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CONTENTS

Isolation, Crystal Structure and Antitussive Activity of 9*S*,9a*S*-neotuberostemonine①

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ABSTRACT The title compound 9*S*,9a*S*-neotuberostemonine (**1**) was isolated from the 95% ethanol extract of the roots of *Stemona tuberosa*. The crystal structure of **1**, $C_{22}H_{33}NO_4$, was determined by single-crystal X-ray diffraction analysis. The crystal belongs to orthorhombic system, space group $P2_12_12_1$, with a = 9.0115(11), b = 10.612(4), c = 22.074(3) Å, V = 2110.9(8) Å^3, Z = 4, M_r = 375.49, D_c = 1.182 g/ cm^3, λ = 0.71079 Å, μ = 0.080 cm^{-1}, $F(000)$ = 816, S = 1.019, R = 0.0579 and wR = 0.1358. A total of 3109 unique reflections were collected, of which 2902 were observed ($I > 2\sigma(I)$). The absolute configuration of **1** could be assigned by referring to the conserved configuration of the methyl groups at C(13) and C(20). In the solid state, the molecules were linked into a chain along the a-axis through weak hydrogen bond C(11)–H(11A)···O(2). Compound **1** shows significant inhibition of cough by 24%, 44% and 65% at doses of 50, 100 and 150 mg/kg, respectively.

Keywords: 9*S*,9a*S*-neotuberostemonine; isolation; crystal structure; antitussive;
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1 INTRODUCTION

The root of three species of *Stemona* has long been used in Chinese traditional medicine as antitussive agents and insecticides[1-2]. Plants in this genus have attracted many phytochemical[3-5] and pharmacological interests[6-8] and over eighty alkaloids have been isolated[9]. These alkaloids could be classified into six structural groups according to the structural skeleton[10].

We have been engaged in the identification of structurally unique alkaloid from Stemona species[11-15], assessment of their antiussive effects[16], and investigation of the in depth mechanism[17]. Our current phytochemical study on the chemical constituents of *Stemona tuberosa* collected in Guangxi province led to the isolation of 9*S*,9a*S*-neotuberostemonine (**1**, scheme 1), which was a new isomer of our previously reported tuberostemonine (**2**), tuberostemonine D (**3**), neotuberostemonine (**4**) and tuberostemonine K(**5**)[14]. Compound **1** was found to show antitussive activity in a dose-dependent manner.

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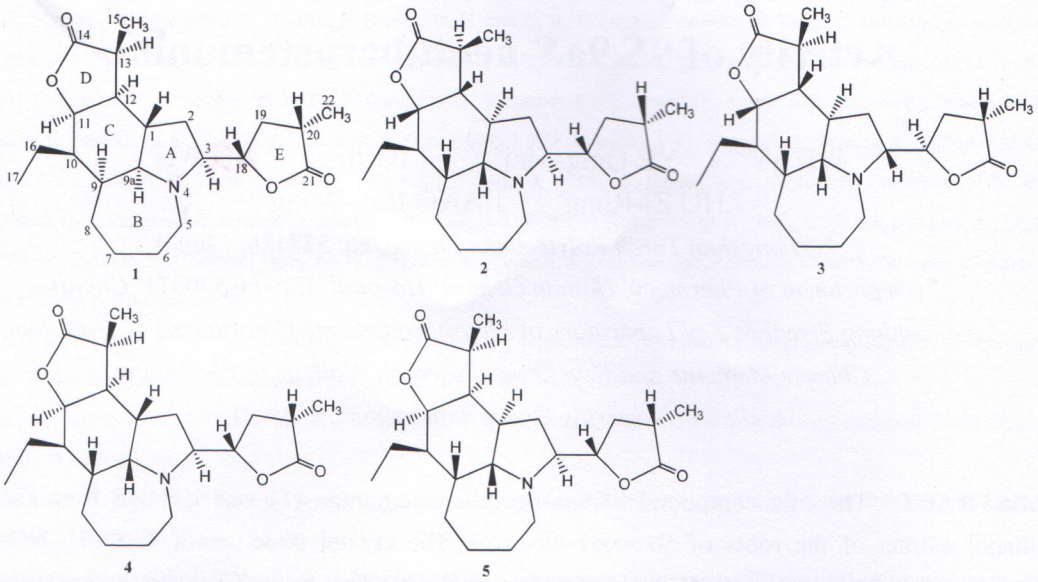
Compound **1** has ten chiral centers, and a precise understanding of their three-dimensional structures would be important for understanding the bioactivities. We report herein the isolation, crystal structure and antitussive activity of compound **1**.



