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Dimeric Matrine-Type Alkaloids from the Roots of *Sophora flavescens* and Their Anti-Hepatitis B Virus Activities

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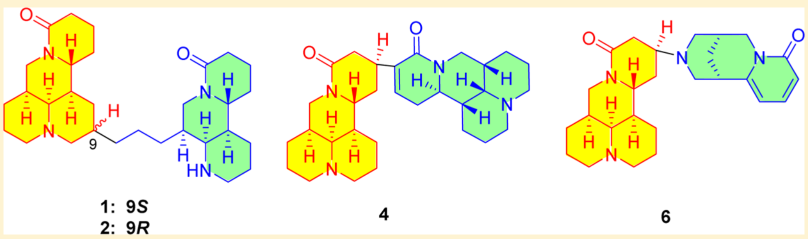
Supporting Information



ABSTRACT: Six unusual matrine-type alkaloid dimers, flavesines A−F (**1**−**6**, respectively), together with three proposed biosynthetic intermediates (**7**−**9**) were isolated from the roots of *Sophora flavescens*. Compounds **1**−**5** were the first natural matrine-type alkaloid dimers, and compound **6** represented an unprecedented dimerization pattern constructed by matrine and (−)-cytisine. Their structures were elucidated by NMR, MS, single-crystal X-ray diffraction, and a chemical method. The hypothetical biogenetic pathways of **1**−**6** were also proposed. Compounds **1**−**9** exhibited inhibitory activities against hepatitis B virus.

■ INTRODUCTION

The plant *Sophora flavescens* (Leguminosae) is a traditional Chinese medicine, which is widely distributed in China, Japan, and India and used for the treatment of pruritus, eczema, dysentery, pyogenic infections of the skin, and trichomonas vaginitis.[1,2] Modern pharmacological studies have shown that the extracts of *S. flavescens* displayed antiviral, antifungal, anti-inflammation, and antitumor effects.[3−6] Phytochemical investigations suggested that matrine-type alkaloids and flavonoids were the main components of this plant.[7−15] More than 40 matrine-type alkaloids had been isolated from *Sophora* plants since 1895.[13−30] Pharmacological studies showed that these alkaloids exhibited potent antiviral activities against hepatitis B (HBV), Coxsackie B3 (CVB3), and influenza A/Hanfang/359/95 (H3N2) viruses.[16,31] In China, some matrine-type alkaloids such as matrine and oxymatrine had been used for the treatment of hepatitis B, dysentery, pyogenic infections of the skin, and trichomonas vaginitis in the clinic.[32,33]

In systematic research of biologically active compounds from Chinese medicinal plants,[34−36] we found six novel dimeric matrine-type alkaloids, flavesines A−F (**1**−**6**, respectively) (Figure 1), together with three proposed biosynthetic intermediates (**7**−**9**) from the roots of *S. flavescens* Aiton. Compounds **1**−**5** were the first natural matrine-type alkaloid dimers, and compound **6** represented an unprecedented dimerization pattern constructed from matrine and (−)-cytisine. Herein, we reported the isolation and structure elucidation of **1**−**6**. In addition, the biogenetic pathway and anti-HBV activity were also discussed.

■ RESULTS AND DISCUSSION

Compound **1** was isolated as colorless crystals (mp 102−103 °C). The molecular formula of **1** was deduced to be $C_{30}H_{48}N_4O_2$ from its HR-ESI-MS at m/z 497.3841 $[M + H]^+$ (calcd for $C_{30}H_{49}N_4O_2$ m/z 497.3850). The IR absorption bands at 3442 and 1620 cm^{-1} indicated the presence of secondary amine and carbonyl groups. The ^1H NMR spectrum of **1** showed the existence of four characteristic protons at δ_H 4.46 (1H, dd, J = 12.8, 4.3 Hz), 4.31 (1H, dd, J = 12.7, 4.1 Hz), 3.87 (1H, dt, J = 9.3, 5.7 Hz), and 3.77 (1H, dt, J = 9.1, 5.9 Hz). The ^{13}C NMR spectrum displayed the presence of 30 carbons, including two carbonyls (δ_C 172.0 and 171.9) and four methines connected to heteroatoms (δ_C 65.0, 58.5, 55.2, and 54.2). These data suggested that **1** was composed of two matrine-type alkaloid units,[14,24] indicating that **1** was a dimeric matrine-type alkaloid. With the aid of ^1H−^1H COSY, HSQC, 2D INADEQUATE, and HMBC experiments (Figures 2 and 3), the ^1H and ^{13}C NMR signals of **1** were assigned as shown in Table 1.

In the ^1H NMR and ^1H−^1H COSY spectra of **1**, the signals in the high-field region were substantially overlapped. 2D

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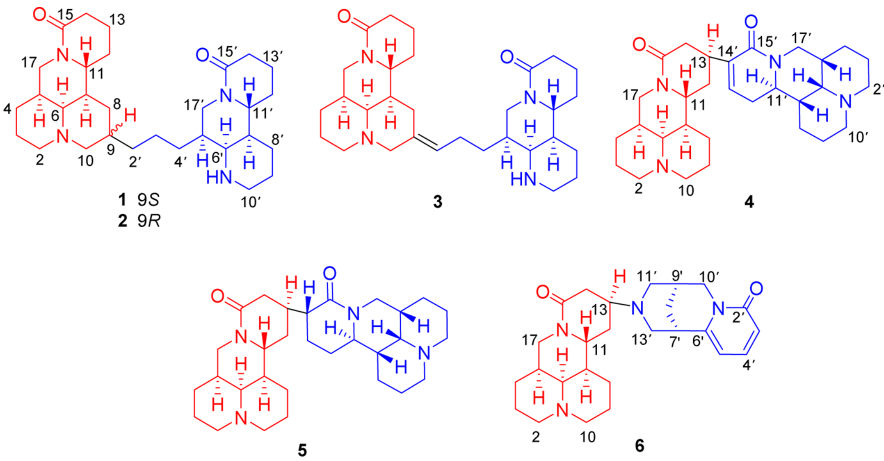

Figure 1. Chemical structures of compounds **1**−**6**.

