22}H_{27}NO_4$ by HR-ESIMS (m/z 392.1840 $[M+Na]^+$) with ten degrees of unsaturation. The strong IR absorption at 1770 cm^{-1} indicated the presence of γ-lactone. The ^{13}C NMR and DEPT spectra of **5** displayed 22 carbon signals, involving 2 carbonyl carbons, 6 olefinic carbons, 6 methine carbons, 5 methylene carbons, and 3 methyl carbons (Table 1). In the ^{1}H NMR spectrum (Table 3) the characteristic signals of 3 methyls at δ 0.92 (3H, t, J=7.4 Hz, H_3-17), 1.36 (3H, d, J=6.9 Hz, H_3-22), and 1.42 (3H, d, J=7.7 Hz, H_3-15), and an olefinic proton at δ 6.00 (1H, s, H-2) strongly resembled the corresponding signals of bisdehydrotuberostemonine.[6] The other olefinic proton at δ 5.44 (1H, t, J=4.4 Hz, H-8), corresponding to two olefinic carbons at δ 124.1 and 125.3, suggested one additional double bond in the molecule. This double bond was assigned at C-8 and C-9 by HMBC correlations between H-8 and C-6, C-7, C-9a, and C-10, as well as correlations between C-9 and H-7, H-8 and H-11. The relative configuration of **5** was also revealed by the ROESY experiment. The correlations of H-11/H-12, H-11/H-16, H-11/H_3-17, and H-12/H_3-15 revealed that H-11, H-12, H_3-15, and the ethyl are cis-related and all β-oriented. Thus the relative configurations of all chiral centers were assigned as rel-(10*R*, 11*S*, 12*R*, 13*R*, and 20*S*).

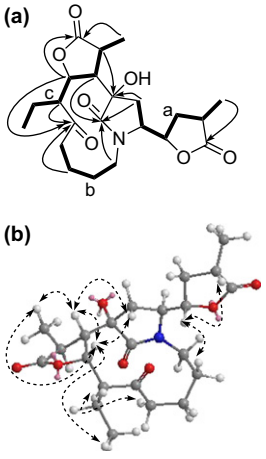

Figure 4. (a) ^{1}H–^{1}H correlations (bold lines) and key HMBC correlations (arrows) of **4**; (b) key NOE correlations and possible conformation of **4**.

9α-Bisdehydrotuberostemonine (**6**) was obtained as a colorless solid. The HR-EIMS (m/z 371.2091 $[M]^+$) indicated its molecular formula as $C_{22}H_{29}NO_4$, the same as that of bisdehydrotuberostemonine.[6] The existence of lactones was indicated by the IR absorption at 1770 cm^{-1}. The ^{1}H and ^{13}C NMR data of **6** and bisdehydrotuberostemonine were almost superimposable (Tables 1 and 3). Compound **6** was deduced to be a stereoisomer of bisdehydrotuberostemonine. The ROESY correlations of H-2/H-12, H-11/H-12, H-11/H-16, H-11/H_3-17, and H-12/H_3-15 revealed that H-11, H-12, H_3-15, and the ethyl were cis-related and β-oriented (Fig. 3). The correlation of H-9/H-10 indicated these two protons were both α-oriented. Thus the relative configurations of all chiral centers of **6** were assigned as rel-(9*S*, 10*R*, 11*S*, 12*R*, 13*R*, and 20*S*).

9α-Bisdehydrotuberostemonine A (**7**) was isolated as a colorless solid ($C_{22}H_{31}NO_4$ by HR-ESIMS). The IR absorption suggested the presence of a lactone (1770 cm^{-1}) and a hydroxyl group (3419 cm^{-1}). The NMR data comparison of **6** and **7** revealed that the signals of their rings A, B, C, and D were almost similar, except a methylene (δ 2.57, 1.27/38.6) instead of a methine was present in **7**. These evidences indicated that the common γ-lactone annexed to C-3 was opened to form a five-carbon carbonic acid side chain in **7**. The NMR data of this side chain resembled those of bisdehydrostemoninine B,[9] which indicated that **7** was the first sample of tuberostemonine-type alkaloid with an opened ring E. The relative configuration of **7** was determined as the same to that of **6** by the ROESY experiment.