Scheme 1. Structure of compound 1, tuberostemonine (2), tuberostemonine D (3), neotubersostemonine (4) and tubersostemonine K(5)

2 EXPERIMENTAL

2. 1 Materials and instrumentations

Optical rotations were recorded on a Perkin-Elmer 341 Polarimeter in MeOH solution. Melting points were determined using a Fisher scientific and uncorrected. The UV spectra were obtained on a Beckman Du650 spectrophotometer in MeOH. IR spectra were recorded on a Nicolet impact 420 FT-IR spectrometer. The NMR spectra were obtained on a Bruker 300 spectrometer with chemical shift reported in δ (ppm) using TMS as the internal stand. ESIMS was recorded on a Finnigan MAT TSQ 7000 instrument. X-ray diffraction of compound **1** was conducted on a Bruker SMART1000 CCD diffractometer.

2. 2 Plant material

Roots of *Stemona tuberosa* were collected in Guangxi province, and were identified by Prof. Guangzhou Zhou in College of Pharmacy, Jinan University. A voucher specimen (ST-GX1) is deposited in College of Pharmacy, Jinan University.

2. 3 Extraction and isolation

Dry ground roots of *Stemona tuberosa* (1 kg) were refluxed with 95% ethanol. After the evaporation of ethanol, the residue was acidified with dilute HCl (4%) and centrifuged at 5 °C, 3500RPM for 30 min. The supernatant was basified with aqueous amonia to pH = 9 and extracted with Et_2O to afford the total alkaloid (6.3 g). The total alkaloids were subjected to silica gel chromatography; eluted with gradient solvent of $CHCl_3$:MeOH:NH_4OH to give compound **1**, which was further purified by re-crystallization from hexane and ethyl acetate mixture (2:1) at room temperature.

2. 4 Spectral structure determination

Compound **1**: $C_{22}H_{33}NO_4$, m.p.: 183～185 ℃, $[\alpha]_D^{20}$ +77.6° (c 0.1 in CH_3OH); IR (KBr) ν_{max} 2945, 1769, 1454, 1174 and 1016 cm^{-1}. ESI-MS 375$[M]^+$, 276$[M\text{-}C_5H_7O_2]^+$. ^1H NMR (CD_3OD, 300 MHz) δ: 1.79 [m, H(1)], 1.57 [m, H_2(2)], 3.20 [dd, J = 6.5, 11.8 Hz, H(3)], 2.61 [m, H(5α)], 2.83 [m, H(5β)], 1.42 [m, H(6α)], 1.60 [m, H(6β)], 1.43 [m, H(7α)], 1.82 [m, H(7β)], 1.63 [m, H(8α)], 1.93 [m, H(8β)],1.85 [m, H(9)], 3.01 [dd, J = 6.6, 7.2 Hz, H(9a)], 1.71 [m, H(10)], 4.57 [dd, J = 4.3, 3.6 Hz, H(11)], 1.97 [m, H(12)], 2.75 [dq, J = 6.5, 12.3 Hz, H(13)], 1.18 [d, J = 6.5 Hz, H_3(15)], 1.38 [m,

H(16α)], 1.67 [m, H(16β)], 1.02 [t, J = 7.2 Hz, H(17)], 4.37 [ddd, J = 4.6, 6.5, 11.8 Hz, H(18)], 1.43 [m, H(19α)], 2.35 [ddd, J = 4.1, 6.5, 14.5 Hz, H(19β)], 2.46 [ddd, J = 5.2, 7.2, 10.8 Hz, H(20)], 1.22 [d, J = 7.2 Hz, H_3(22)]. ^{13}C NMR [CD_3OD, 75 MHz] δ: 41.9 [C(1)], 31.2[C(2)], 78.0[C(3)], 54.7[C(5)], 27.3[C(6)], 24.1[C(7)], 27.1[C(8)], 41.1[C(9)], 67.5[C(9a)], 35.3[C(10)], 80.7[C(11)], 44.1[C(12)], 47.2[C(13)], 179.5[C(14)], 11.6[C(15)], 21.2[C(16)], 11.9[C(17)], 79.2[C(18)], 33.4[C(19)], 44.8[C(20)], 179.1[C(21)], 15.1[C(22)].