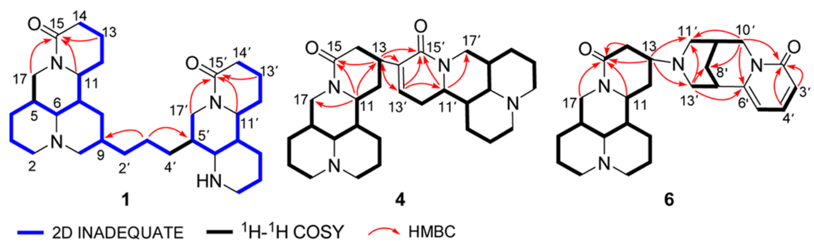

Figure 2. Key 2D INADEQUATE, $^{1}H-^{1}H$ COSY, and HMBC correlations of **1**, **4**, and **6**.

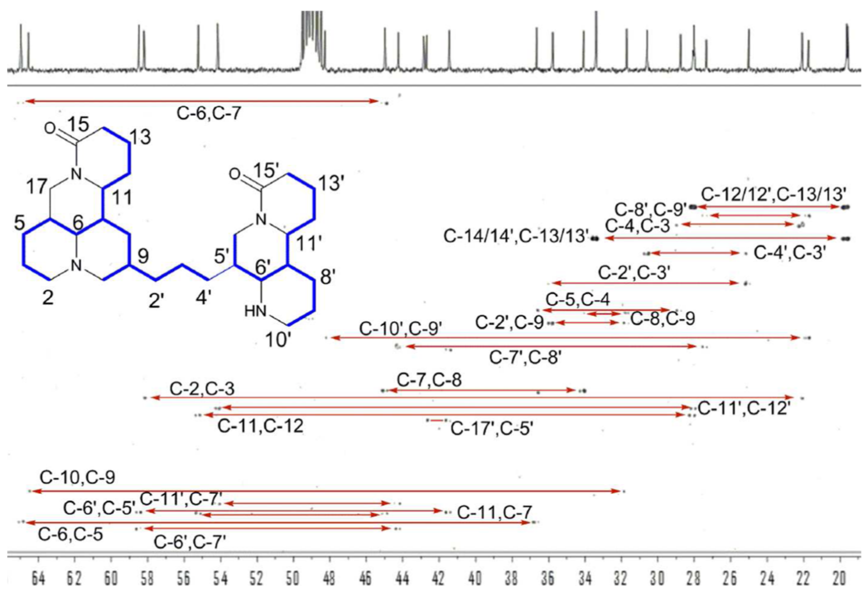

Figure 3. 2D INADEQUATE data of **1** with diagnostic correlations.

INADEQUATE experiments could be used to deduce the planar structure of organic compounds by detecting a pair of ^{13}C atoms in natural abundance coupled to each other through J_{C-C}.[37,38] Hence, the $^{13}C-^{13}C$ connections of C-2 to C-10, C-14, and C-4′ and connections of C-17′ to C-10′ and C-14′ were established by the 2D INADEQUATE spectrum (Figures 2 and 3). The weak 2D INADEQUATE correlations between C-5 and C-17 and between C-4′ and C-5′ were further confirmed by the $^{1}H-^{1}H$ COSY correlations between H-5 (δ_H 1.73) and H_2-17 (δ_H 4.31/3.04) as well as between H_2-4′ (δ_H 1.30) and H-5′ (δ_H 1.57).

The relative stereochemistry of **1** was deduced through the ROESY correlations between H-5 and H-7, between H-6 and H-10α, between H-11 and H-9/H-17β, between H-5′ and H-7′, between H-6′ and H-10′α, and between H-11′ and H-17′β (Figure 4). Finally, the complete structure and stereochemistry were established by single-crystal X-ray diffraction (Cu Kα radiation). The result of X-ray diffraction in a reasonable Flack parameter of 0.10(17) allowed the unambiguous assignment of the absolute configuration as 5*S*, 6*S*, 7*R*, 9*S*, 11*R*, 5′*S*, 6′*S*, 7′*S*, and 11′*R* (Figure 5).[39] Accordingly, compound **1** was elucidated and named flavesine A.

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Table 1. NMR Data of 1−3 (δ in parts per million and J in hertz)

	1[a]		2[a]		3[b]	
	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C
2α	2.01	58.2	1.94	58.8	1.77	57.2
2β	2.82		2.82		2.66	
3a	1.73	22.1	1.75	21.9	1.24	21.5
3b	1.52		1.50		1.24	
4	1.65	28.8	1.67	29.1	1.46	28.3
5	1.73	36.7	1.72	37.0	1.59	35.9
6	2.16	65.0	2.11	65.6	1.98	64.0
7	1.57	45.0	1.55	45.0	1.41	44.7
8α	1.06 (m)	34.1	1.71	30.9	1.82	29.6
8β	1.98		1.90		2.72	
9	1.65	31.7	1.64	35.2	–	132.5
10α	1.65	64.5	2.11	62.1	2.48	64.9
10β	2.86		2.78		3.06 (m)	
11	3.77 (dt, 9.1, 5.9)	55.2	3.83 (dt, 11.4, 6.2)	57.3	3.50 (dt, 9.8, 6.0)	54.0
12a	2.16	28.1	2.16	28.6	1.95	28.0
12b	1.52		1.60		1.34	
13α	1.86	19.6	1.82	19.5	1.65	19.6
13β	1.65		1.62		1.44	
14a	2.33	33.4	2.34	33.3	2.44	33.5
14b	2.28		2.26		2.27	
15	–	171.9	–	172.0	–	169.3
17α	4.31 (dd, 12.7, 4.1)	42.7	4.27 (dd, 12.7, 3.9)	43.5	4.58 (dd, 12.5, 4.3)	41.7
17β	3.04 (t, 12.7)		3.05 (t, 12.7)		3.08 (t, 12.5)	
2′	1.21 (m)	35.8	1.43	35.9	5.31 (t, 7.0)	125.0
3′	1.40	25.0	1.54	27.5	2.12	25.3
4′a	1.30	30.6	1.30	30.6	1.36	30.5
4′b	1.30		1.30		1.25	
5′	1.57	41.4	1.55	41.8	1.59	40.6
6′	2.86	58.5	2.84	58.4	2.72	58.2
7′	1.57	44.2	1.52	44.4	1.29	43.7
8′α	1.52	27.3	1.50	27.5	1.34	27.3
8′β	1.98		1.98		1.77	
9′a	1.52	21.7	1.50	22.1	1.46	21.9
9′b	1.45		1.43		1.28	
10′α	2.67 (m)	48.3	2.63 (m)	48.3	2.59 (td, 11.7, 2.3)	48.1
10′β	3.14 (m)		3.12 (m)		3.15 (m)	
11′	3.87 (dt, 9.3, 5.7)	54.2	3.90 (dt, 9.3, 6.0)	54.2	3.94 (dt, 9.4, 6.1)	53.0
12′a	2.16	28.0	2.16	28.0	1.86	27.4
12′b	1.52		1.60		1.29	
13′α	1.86	19.6	1.82	19.6	1.65	19.6
13′β	1.65		1.62		1.44	
14′a	2.33	33.4	2.34	33.4	2.44	33.5
14′b	2.28		2.26		2.27	
15′	–	172.0	–	171.7	–	169.2
17′α	4.46 (dd, 12.8, 4.3)	42.8	4.44 (dd, 12.9, 4.2)	42.8	4.83 (dd, 12.2, 4.2)	41.8
17′β	2.58 (t, 12.8)				2.77 (t, 12.2)	

