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(+)-12α-Hydroxysophocarpine, a new quinolizidine alkaloid and related anti-HBV alkaloids from *Sophora flavescens*

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Abstract—(+)-12α-Hydroxysophocarpine (**8**), a new quinolizidine alkaloid was isolated from the roots of *Sophora flavescens*, together with 10 known quinolizidine alkaloids, (+)-oxymatrine (**1**), (+)-matrine (**2**), (+)-9α-hydroxymatrine (**3**), (+)-allomatrine (**4**), (+)-oxysophocarpine (**5**), (−)-sophocarpine (**6**), (−)-9α-hydroxysophocarpine (**7**), (+)-lehmannine (**9**), (−)-13,14-dehydrosophoridine (**10**), and (−)-anagyrine (**11**). Their structures were elucidated by spectroscopic methods, and the stereochemistry of **8** was confirmed by X-ray analysis. These alkaloids were tested for anti-hepatitis B virus (HBV) activity in vitro, compounds **5**, **6**, **9**, and **10** showed significant anti-HBV activity with inhibitory potency against HBsAg secretion at 48.3–79.3% and that against HBeAg secretion at 24.6–34.6%.

Sophora species (Leguminosae) are important sources of Chinese herbal drugs. They accumulate quinolizidine alkaloids as principal constituents with potentially useful pharmacological effects such as analgesic, antipyretic, anti-inflammatory, anti-tumor, and notable antiviral activities.[1,2] The major quinolizidine alkaloids oxymatrine and matrine were reported to exhibit anti-hepatitis B virus (HBV) activity, oxymatrine could downregulate HBV gene expression and decrease HBsAg and HBeAg content in HBV transgenic mice,[3] and protect mice from fulminant hepatitis induced by GalN (galactosamine)/LPS (lipopolysaccharide) and block hepatocyte apoptosis as well,[4] and matrine could protect the D-GalN-treated mice from the development of fatal hepatitis induced by LPS.[5]

The roots of *Sophora flavescens* Ait., a species widely distributed throughout China, are commonly used as the traditional Chinese medicine 'Kushen' for the treatment of skin diseases and gynecological diseases, such as eczema, dermatitis, and colpitis.[6] Pharmacological studies showed that the alkaloids of *S. flavescens* inhibited CBV_3 (coxsackie B_3 virus) replication and possessed protective effect on infected myocardial cells,[7] and its components anagyrine, oxymatrine, and sophoranol also exhibited potent antiviral activities against RSV (respiratory syncytial virus).[8] During the course of our screening for anti-HBV agents from *Sophora* plants, a phytochemical investigation on the alkaloid constituents of *S. flavescens* was carried out and resulted in the isolation of a new quinolizidine alkaloid, (+)-12α-hydroxysophocarpine (**8**), together with 10 known quinolizidine alkaloids (**1**–**7**, **9**–**11**) (Fig. 1). This paper reports the isolation and structure elucidation of the new alkaloid, as well as the in vitro anti-HBV activity of these isolated alkaloids.

The roots of *S. flavescens* Ait.[9] (14 kg) were extracted with aqueous 1% (v/v) H_2SO_4, followed by partition with $CHCl_3$ after being basified with Na_2CO_3 to give the crude alkaloids, which were subjected to repeated silica gel column chromatography and prep. TLC to give a new quinolizidine alkaloid, (+)-12α-hydroxysophocarpine (**8**), as well as 10 known ones (**1**–**7**, **9**–**11**).[10] These known alkaloids were identified as (+)-oxymatrine (**1**),[11] (+)-matrine (**2**),[11] (+)-9α-hydroxymatrine (**3**),[12] (+)-allomatrine (**4**),[13] (+)-oxysophocarpine (**5**),[14] (−)-sophocarpine (**6**),[15,16] (−)-9α-hydroxysophocarpine (**7**),[15] (+)-lehmannine

Keywords: *Sophora flavescens*; Quinolizidine alkaloids; (+)-12α-Hydroxysophocarpine; Anti-HBV.
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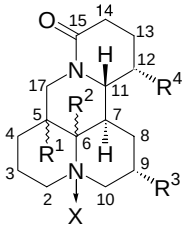