2.5 X-ray structure determination

The crystals suitable for X-ray structure determination were obtained by slow evaporation of hexane and ethyl acetate mixture (2:1) at room temperature. A colorless prism-like crystal of the title compound with dimensions of 0.38mm × 0.36mm × 0.24mm was selected and mounted on a thin glass fiber. Intensity data were collected at room temperature (298 K) on a Smart1000 CCD diffractometer using Mo$K\alpha$ radiation λ = 0.71079 Å. The data frames with a maximum 2θ value of ~52° were processed using the program SAINT. The data were corrected for absorption and beam corrections based on the multi-scan technique as implemented in SADABS.

A total of 3109 reflections were collected in the range of $1.85 \leqslant \theta \leqslant 26.00°$ (index ranges: $-1 \leqslant h \leqslant 11$, $-1 \leqslant k \leqslant 13$, $-1 \leqslant l \leqslant 27$) by using an ω scan mode, of which 2902 independent reflections (R_{int} = 0.0133) with $I > 2\sigma(I)$ were considered as observed and used in the succeeding refinements. The structure was solved by direct methods with SHELXS-97 and expanded by using Fourier difference techniques. The non-hydrogen atoms were refined anisotropically, and the hydrogen atoms were added according to theoretical models. The structure was refined by full-matrix least-squares techniques on F^2 with SHELXL-97. The final refinement gave R = 0.0579, wR = 0.1358 ($I > 4\sigma(I)$), $w = 1/[\sigma^2(F_o^2) + (0.0743P)^2 + 0.5547P]$, where $P = (F_o^2 + 2F_c^2)/3$. $(\Delta/\sigma)_{max}$ = 0.000, S = 1.019, $(\Delta\rho)_{max}$ = 0.457 and $(\Delta\rho)_{min}$ = –0.235 e/Å^3.

2.6 Antitussive assay

Antitussive assay was carried out as previously reported[18]. Briefly, unrestrained conscious guinea pigs were individually placed in a transparent Perspex airtight chamber and exposed to 0.5 M citric acid aerosol produced by an ultrasonic nebulizer (NEU12, Omron, Tokyo, Japan) for 10 min with a flow rate of 0.6 mL/min. During the 10 min observation period, the animals were continuously observed. Animals producing cough more than 18 times but less than 30 in the first challenge were selected for further antitussive tests. Cough episodes during the first challenge were recorded as the control data. After 72 h recovery, the selected sensitive animals were randomly divided into several groups with six animals in each group. Compound **1** was suspended in 0.1% CMC. The samples were intragastrically administered at 60 min before the second challenge.

Table 1. Selected Bond Lengths (Å) and Bond Angles (°)

Bond	Dist.	Bond	Dist.
O(1)–C(14)	1.334(6)	O(4)–C(21)	1.197(5)
O(2)–C(11)	1.484(5)	C(3)–N(4)	1.452(5)
O(2)–C(14)	1.219(5)	N(4)–C(5)	1.455(6)
O(3)–C(21)	1.346(6)	N(4)–C(9A)	1.457(5)
O(3)–C(18)	1.459(5)	C(1)–C(9A)	1.498(6)
Angle	(°)	Angle	(°)
C(14)–O(1)–C(11)	109.6(4)	O(1)–C(11)–C(10)	111.2(4)
C(21)–O(3)–C(18)	110.7(3)	O(1)–C(11)–C(12)	102.4(3)
N(4)–C(3)–C(18)	110.0(3)	O(2)–C(14)–O(1)	122.4(4)
N(4)–C(3)–C(2)	103.6(3)	O(2)–C(14)–C(13)	127.8(5)
C(3)–N(4)–C(5)	120.4(3)	O(1)–C(14)–C(13)	109.7(4)
C(3)–N(4)–C(9A)	106.2(3)	O(3)–C(18)–C(3)	108.2(4)