[a]Measured at 500 (^{1}H) and 125 (^{13}C) MHz in CD_3OD. [b]Measured at 500 (^{1}H) and 125 (^{13}C) MHz in C_5D_5N. Overlapped signals are reported without designating multiplicity.

Compound **2** displayed the same molecular formula ($C_{30}H_{48}N_4O_2$) as that of **1** by its HR-ESI-MS (m/z 497.3841 $[M + H]^+$, calcd for $C_{30}H_{49}N_4O_2$ m/z 497.3850). Comparison of the ^{1}H and ^{13}C NMR data of **2** with those of **1** (Table 1) showed that they were very similar except for the signals assigned to C-8−C-10, suggesting that **2** might be an epimer of **1**. The planar structure of **2** was also deduced by the 2D INADEQUATE experiment and verified by ^{1}H−^{1}H COSY and HMBC spectra. The relative stereochemistry of **2** was determined by analysis of the ROESY spectrum. The NOE correlation between H-7 and H-9 indicated the stereochemistry of C-9 in **2** was different from that in **1**, suggesting that the absolute configuration of C-9 in **2** was 9*R*. Compound **2** was named flavesine B.

The molecular formula of **3** was determined to be $C_{30}H_{46}N_4O_2$ on the basis of HR-ESI-MS at m/z 495.3694 $[M + H]^+$ (calcd for $C_{30}H_{47}N_4O_2$ m/z 495.3694). The IR spectrum showed the presence of secondary amine (3417 cm^{-1}) and carbonyl (1619 cm^{-1}) groups. The ^{1}H and ^{13}C NMR spectroscopic data of **3** (Table 1) were very similar to those of **1** except for the presence of an additional double bond (δ_C 132.5 and 125.0), suggesting the presence of an olefinic double bond in **3**. The HMBC correlations between H-10 and C-8, C-9, and C-2′, between H-2′ and C-8, C-10, and C-4′, and between H-3′ and C-9 and C-5′ allowed the assignment of the planar structure of **3**. The ROESY correlation between H-2′ and H-10β suggested the configuration of the double bond at C-9 and C-2′ was of the *E* type. The structure of **3** was further verified by hydrogenation to afford **1** and **2** (see the Supporting Information). Thus, the structure of **3** was established, and **3** was named flavesine C.

Compound **4** was obtained as colorless crystals (mp 127−128 °C). A $C_{30}H_{44}N_4O_2$ molecular formula was established by its HR-ESI-MS (m/z 493.3536 $[M + H]^+$, calcd for $C_{30}H_{45}N_4O_2$ m/z 493.3537). The ^{1}H and ^{13}C NMR spectra of **4** (Table 2) showed four characteristic protons [δ_H 4.28 (1H, dd, J = 12.7, 4.5 Hz), 4.06 (1H, dd, J = 13.2, 4.8 Hz), 3.93 (1H, dt, J = 10.6, 5.4 Hz), and 3.88 (1H, dt, J = 10.1, 6.5 Hz)], two carbonyls (δ_C 171.0 and 167.5), four methines connected to heteroatoms (δ_C 65.5, 64.5, 53.5, and 52.5), and a double bond (δ_C 136.1 and 133.8), indicating that **4** was also a dimer with two matrine-type alkaloid units.[24] Detailed analysis of the 1D NMR data showed ring D of two matrine-type alkaloid units in **4** was substituted. The ^{1}H−^{1}H COSY correlations between H-13 and H-12 and H-14 and the HMBC correlations between H-11 and C-13, between H-13 and C-15, C-13′, C-14′, and C-15′, and between H-13′ and C-13, C-11′, and C-15′ revealed that the two matrine units were connected by a bond between C-13 and C-14′ (Figure 2). Furthermore, the relative configuration of **4** was established by its ROESY correlations (Figure 4). Finally, the complete structure of **4** was confirmed by a single-crystal X-ray diffraction analysis.[40] Thus, compound **4** was identified and named flavesine D.