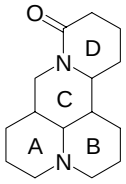

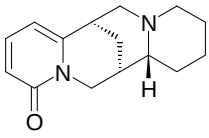

11

	X	R^1	R^2	R^3	R^4	C-12–C-13	C-13–C-14
1	O	α-H	α-H	H	H	single bond	single bond
2	lone pair	α-H	α-H	H	H	single bond	single bond
3	lone pair	α-H	α-H	OH	H	single bond	single bond
4	lone pair	α-H	β-H	H	H	single bond	single bond
5	O	α-H	α-H	H	H	single bond	double bond
6	lone pair	α-H	α-H	H	H	single bond	double bond
7	lone pair	α-H	α-H	OH	H	single bond	double bond
8	lone pair	α-H	α-H	H	OH	single bond	double bond
9	lone pair	α-H	α-H	H	H	double bond	single bond
10	lone pair	β-H	α-H	H	H	single bond	double bond

Figure 1. Chemical structures of compounds **1**–**11**.

(**9**),[17,18] (−)-13,14-dehydrosophoridine (**10**),[13] and (−)-anagyrine (**11**),[19] respectively, by comparison of their $[\alpha]_D$, IR, EIMS, ^{1}H NMR, and ^{13}C NMR spectroscopic data with those reported.

Compound **8**[20] was obtained as white plates, mp 90–92 °C, $[\alpha]_D^{24}$ +137.3° (*c* 0.10, MeOH). The quasi-molecular ion $[M+H]^+$ was detected by HRESIMS at *m*/*z* 263.1761, consistent with the formula of $C_{15}H_{22}N_2O_2$. Its IR spectrum showed absorption bands characteristic of a hydroxyl group (3311 cm^{-1}), an α,β-unsaturated lactam system (1665 and 1598 cm^{-1}), and *trans*-quinolizidine functionalities (2811 and 2783 cm^{-1}). The EI mass spectrum of **8** showed a base peak at *m*/*z* 245 (100), corresponding to $[M-OH]^+$, and fragmentations similar to those of (−)-sophocarpine (**6**) and (−)-12β-hydroxysophocarpine,[15] indicating it might be a hydroxyl derivative of **6**. The ^{1}H NMR spectrum of **8** agreed well with that of **6**, except that there was an additional isolated signal at δ_H 4.17 (1H, m) in the spectrum of **8** which could be assigned to a methine proton bearing a hydroxyl group because of its low chemical shift, and that the signals due to the olefinic protons H-13 and H-14 simplified in splitting pattern from octets and sextets in **6** to quartets and doublets in **8**, and shifted to downfield for 0.29 and 0.10 ppm, respectively (Table 1). These differences were very similar to those of (−)-12β-hydroxysophocarpine as compared with **6**.[15] The hydroxyl group was thus deduced to be located on C-12, which was further supported by the HMBC correlations of the methine proton at δ_H 4.17 (H-12) bearing a hydroxyl group with C-13 (δ_C 138.7) and C-14 (δ_C 126.6), and of the olefinic protons at δ_H 6.74 (H-13) and δ_H 5.99 (H-14) with the carbon at δ_C 60.9 which correlated with the methine proton at δ_H 4.17 in the HMQC spectrum. In the ^{13}C NMR spectrum of **8**, the signals corresponding to C-2–C-6, C-8–C-10, C-15, and C-17 were consistent with those of **6** with $\Delta\delta_C \leqslant 1.2$ ppm, and the olefinic carbons C-13 and C-14 shifted to downfield for 1.3 and 1.9 ppm,

Table 1. Selected ^{1}H NMR data of compounds **6** and **8** (in $CDCl_3$, δ_H in ppm, *J* in Hz)

Compound	11β	12β	13	14	17α	17β
6[a]	3.98 (dd, 16.9, 9.6)	—[c]	6.45 (ddd, 9.8, 5.0, 3.8)	5.89 (dt, 9.8, 2.2)	4.14 (dd, 12.9, 4.7)	3.17 (t, 12.9)
8[b]	3.73 (br d, 10.4)	4.17 (m)	6.74 (dd, 9.6, 6.0)	5.99 (d, 9.6)	3.98 (dd, 13.2, 4.6)	3.22 (t, 13.2)

[a] 400 MHz.
[b] 500 MHz.
[c] Not assigned.