All alkaloids isolated from the roots of *S. tuberosa* collected from Yunnan province in this study can be classified into croomine-type and tuberostemonine-type compounds according to their structural features. This result is consistent with the previous reports.[12] Additionally the chemical diversity of the constituents of this species is further verified by the new alkaloidal constituents. For example, tuberocrooline (**1**) was the first sample of 3,4-*seco Stemona* alkaloid, which contained an azepine nucleus instead of the normal 4-azaazulene nucleus. Tridehydrotuberostemonine (**5**) was considered as dehydrogenated derivate of bisdehydrotuberostemonine. 9α-Bisdehydrotuberostemonine A (**7**) was the first tuberostemonine-type alkaloid containing a five-carbon carbonic acid side chain, which might imply a common existence of an opened ring E in *Stemona* alkaloids.

Croomine, the representative of croomine-type alkaloid, was isolated with a sufficient quantity for the antitussive study via

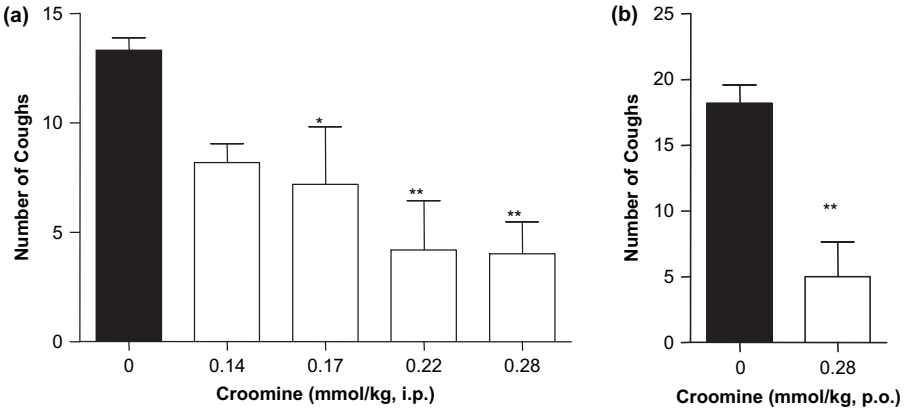

Figure 5. Antitussive activity of croomine in guinea pigs treated with a single ip (a) and oral (b) dose. Data are expressed as mean±SEM (n=4–6). $^{**}P<0.01$, $^{***}P<0.001$ compared with the corresponding vehicle control.

intraperitoneal (ip) and oral administration using the citric acid-induced guinea pig cough model. After ip administration, croomine exhibited a dose-dependent inhibition of citric acid-induced coughing with an ID_{50} value of 0.18 mmol/kg (CI: 0.87–0.30 mmol/kg). At 0.17, 0.22, and 0.28 mmol/kg ip dosing, it inhibited coughing significantly by 46% ($P<0.05$), 69% ($P<0.01$), and 70% ($P<0.01$), respectively (Fig. 5a). Croomine was also administered orally and at 0.28 mmol/kg it significantly inhibited coughing by about 70% ($P<0.01$) (Fig. 5b). There was no significant difference in the potency of antitussive activity between oral and ip routes of administration at 0.28 mmol/kg ($P>0.05$), suggesting that the compound has a good oral absorption profile.

10-Hydroxycroomine, dehydrocroomine, tuberospironine, and 6-hydroxycroomine were only available in small quantities and were only evaluated for antitussive activity following intracerebroventricular (icv) administration. Compared with the vehicle control, dehydrocroomine, 6-hydroxycroomine, and 10-hydroxycroomine decreased coughing by 15–33%, but the level of inhibition was not statistically significant ($P>0.05$; Fig. 6). The relatively lower antitussive activity via icv administration might be due to either the low dose that was administered, and/or that the compounds only possess weak central antitussive properties. Further investigations are required to resolve the precise mechanism. The result revealed that croomine-type alkaloids, especially crominie, may contribute to the antitussive activity of *S. tuberosa* growing in Yunnan province.

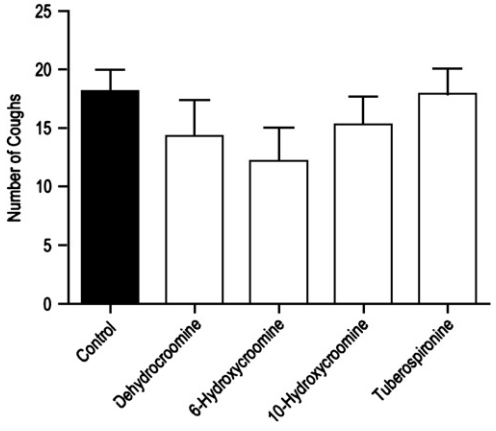

Figure 6. Antitussive of dehydrocroomine, 6-hydroxycroomine, 10-hydroxycroomine, and tuberospironine in guinea pigs treated with a single icv dose. Data are expressed as mean±SEM (n=3).