To be continued

C(5)–N(4)–C(9A)	118.2(4)	O(3)–C(18)–C(19)	104.1(4)
N(4)–C(5)–C(6)	109.8(4)	O(4)–C(21)–O(3)	121.9(5)
N(4)–C(9A)–C(1)	99.1(4)	O(4)–C(21)–C(20)	128.5(5)
N(4)–C(9A)–C(9)	115.1(4)	O(3)–C(21)–C(20)	109.6(4)
C(18)–C(3)–C(2)	114.9(4)	C(16)–C(10)–C(9)	113.0(5)

Table 2. Hydrogen Bond Lengths (Å) and Bond Angles (°)

D–H···A	d(D–H)	d(H···A)	d(D···A)	∠DHA
C(11)–H(11A)···O(2)[a]	0.98	2.57	3.482(4)	155

Symmetry code: (a) 0.5+*x*, 0.5–*y*, –*z*

3 RESULTS AND DISCUSSION

Compound **1** was obtained by silica gel column chromatography of the 95% ethanol extract followed by open silica gel column chromatography, and recrystallized as colorless prism-like crystals from hexane-ethyl acetate mixture (2:1). High resolution ESIMS analysis of **1** showed a quasi-molecular ion peak at $[M+H]^+$ 376.2476, corresponding to a molecular formula $C_{22}H_{33}NO_4$ (calculated 376.2482). Its IR spectrum (KBr) showed the presence of a γ-lactone ring (1763 cm^{-1}). The ESI mass spectrum showed $[M+H]^+$ at *m/z* 376 and the base peak at *m/z* 276 [M-$C_5H_7O_2$] indicated the presence of the typical β-methyl-γ-lactone ring annexed to C(3) of the azepine ring.

The ^{1}H-NMR spectrum of compound **1** showed a triplet (3H) at δ 1.02 for the C(17) methyl group and two doublets (3H each) at δ 1.18 and 1.22, corresponding to two secondary methyl groups at C(13) and C(20). The ^{13}C-NMR spectrum showed two lactone carbonyls at δ179.10 and 179.45, corresponding to C(21) and C(14). These signals indicated that compound **1** belongs to the tuberostemonine-type of alkaloids[14].

The complete structure and stereochemistry were determined unambiguously by X-ray diffraction analysis. Selected bond lengths and bond angles of compound **1** are given in Table 1. Fig. 1 shows the molecular structure of the title compound, and Fig. 2 depicts the packing diagram.

The crystal belongs to orthorhombic system with space group $P2_12_12_1$. The crystal data are listed Table 1. The skeleton consists of two γ-lactone rings, a pyrrolidine, a cyclohexane, and an azepine ring. The lactone rings D and E adopt an envelope conformation with C(12) and C(19) displaced by 0.607 and 0.350 Å from the corresponding least-squares plane of the remaining four atoms, respectively. The pyrrolidine ring A fused to the cyclohexane and azepine rings has a twist envelope conformation. The cyclohexane ring has a distorted chair conformation as indicated by the smaller torsion angle C(10)–C(11)–C(12)–C(1) 39.3°. The azepine ring adopts a chair conformation. The groups of atoms C(5), N(4), C(8) and C(9) form a plane with mean deviation of 0.0035 Å. The deviations of C(9A), C(6) and C(7) from this plane are –0.6403, 1.1532 and 1.0563 Å, respectively. The stereochemisty of ring juncture is A/B *trans*, B/C *trans*, A/C *trans* and C/D *cis*. The configurations at the ten chiral centers are determined as follows: H(1), H(9), H(18) and H(20) are β-oriented. H(3), H(9a), H(10), H(11), H(12) and H(13) are α-oriented.

Accordingly, the relative configurations of the chiral centers C(1), C(3), C(9), C(9a), C(10), C(11), C(12), C(13), C(18) and C(20) were established to be *rel*-(*S*, *S*, *S*, *S*, *R*, *S*, *S*, *S*, *S* and *S*), respectively. Compound **1** is an isomer of neotuberostemonine[14] at C(9) and C(9a). It is here named 9S,9aS-neotuberostemonine. Compound **1** is also an isomer of tuberostemonine[14] at C(1), C(9), C(9a), C(11) and C(12), and an isomer of tuberstemonine K[14] at C(1), C(9), and C(9a). Due to the absence of heavy atom in the molecule, the final refinement resulted in a non-significant Flack parameter 0(3); however, con-

sidering the conserved C(13) β- and C(20) α-orientated methyl groups in Stemona alkaloids[9, 10], the absolute configuration of compound **1** could be assigned as shown in Fig. 1.