Compound **5** has a molecular formula of $C_{30}H_{46}N_4O_2$ as determined by the HR-ESI-MS (m/z 495.3691 $[M + H]^+$, calcd for $C_{30}H_{47}N_4O_2$ m/z 495.3694). The ^{1}H and ^{13}C NMR data of **5** (Table 2) were in good agreement with those of **4**, except for the absence of a double bond at C-13′ and C-14′, as well as the presence of methylene (δ_C 20.3) and methine (δ_C 45.6) carbons. These differences suggested that the double bond was reduced in **5**, which was confirmed by the ^{1}H−^{1}H COSY correlations between H-13 and H-12, H-14, and H-14′ and the HMBC correlations between H-14′ and C-12, C-14, C-12′, and C-15′. To confirm the stereochemistry of **5**, the double bond of compound **4** was successfully hydrogenated to yield **5** (see the

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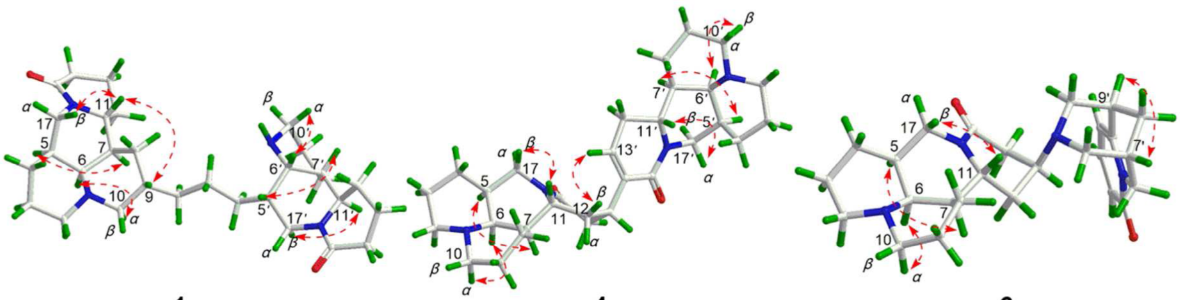
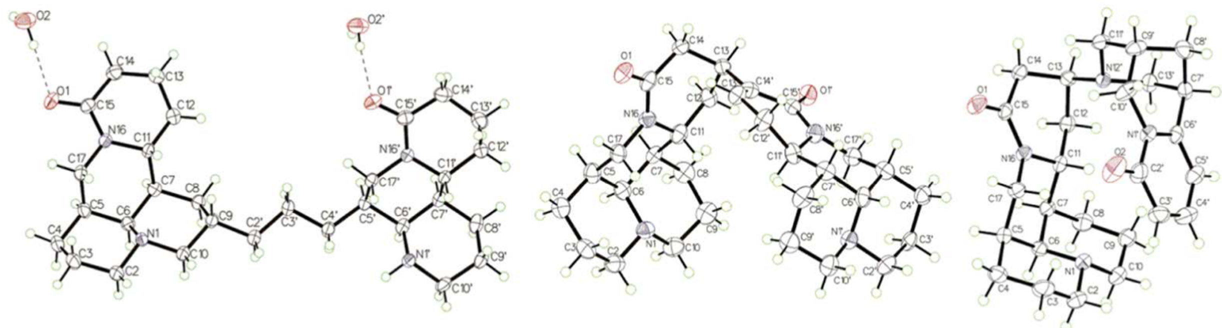


1 4 6

Figure 4. Key ROESY correlations of **1**, **4**, and **6**.



1 4 6

Figure 5. X-ray ORTEP drawings of **1**, **4**, and **6**. The thermal ellipsoids are scaled to the 50% probability level.

Table 2. NMR Data of 4−6 (δ in parts per million, J in hertz)

	4[a]		**5**[a]		**6**[b]	
	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C
2α	2.04	58.4	2.07	58.2	1.84	57.3
2β	2.86		2.86		2.70	
3a	1.75	21.6	1.65	21.6	1.76	21.2
3b	1.48		1.48		1.36	
4α	1.67	28.7	1.66	28.9	1.39 (m)	27.9
4β	1.67		1.66		1.56	
5	1.74	37.3	1.74	37.6	1.57	35.9
6	2.30	65.5	2.31	65.6	1.95 (m)	63.8
7	1.75	42.8	1.74	42.1	1.30	42.3
8α	1.48	27.5	1.46	27.1	1.26	26.8
8β	2.05		1.94		1.66 (m)	
9a	1.81	22.1	1.77	22.1	1.76	20.8
9b	1.48		1.50		1.27	
10α	2.04	58.4	2.07	58.3	1.80	57.4
10β	2.86		2.84		2.66	
11	3.93 (dt, 10.6, 5.4)	53.5	4.09 (dt, 10.2, 5.1)	53.7	3.42 (m)	50.5
12α	1.98	30.7	2.06	28.6	1.87	28.9
12β	1.88		1.84		1.44	
13	3.11	29.9	2.36	30.2	2.58 (m)	53.6
14a	2.52 (ddd, 17.2, 5.1, 1.6)	37.7	2.36	36.0	2.42	35.9
14b	2.30		2.20 (m)		2.29	
15	–	171.0	–	170.4	–	167.4
17α	4.28 (dd, 12.7, 4.5)	43.5	4.24 (dd, 12.6, 4.5)	43.9	4.17 (dd, 12.5, 4.2)	41.8
17β	3.09 (t, 12.7)		3.11 (t, 12.6)		2.81 (t, 12.5)	
2′α	2.86	58.3	2.86	58.2	–	163.6
2′β	2.06		2.07			
3′a	1.75	21.6	1.65	21.5	6.36 (dd, 9.0, 1.4)	117.1
3′b	1.48		1.48			
4′	1.65	28.7	1.66	28.8	7.19 (dd, 9.0, 6.8)	138.4
5′	1.88	35.9	1.67	37.8	5.92 (dd, 6.8, 1.4)	104.4
6′	2.21	64.5	2.27	65.3	–	151.4
7′	1.75	42.5	1.58	43.9	2.94	35.8
8′α	1.48	27.1	1.46	27.4	1.73	26.1
8′β	1.90		2.00		1.84	
9′a	1.75	22.0	1.77	22.1	2.41	28.3
9′b	1.48		1.50			
10′α	2.86	58.3	2.84	58.2	3.85 (d, 3.5)	50.1
10′β	2.06		2.07		3.85 (d, 3.5)	
11′α	3.88 (dt, 10.1, 6.5)	52.5	3.87 (dt, 11.3, 6.1)	54.7	2.33	56.1
11′β					2.98	
12′a	2.72 (dt, 18.1, 5.9)	28.4	1.98	23.3	–	–
12′b	2.23		1.78			
13′α	6.30 (ddd, 5.2, 3.6, 1.3)	133.8	1.76	20.3	2.37	57.7
13′β			1.83		2.94	
14′	–	136.1	2.28	45.6		
15′	–	167.5	–	172.3		
17′α	3.16 (t, 13.2)	43.4	3.06 (t, 12.6)	43.6		
17′β	4.06 (dd, 13.2, 4.8)		4.28 (dd, 12.6, 4.0)			

[a]Measured at 500 (^{1}H) and 125 (^{13}C) MHz in CD_3OD. [b]Measured at 300 (^{1}H) and 75 (^{13}C) MHz in $CDCl_3$. Overlapped signals are reported without designating multiplicity.