3. Conclusion

As one of three authentic plant resources for Baibu documented in the China Pharmacopoeia, *S. tuberosa* is the most widely distributed *Stemona* species in China. The alkaloids in this species are believed to be responsible for its bioactivity, but they vary significantly in types and amounts with different localities. Tuberostemonine-, stemoninine-, and croomine-type alkaloids were the three major type compounds isolated from *S. tuberosa* so far. Previous studies have revealed that tuberostemonine-type[5] and stemoninine-type[9,10] alkaloids exhibit significant antitussive activity. In this study, we demonstrated that croomine-type alkaloids, especially croomine, showed also strong antitussive activity for the first time. On the basis of the previous studies and our current findings, we can conclude that the roots of *S. tuberosa* are good enough to be used as medicinal material of Baibu.

4. Experimental section

4.1. General experimental procedures

Optical rotations were taken on a Perkin–Elmer 341 polarimeter. IR spectra were recorded on Nicolet Magna FT-IR 750 spectrophotometer with KBr disks. NMR spectra were recorded on Bruker AM-400 and INVOR-600 NMR spectrometers. The chemical shift (δ) values are given in parts per million with TMS as internal standard and coupling constants (J) are in hertz. EIMS and HR-EIMS spectra were recorded on Finnigan MAT-95 mass spectrometer. ESIMS and HR-ESIMS spectra were recorded on Micromass LC-MS-MS mass spectrometer. Analytical HPLC was performed on a Waters 2690 instrument equipped with a 996 PAD (Photodiode Array Detector) and coupled with an Alltech ELSD 2000 detector. Chromatographic separation was carried out on a C18 column (125×4.0 mm, 5 μm, Merck), using a gradient solvent system comprised of H_2O (A) and CH_3CN (B), with a flow rate of 1.0 mL/min. Temperature for the ELSD drift tube was set at 105 °C and the air flow was 3.2 L/min. Preparative HPLC was performed on a Varian SD1 instrument equipped with 320 single wave detector. Chromatographic separation was carried out on C18 columns (220×25 mm, 10 μm, Merck), using a gradient solvent system comprised of H_2O (A) and CH_3CN (B), with a flow rate of 15 mL/min. Silica gel was used for flash chromatography and was produced by Qingdao Marine Chemical Industrials. TLC was carried out on precoated silica gel GF_{254} plates (Yantai Chemical Industrials) and the TLC spots were viewed at 254 nm and visualized by spraying Dragendorff reagent.

4.2. Plant material

The plant material was collected from Wenshan county Yunnan province, southwest of China in May 2005 and was identified by Associate Professor Jin-gui Shen. A voucher of this plant (No. SIMM-YYE-050401) was deposited at herbarium of Shanghai Institute of Materia Medica, Chinese Academy of Sciences.