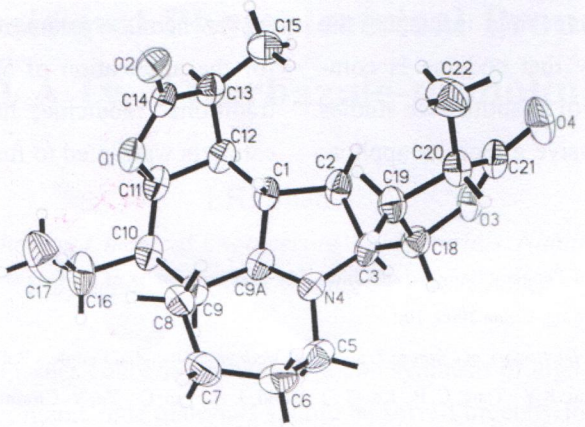


Fig. 1. Molecular structure of 1 showing 30% probability displacement ellipsoids and the atom-numbering scheme

Normally, drug molecules exert pharmacological effects in solution state. It is necessary to compare the conformations in the solution and solid state. The ^{1}H-NMR spectrum of **1** showed that the coupling constants between H(11) and H(12) and H(10) are 4.3 and 3.6 Hz, respectively. These coupling constants are consistent with the torsion angles H(11)–C(11)–C(12)–H(12) of 42.3° and H(10)–C(10)–C(11)–H(11) of 54.5° in the crystal structure. Thus, the conformation of **1** in methanol might be consistent with that in crystalline state, which is similar to 1β-hydroxydigitoxigenin[19].

In solid state, the molecules were linked into a chain along the *a*-axis through weak hydrogen bond C(11)−H(11A)···O(2) (3.482(4) Å, symmetry code: x+0.5, 0.5–y, –z), as shown in Fig. 2.

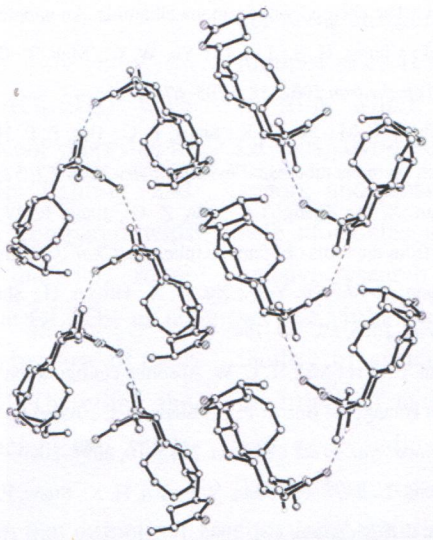


Fig. 2. A packing diagram for compound 1. Hydrogen-bonding network of 1 viewed roughly down the *b*-axis. Selected hydrogen atoms highlight the scheme of hydrogen bonding

The antitussive property of **1** was studied using a citric acid induced guinea pig cough model. Compound **1** showed significant inhibition of cough by 24%, 44% and 65% at doses of 50, 100 and 150 mg/kg, respectively, which is comparable to the positive control codeine at 15 mg/kg (68% cough reduction). The potency of **1** was lower than the reported value of neotuberostemonine (**4**, 85% inhibition at 50 mg/kg)[20], but much stronger than epi-bisdehydrotuberostemonine J (no significant

antitussive activity)[19], indicating that the configurations at C(9) and C(9a) and the presence of double bonds in the pyrrole ring influence the bioactivity. It is noteworthy that codeine is commonly used as positive control in antitussive studies. Though it has potent antitussive effect, its application in clinics is limited due to the strong addiction side effect. Discovery of the antitussive effect of 9*S*,9a*S*-neotuberostemonine (**1**) partially accounts for the application of *Stemona* species in Chinese traditional medicine; however, structural modification is warranted to further improve the potency.

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