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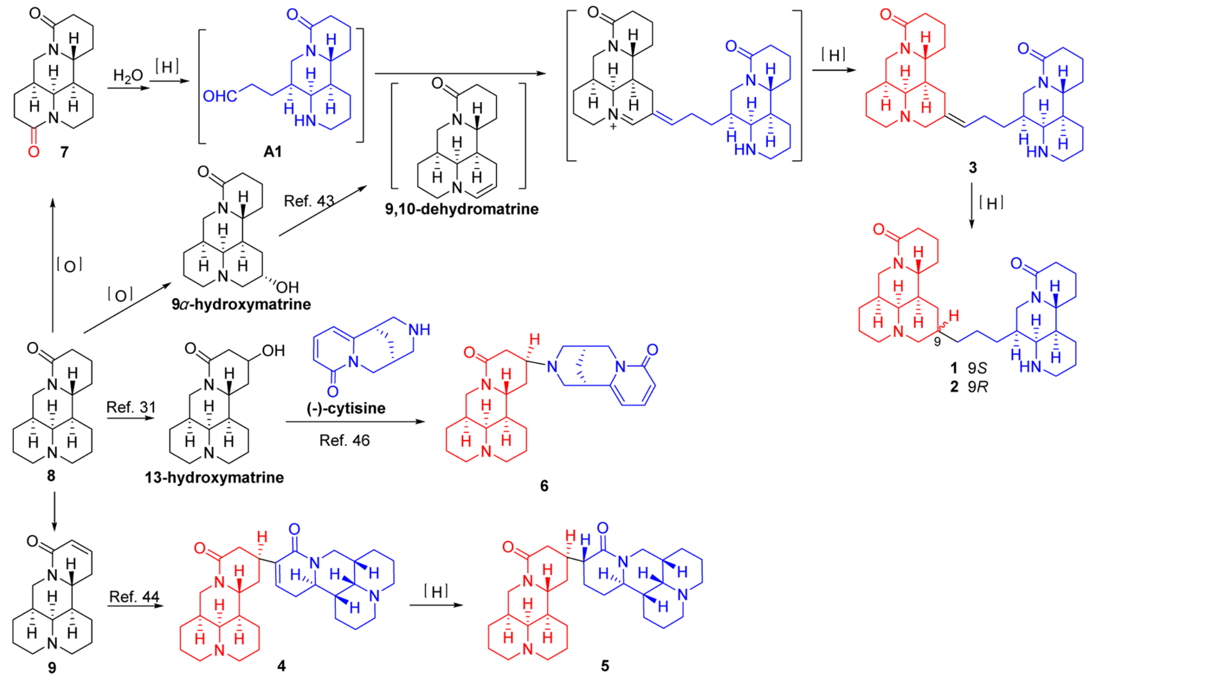
Scheme 1. Hypothetical Biosynthetic Pathways for 1−6



Supporting Information). Therefore, all the configurations of **5** were the same as those of **4** except for C-14′. Via combination with the ROESY correlations of H-13, H-13′α, H-11′, and H-17′α, the structure of **5** was elucidated.

A $C_{26}H_{36}N_4O_2$ molecular formula was assigned to compound **6** by interpretation of HR-ESI-MS data. The NMR spectra of **6** displayed 26 carbon signals, which consisted of three quaternary carbons, 10 tertiary carbons, and 13 secondary carbons. The ^{1}H NMR spectrum of **6** showed seven characteristic protons [δ_H 7.19 (1H, dd, J = 9.0, 6.8 Hz), 6.36 (1H, dd, J = 9.0, 1.4 Hz), 5.92 (1H, dd, J = 6.8, 1.4 Hz), 4.17 (1H, dd, J = 12.5, 4.2 Hz), 3.85 (2H, d, J = 3.5 Hz), and 2.81 (1H, t, J = 12.5 Hz)]. The ^{13}C NMR spectrum displayed two carbonyls (δ_C 167.4 and 163.6), four olefinic carbons (δ_C 151.4, 138.4, 117.1, and 104.4), and three methines connected to heteroatoms (δ_C 63.8, 53.6, and 50.5). The 1D NMR spectra of **6** showed a set of resonances similar to those of matrine[24] and (−)-cytisine[41] (Table 2). The most notable difference was that the methylene (δ_C 19.2) at C-13 in matrine was replaced by a methine (δ_C 53.6) in **6**, suggesting the matrine and (−)-cytisine units were connected by the bond between C-13 and N-12′. This was further confirmed by the HMBC correlations between H-13 and C-15, C-11′, and C-13′. Thus, the structure of **6** was established as shown in Figure 2. The relative configuration of **6** was determined by the ROESY correlations of H-5 and H-7, H-6 and H-10α, H-11 and H-17β, and H-7′ and H-9′ (Figure 4). Finally, the absolute configuration of **6** was defined by single-crystal X-ray diffraction (Cu Kα radiation) with a Flack parameter of 0.0(2), allowing the assignment of the absolute configuration of **6** as 5*S*, 6*S*, 7*R*, 11*R*, 13*S*, 7′*R*, and 9′*S* (Figure 5).[42]

2-Oxymatrine (**7**) was isolated as a brown oil. The molecular formula of **7** was calculated as $C_{15}H_{22}N_2O_2$ with the aid of its HR-ESI-MS (m/z 263.1755 $[M + H]^+$, calcd for $C_{15}H_{23}N_2O_2$ m/z 263.1754). The ^{13}C NMR data of **7** were similar to those of matrine,[24] except for the absence of the C-2 (δ_C 57.6) signal in matrine, the presence of an additional carbonyl at δ_C 173.4 in **7**, and the chemical shifts of C-6 and C-10 at δ_C 64.0 and 57.4 shifted to δ_C 59.1 and 43.4 in **7**, respectively, implying that the methylene at C-2 was oxidized to a carbonyl in **7**. This was confirmed by the ^{1}H−^{1}H COSY correlations of H$_2$-3, H$_2$-4, H-5, and H$_2$-17, together with the HMBC correlations from H$_2$-4 to C-2, C-6, and C-17 and from H$_2$-10 to C-2. The relative configuration of **7** was elucidated by interpretation of the ROESY NMR data.