4.3. Extraction and isolation

The roots of *S. tuberosa* (7.6 kg) were ground into powder and extracted with 95% ethanol. After evaporation of the collected percolate, the crude extract was acidified with dilute HCl (4%) to pH 1–2 and partitioned between CH_2Cl_2 and water. The aqueous part was basified with aqueous NH_3 to pH 9–10 and extracted with CH_2Cl_2 to afford 82 g of crude alkaloids. Then the crude alkaloids (70 g) was subjected to column chromatography over silica gel and eluted with petroleum ether–acetone (8:1 to 1:2) and acetone (each 4 L) to yield nine fractions. Fraction 3 (520 mg) was subjected to column chromatography over silica gel with petroleum–acetone (9:1 to 4:1). Further purification with Sephadex LH-20 (chloroform–methanol 1:1) yielded bisdehydrotuberostemonine (245 mg), 9α-bisdehydrotuberostemonine (**6**, 12 mg), and 9α-bisdehydrotuberostemonine A (**7**, 3 mg). Colorless needle crystals were filtrated from fraction 4 and then recrystallized to yield dehydrocroomine (**3**, 68 mg). The remainder of fraction 4 (1.52 g) was subjected to column chromatography over silica gel with petroleum–acetone (4:1 to 2:1) and then purified with Sephadex LH-20 (chloroform–methanol 1:1) to yield bisdehydrocroomine (36 mg), stemotinine (32 mg), and isostemotinine (12 mg). Fraction 5 (850 mg) was subjected to column chromatography over silica gel with petroleum–acetone (4:1 to 2:1) and then purified with preparative HPLC (CH_3CN–H_2O from 30% to 50% in 0–60 min and then from 50% to 70% in 60–180 min), affording tuberostemoline (**4**, 26 mg) and tridehydrotuberostemonine (**5**, 105 mg). Fraction 7 (8.25 g) was separated on silica gel column with petroleum–acetone (2:1 to 1:2) to yield croomine (**8**, 6.48 g). The remainder fraction (800 mg) was separated by Sephadex LH-20 column and eluted with chloroform–methanol (1:1) to yield tuberostemoninoamide (35 mg). Further purification of the remainder (350 mg) was carried out by preparative HPLC (CH_3CN–H_2O from 30% to 55% in 0–180 min) to yield tuberostemospironine (120 mg) and 6α-hydrocroomine (105 mg). Fraction 8 (1.80 g) was subjected to a MCI column, eluted with methanol–water (1:1 to 1:0) to give two subfractions. The first subfraction (860 mg) was subjected to column chromatography over silica gel and eluted with petroleum–acetone (2:1 to 1:3) to afford 10-hydrocroomine (**2**, 210 mg). The second subfraction (105 mg) was purified by preparative HPLC (CH_3CN–H_2O from 20% to 50% in 0–180 min) to yield tuberocrooline (**1**, 12 mg) and tuberospironine (8 mg).

4.3.1. Tuberocrooline (*1*)

Colorless oil; $[\alpha]_D^{20}$ −24 (*c* 0.10, CH_3OH); IR ν_{max} (Film) 3396, 2937, 1768, 1632, 1456, 1194, 1016, 754 cm^{-1}; ^1H and ^{13}C NMR data see Tables 1 and 2; EIMS *m/z* 339 $[M]^+$, 321 $[M-H_2O]^+$, 306, 240 $[M-C_5H_7O_2]^+$, 222 $[M-C_5H_7O_2-H_2O]^+$, 194, 168; HR-EIMS *m/z* 339.2042 (calcd for $C_{18}H_{29}NO_5$, 339.2046).

4.3.2. 10-Hydrocroomine (*2*)

Yellow amorphous solid; $[\alpha]_D^{20}$ 0 (*c* 0.08, $CHCl_3$); IR ν_{max} (KBr) 3431, 2931, 1768, 1456, 1194, 999 cm^{-1}; ^1H and ^{13}C NMR data see Tables 1 and 2; EIMS *m/z* 337 $[M]^+$, 335, 320 $[M-OH]^+$, 238 $[M-C_5H_7O_2]^+$, 220 $[M-C_5H_7O_2-H_2O]^+$, 208; ESIMS *m/z* 338.2 $[M+H]^+$, 360.3 $[M+Na]^+$; HR-EIMS *m/z* 337.1892 (calcd for $C_{18}H_{27}NO_5$, 337.1889).

4.3.3. Dehydrocroomine (*3*)

Colorless needle crystal (petroleum ether–acetone); mp 122–124 °C; $[\alpha]_D^{20}$ −31.4 (*c* 0.32, $CHCl_3$); IR ν_{max} (Film) 2928, 1767, 1454, 1163, 1016, 754 cm^{-1}; ^1H and ^{13}C NMR data see Tables 1 and 2; ESIMS *m/z* 320.2 $[M+H]^+$, 661.3 $[2M+Na]^+$; HR-ESIMS *m/z* 342.1692 (calcd for $C_{18}H_{25}NO_4Na$, 342.1681).

4.3.4. Tuberostemoline (*4*)

Yellow amorphous powder; $[\alpha]_D^{20}$ −76 (*c* 0.15, $CHCl_3$); IR ν_{max} (KBr) 3435, 1776, 1703, 1456, 1173, 1020 cm^{-1}; ^1H and ^{13}C NMR data see Tables 1 and 3; EIMS *m/z* 421 $[M]^+$, 403 $[M-H_2O]^+$, 375, 348, 322 $[M-C_5H_7O_2]^+$, 304 $[M-C_5H_7O_2-H_2O]^+$, 294, 267, 236, 83; ESIMS *m/z* 422.3 $[M+H]^+$, 444.2 $[M+Na]^+$; HR-EIMS *m/z* 421.2096 (calcd for $C_{22}H_{31}NO_7$, 421.2101).