In addition, the other two known compounds were identified as matrine (**8**)[24] and 13,14-dehydromatrine (**9**)[29] by comparison of their spectroscopic data with those from the related literature.

Hypothetical Biogenetic Pathway. Compounds **1**−**6** are six novel dimeric matrine-type alkaloids. As outlined in Scheme 1, compounds **1**−**3** could be considered to be derived from matrine (**8**). Matrine could be oxidized to yield the intermediates **7** and 9α-hydroxymatrine.[16] Then, 9α-hydroxymatrine was dehydrated to afford 9,10-dehydromatrine,[43] and **7** could further generate **A1** through hydrolysis and reduction reactions. **A1** was combined with 9,10-dehydromatrine and then reduced to give **3**, which was further reduced to yield **1** and **2** with different configurations at C-9. Compounds **4** and **5** were also considered to originate from the same precursor matrine. First, matrine was dehydrogenated to yield 13,14-dehydromatrine (**9**). After an intermolecular addition reaction, **9** could afford **4**,[44] which could be further reduced to give **5**. Compound **6** was constructed from matrine and (−)-cytisine.[45] The key biogenetic intermediate, 13-hydroxymatrine,[31] could be derived from matrine through an oxidation reaction. Then, 13-hydroxymatrine was coupled with (−)-cytisine to afford **6**.[46]

Biological Activity. On the basis of previous research results,[31] some matrine-type alkaloids could reduce HBV DNA levels in HepG2.2.15 cells to different extents. Therefore, compounds **1**−**9** were also evaluated for their anti-HBV activities in HepG2.2.15 cells with real time PCR. The result (see the Supporting Information) revealed that compounds **1**−**9** exhibited inhibitory effects on the expression of HBV DNA in

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J. Org. Chem. 2016, 81, 6273−6280

HepG2.2.15 cells with IC_{50} values of 44.85 ± 7.30, 86.60 ± 4.30, 32.11 ± 2.83, 74.28 ± 0.31, 70.62 ± 0.93, 17.16 ± 0.38, 14.90 ± 0.53, 7.37 ± 0.17, and 15.69 ± 0.64 μM, respectively, while the IC_{50} value of positive control PFA (foscarnet) was 105.53 ± 8.57 μM.

■ CONCLUSION

In summary, five unusual matrine-type alkaloid dimers (**1**−**5**) and the first matrine cytisine alkaloid (**6**) were isolated from *S. flavescens*. Their structures were established on the basis of comprehensive spectroscopic analyses as well as X-ray crystallographic and chemical methods. In addition, the results of the bioassay of the expression of HBV DNA in HepG2.2.15 cells showed all tested matrine-type alkaloids, including dimers, showed activities more potent than that of the positive control PFA.

■ EXPERIMENTAL SECTION

General Experimental Procedures. Melting points were measured on a micro-melting point apparatus. Optical rotations were recorded on a polarimeter. IR spectra were recorded with an IR spectrometer (KBr pellets). UV spectra were recorded with a spectrophotometer. The NMR spectra were recorded on 300, 400, and 500 MHz spectrometers with TMS as an internal standard. HR-ESI-MS spectra were recorded on a TOF mass spectrometer. Analytical HPLC was performed using a solvent delivery system with a DAD detector and an analytical column (5 μm, 4.6 mm × 250 mm). Preparative HPLC was performed using a solvent delivery system equipped with UV detectors and a preparative column (5 μm, 20 mm × 250 mm). Thin-layer chromatography (TLC) was performed using precoated silica gel plates (GF254). Open column chromatography (CC) was performed using macroporous resin (Diaion HP-101), silica gel (200−300 mesh), ODS silica gel (50 μm), and Sephadex LH-20. All reagents and solvents used were of analytical or chromatographic grade.

Plant Material. The roots of *S. flavescens* that had been cultivated over 3 years were collected in October 2011 from Xi'an, Shaanxi Province, China, and were authenticated by Z.-Q. Mai, a senior herbalist at the Chinese Medicinal Material Co. (Guangzhou, China). A voucher specimen (no. 20120712) was deposited in the Institute of Traditional Chinese Medicine and Natural Products of Jinan University.

Extraction and Isolation. Dried roots of *S. flavescens* (25.0 kg) were pulverized and extracted with an EtOH/H_2O mixture [95:5 (v/v)] at room temperature, and the combined solution was concentrated to afford a crude extract (1.6 kg). The crude extract was dissolved in H_2O and acidified with 1% HCl to pH 4. The acidic suspension was extracted with $CHCl_3$ to remove the neutral components. Then the aqueous layer was basified with $NH_3{\cdot}H_2O$ to pH 9 and re-extracted with $CHCl_3$ to obtain a total alkaloid fraction (464 g), which was subjected repeatedly to column chromatography over macroporous resin (Diaion HP-101) eluting with an EtOH/H_2O mixture [10:90, 30:70, 50:50, 70:30, and 95:5 (v/v), each 40.0 L] to afford five major fractions (Fr. 1−5). Fr. 2 (43.5 g) was subjected to a silica gel column chromatography with a $CHCl_3$/MeOH mixture [98:2, 95:5, 90:10, 85:15, 80:20, and 70:30 (v/v), each 5.0 L] and further separated by preparative HPLC [30:70:0.01 MeOH/H_2O/Et_2NH (v/v)] to yield compound **7** (10.9 mg). Fr. 4 (87.7 g) was chromatographed over a ODS column using a MeOH/H_2O mixture [30:70, 50:50, 70:30, and 100:0 (v/v), each 7.0 L] as the eluent to yield four subfractions (Fr. 4.1−4.4). Fr. 4.1 (5.7 g) was purified by Sephadex LH-20 [1:1 $CHCl_3$/MeOH (v/v)] and preparative HPLC [60:40:0.01 MeOH/H_2O/Et_2NH (v/v)] to yield **3** (27.3 mg) and **6** (12.1 mg). Fr. 4.3 (21.0 g) was purified by Sephadex LH-20 (MeOH) and then preparative HPLC [60:40:0.01 MeOH/H_2O/Et_2NH (v/v)] to yield **1** (22.1 mg) and **2** (18.4 mg), respectively. Fr. 4.4 (17.4 g) was separated by preparative HPLC [30:70:0.01 MeCN/H_2O/Et_2NH (v/v)] to afford **4** (12.4 mg) and **5** (10.7 mg). Fr. 5 (50.2 g) was successively chromatographed by ODS [50:50, 70:30, and 100:0 (v/v), each 5.0 L], Sephadex LH-20 (MeOH), and preparative HPLC [60:40:0.01 MeOH/H_2O/Et_2NH (v/v)] to yield **8** (37.8 mg) and **9** (12.7 mg).

Acquisition of 2D INADEQUATE Data. The samples (**1**, 22.1 mg; **2**, 18.4 mg; **3**, 27.3 mg) were dissolved in 200 μL of CD_3OD and placed in a 3 mm outside diameter NMR tube. The data were obtained on a 500 MHz spectrometer. The temperature of ∼25 °C was controlled throughout the experiment. To obtain stronger INADEQUATE correlations, the selected measurement range was from δ_C 1 to 70. Compounds **1**−**3** were scanned 128, 512, and 640 times, respectively, by using a standard pulse sequence.

Characterization Data. *Flavesine A (1).* Colorless crystals in MeOH/H_2O: $[\alpha]_D^{25}$ + 33.7 (*c* 1.0, CH_3OH); mp 102−103 °C; HR-ESI-MS *m/z* 497.3841 $[M + H]^+$ (calcd for $C_{30}H_{49}N_4O_2$ *m/z* 497.3850); UV (CH_3OH) λ_{max} 208 nm; IR (KBr) ν_{max} 3442, 2922, 2871, 2808, 1620, 1443, 1341 cm^{-1}; ^1H and ^{13}C NMR data in Table 1.

Flavesine B (2). Brown oil: $[\alpha]_D^{25}$ + 39.0 (*c* 1.0, CH_3OH); HR-ESI-MS *m/z* 497.3841 $[M + H]^+$ (calcd for $C_{30}H_{49}N_4O_2$ *m/z* 497.3850); UV (CH_3OH) λ_{max} 207 nm; IR (KBr) ν_{max} 3425, 2932, 2808, 2765, 1616, 1443, 1339 cm^{-1}; ^1H and ^{13}C NMR data in Table 1.

Flavesine C (3). Brown oil: $[\alpha]_D^{25}$ + 9.9 (*c* 1.0, CH_3OH); HR-ESI-MS *m/z* 495.3694 $[M + H]^+$ (calcd for $C_{30}H_{47}N_4O_2$ *m/z* 495.3694); UV (CH_3OH) λ_{max} 206 nm; IR (KBr) ν_{max} 3417, 2933, 2868, 2794, 1619, 1444, 1416, 1340 cm^{-1}; ^1H and ^{13}C NMR data in Table 1.

Flavesine D (4). Colorless crystals in MeOH/H_2O: $[\alpha]_D^{25}$ + 19.3 (*c* 1.0, CH_3OH); mp 127−128 °C; HR-ESI-MS *m/z* 493.3536 $[M + H]^+$ (calcd for $C_{30}H_{45}N_4O_2$ *m/z* 493.3537); UV (CH_3OH) λ_{max} 207, 262 nm; IR (KBr) ν_{max} 2938, 2772, 1616, 1581, 1436, 1344, 1291, 1103 cm^{-1}; ^1H and ^{13}C NMR data in Table 2.

Flavesine E (5). Brown oil: $[\alpha]_D^{25}$ − 32.4 (*c* 0.3, CH_3OH); HR-ESI-MS *m/z* 495.3691 $[M + H]^+$ (calcd for $C_{30}H_{47}N_4O_2$ *m/z* 495.3694); UV (CH_3OH) λ_{max} 206 nm; IR (KBr) ν_{max} 2934, 2877, 1602, 1474, 1443, 1448, 1331, 1250, 1122, 1081 cm^{-1}; ^1H and ^{13}C NMR data in Table 1.

Flavesine F (6). Colorless crystals in MeOH/H_2O: $[\alpha]_D^{22}$ − 36.1 (*c* 0.5, CH_3OH); mp 245−246 °C; HR-ESI-MS *m/z* 437.2938 $[M + H]^+$ (calcd for $C_{26}H_{37}N_4O_2$ *m/z* 437.2911); UV (CH_3OH) λ_{max} 208, 232, 310 nm; IR (KBr) ν_{max} 2933, 2800, 2761, 1631, 1543, 1465, 1441, 1345, 1158, 808 cm^{-1}; ^1H and ^{13}C NMR data in Table 2.