4.3.5. Tridehydrotuberostemonine (*5*)

Yellow amorphous powder; $[\alpha]_D^{20}$ +56.2 (*c* 0.36, $CHCl_3$); IR ν_{max} (KBr) 2933, 1770, 1456, 1182, 1165, 995, 924 cm^{-1}; ^1H and ^{13}C NMR data see Tables 1 and 3; ESIMS *m/z* 370.3 $[M+H]^+$, 392.2 $[M+Na]^+$, 761.4 $[2M+Na]^+$; HR-ESIMS *m/z* 392.1840 (calcd for $C_{22}H_{27}NO_4Na$, 392.1842).

4.3.6. 9α-Bisdehydrotuberostemonine (*6*)

Colorless solid; $[\alpha]_D^{20}$ −15.7 (*c* 0.30, $CHCl_3$); IR ν_{max} (KBr) 2935, 1770, 1167, 1010, 924 cm^{-1}; ^1H and ^{13}C NMR data see Tables 1 and 3; EIMS *m/z* 371 $[M]^+$, 343, 327, 262, 298, 272, 228, 99; HR-EIMS *m/z* 371.2091 (calcd for $C_{22}H_{29}NO_4$, 371.2096).

4.3.7. 9α-Bisdehydrotuberostemonine A (*7*)

Colorless solid; $[\alpha]_D^{20}$ −3 (*c* 0.10, $CHCl_3$); IR ν_{max} (KBr) 3419, 2962, 1770, 1261, 1093, 1020, 800 cm^{-1}; ^1H and ^{13}C NMR data see Tables 1 and 3; ESIMS *m/z* 374.3 $[M+H]^+$, 396.2 $[M+Na]^+$, 769.4 $[2M+Na]^+$, 372.2 $[M-H]^-$, 745.5 $[2M-H]^-$; HR-ESIMS *m/z* 396.2119 (calcd for $C_{22}H_{31}NO_4Na$, 396.2087).

4.4. Antitussive activity of selected compounds

Our previously developed the citric acid-induced guinea pig cough model, which is generally recognized as the most relevant model for predicting the clinical efficacy of drugs treating cough in man,[5,9,17,18] was adopted in the present study. Briefly, unrestrained conscious Dunkin-Hartley guinea pigs were randomly divided into groups for different treatments. A single dose of croomine (45, 55, 70, and 90 mg/kg) was given intraperitoneally (ip) or orally (90 mg/kg). Furthermore, due to the limited amounts of the isolated alkaloids, a single intracerebroventricular (icv) injection (0.25 μmol in 10 μL) of 10-hydroxycroomine, dehydrocroomine, tuberospironine, and 6-hydroxycroomine was given to guinea pigs. A standard stereotaxic procedure[19] was used for the icv injection. Briefly, anaesthetized animals were placed into a stereotaxic apparatus. A 22-gauge stainless steel guide cannula was inserted into the lateral ventricle and anchored to the skull by two 3.2 mm stainless steel mounting screws and dental acrylic. After 4 days recovery, the animal was subjected to icv injection of individual compounds through the cannula with a maximum volume of 10 μL.

Treated animals were individually placed into a transparent Perspex air-tight chamber. At 30 min after ip and oral treatment or 3 min after icv injection, each animal was exposed to 0.5 M citric acid aerosols for 8 min with a flow rate of 0.5 mL/min. During the aerosol exposure, the animal was continuously monitored, and cough sounds were recorded and analyzed by Cool Edit 2000 software (Syntrillium, Phenix, USA). Cough episodes were determined using our previously developed software CoughCount-CHHK-2003-Copyright.[5,9] The vehicle control (5% Tween 80 in saline) was conducted in parallel. Antitussive activity was evaluated and based on the comparison of numbers of cough episodes

recorded in the treated group with the corresponding vehicle control group. Statistical analysis between control and treated groups were conducted using a one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test or Student's *t*-test as appropriate. Statistical analysis of differences between ip and oral effect (weighted mean differences) was calculated using *z*-tests. *P* values less than 0.05 were considered significant. ID_{50} values were calculated by linear regression analysis.

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Supplementary data

Supplementary data associated with this article can be found in the online version, at doi:10.1016/j.tet.2008.08.046.

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