2-Oxymatrine (7). Brown oil: $[\alpha]_D^{25}$ + 6.47 (*c* 0.82, CH_3OH); HR-ESI-MS *m/z* 263.1755 $[M + H]^+$ (calcd for $C_{15}H_{23}N_2O_2$ *m/z* 263.1754); UV (CH_3OH) λ_{max} 206 nm; IR (KBr) ν_{max} 2946, 2867, 1604, 1448, 1421, 1337, 1267, 692 cm^{-1}; ^1H NMR (500 MHz, CD_3OD) δ 4.68 (1H, m, H-10β), 4.33 (1H, dd, *J* = 12.7, 4.2 Hz, H-17α), 3.67 (1H, br t, *J* = 3.4 Hz, H-6), 3.62 (1H, ddd, *J* = 11.1, 8.8, 6.1 Hz, H-11), 2.79 (1H, t, *J* = 12.7 Hz, H-17β), 2.56 (1H, m, H-10α), 2.46 (1H, m, H-3a), 2.37 (1H, m, H-14a), 2.36 (1H, m, H-3b), 2.28 (1H, m, H-14b), 2.20 (1H, m, H-12a), 2.03 (1H, m, H-4a), 1.90 (1H, m, H-7), 1.88 (1H, m, H-13a), 1.87 (1H, m, H-5), 1.83 (1H, m, H-4b), 1.75 (2H, m, H-8), 1.66 (1H, m, H-13b), 1.59 (2H, m, H-9), 1.55 (1H, m, H-12b); ^{13}C NMR (125 MHz, CD_3OD) δ 173.3 (C-2), 172.1 (C-15), 59.0 (C-6), 53.9 (C-11), 43.2 (C-10), 42.0 (C-17), 41.9 (C-7), 36.3 (C-5), 33.3 (C-14), 28.7 (C-3), 28.1 (C-8), 27.9 (C-12), 22.5 (C-4), 21.1 (C-9), 19.5 (C-13).

Matrine (8). White powder: $[\alpha]_D^{25}$ + 3.6 (*c* 0.75, CH_3OH); mp 76−77 °C; HR-ESI-MS *m/z* 249.1961 $[M + H]^+$ (calcd for $C_{15}H_{25}N_2O$ *m/z* 249.1961); UV (CH_3OH) λ_{max} 208 nm; IR (KBr) ν_{max} 2937, 2863, 2797, 2757, 1624 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 4.27 (1H, dd, *J* = 12.8, 4.4 Hz, H-17α), 3.72 (1H, dt, *J* = 9.6, 6.0 Hz, H-11), 2.92 (1H, t, *J* = 12.8 Hz, H-17β), 2.67−2.98 (2H, m, H-2, 10), 2.30 (1H, m, H-14a), 2.12 (1H, m, H-14b); ^{13}C NMR (100 MHz, $CDCl_3$) δ 169.5 (C-15), 63.8 (C-6), 57.3 (C-10), 57.2 (C-2), 53.2 (C-11), 43.3 (C-7), 41.5 (C-17), 35.4 (C-5), 32.9 (C-14), 27.8 (C-12), 27.2 (C-4), 26.5 (C-8), 21.2 (C-9), 20.8 (C-3), 19.0 (C-13).

13,14-Dehydromatrine (9). Brown oil: HR-ESI-MS *m/z* 247.1805 $[M + H]^+$(calcd for $C_{15}H_{23}N_2O$ *m/z* 247.1805); ^1H NMR (300 MHz, CD_3OD) δ 6.63 (1H, ddd, *J* = 9.8, 5.0, 3.7 Hz, H-13), 5.81 (1H, ddd, *J* = 9.8, 2.3, 1.6 Hz, H-14), 4.05 (1H, dd, *J* = 13.2, 4.8 Hz, H-17α), 3.97 (1H, ddd, *J* = 12.7, 7.5, 7.0 Hz, H-11), 3.15 (1H, t, *J* = 13.0 Hz, H-

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J. Org. Chem. 2016, 81, 6273−6280

17β), 2.84 (2H, overlapped, H-2, H-10); ^{13}C NMR (75 MHz, CD_3OD) δ 167.7 (C-15), 140.9 (C-13), 124.1 (C-14), 64.6 (C-6), 58.2 (C-2), 58.2 (C-10), 52.8 (C-11), 43.0 (C-17), 42.7 (C-7), 35.9 (C-5), 28.7 (C-4), 28.3 (C-12), 27.2 (C-8), 22.0 (C-3), 21.5 (C-9).

Hydrogenation Reactions of 3 and 4. Compound **3** (8.0 mg) was dissolved in MeOH (3.0 mL). Then, the catalyst Pd/C (10.0 mg) was added to the solution, which was stirred at room temperature under hydrogen conditions. After 3 h, the reaction was terminated, Pd/C was filtered, and the filtrate was concentrated *in vacuo*. Finally, compounds **1** and **2** were found in the filtrate by analytical HPLC [30:70:0.01 MeCN/H_2O/Et_2NH (v/v)]. Compound **4** (8.0 mg) was treated under the same condition as **3**. Then, the reaction solution of **4** was purified by preparative HPLC [30:70:0.01 MeCN/H_2O/Et_2NH (v/v)] to obtain **5** (4.26 mg).

Cell Lines and Cell Culture. HepG2.2.15 cells (a HBV-transfected human HepG2 cell line) were kindly provided by Z.-q. Liu (Guangzhou University of Chinese Medicine, Guangzhou, China). The HepG2.2.15 cells were cultivated in basic MEM supplemented with 10% FBS (v/v) at 37 °C in a humidified atmosphere of 5% CO_2 (v/v).

Anti-HBV Effect *in Vitro*. The anti-HBV assay of compounds was performed in triplicate.[31] HepG2.2.15 cells were inoculated at a density of 2×10^4 cells/well and after incubation for 1 day were treated with compounds **1**−**9** (0−500 μM) at 37 °C for 9 days. The medium was replaced with fresh drug-containing medium daily. The control group was treated with medium without compounds. PFA (foscarnet) was used as a positive control. A commercial kit was used to extract cellular DNA. The HBV DNA levels of cells were quantified via real time PCR. Results of the experiment were calculated by regression analysis of the dose−response curve generated from the data, indicated as means ± the standard deviation for three independent experiments. The IC_{50} value was calculated from the relative HBV DNA level using Prism software.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.joc.6b00804.

Detailed NMR, HR-ESI-MS, UV, and IR spectra of **1**−**7**; chemical structure of **7**; bioassay results for **1**−**9**; and HPLC chromatograms of the reaction mixtures of **3** and **4** (PDF)

X-ray crystallography data for **1**, **4**, and **6** (ZIP)

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Notes

The authors declare no competing financial interest.

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