Table 2. ^{13}C NMR data of compounds **6** and **8** (in $CDCl_3$, δ_C in ppm)

Compound	2	3	4	5	6	7	8	9	10	11	12	13	14	15	17
6[a]	57.3	21.1	27.8	34.7	63.6	41.6	26.6	20.8	57.3	51.5	27.4	137.4	124.7	165.7	42.1
8[b]	57.4	21.2	27.7	33.5	62.7	34.7	26.3	21.0	57.3	54.7	60.9	138.7	126.6	165.8	41.0
Δδ (**8**–**6**)	+0.1	+0.1	−0.1	−1.2	−0.9	−6.9	−0.3	+0.2	0	+3.2	+33.5	+1.3	+1.9	+0.1	−1.1

[a] 100 MHz.
[b] 125 MHz.

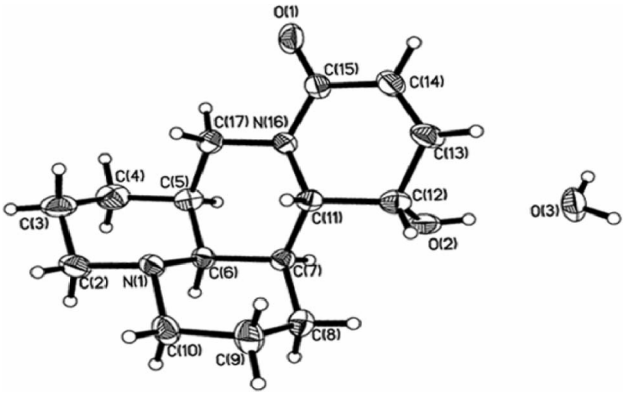

Figure 2. Perspective structure of compound **8** established by single crystal X-ray analysis.

respectively (Table 2). Based on the HMQC and HMBC experiments, the remaining signals at δ_C 34.7, 54.7, and 60.9 were reasonably assigned to C-7, C-11, and C-12, which shifted to upfield for 6.9 ppm and to downfield for 3.2 and 33.5 ppm, respectively, in comparison with those of **6** (Table 2), by considering the substituent effects of a hydroxyl group at C-12. The coupling characteristics of the methine proton at δ_H 4.17 (1H, m) (signal width ca. 16 Hz) indicated that the hydroxyl group was axially oriented. Subsequently the upfield shift ($\Delta\delta_C = -6.9$ ppm) of C-7 could be explained by a γ-gauche effect of the axial hydroxyl group on C-12 in **8**.[21] Therefore, **8** was presumed to be (+)-12α-hydroxysophocarpine. A single crystal X-ray crystallography was thus carried out and the confirmation of the molecule was established as shown in Figure 2.[22]

Compounds **1**–**11** were investigated for in vitro antiviral activity against hepatitis B virus (HBV),[23] and the results are summarized in Table 3. The tested alkaloids showed potent anti-HBV activity, with higher potency against HBsAg secretion than HBeAg secretion. Among them, compounds **5**, **6**, **9** and **10** were the most potent, they significantly inhibited HBsAg secretion by more than 48.3% at the noncytotoxic concentration of 0.2 μmol/mL, which were much stronger than that of the positive control lamivudine (3TC) (29.6% at the concentration of 1.0 μmol/mL), and they also exhibited potent inhibitory activity against HBeAg secretion at 24.6–34.6% (35.4% for 3TC control). Compounds **1** and **3** displayed a little weaker HBsAg inhibitory effect at 31.6–39.7% and HBeAg inhibitory activity at 17.1–19.5%. In addition, it needs to be mentioned that the inhibitory activities against HBsAg and HBeAg secretion of compounds **5**, **6**, **9**, and **10** were much stronger than those of compounds **1** and **2**, which have already been successfully used to treat hepatitis B in the clinic.[24] These four alkaloids are of great value to be further investigated. As to the new compound, its anti-HBV activity was a little stronger than that of compound **2**. While compared to compound **1** and lamivudin, the activity of the new alkaloid against HBsAg secretion (40.8%) was a little stronger, its potency against HBeAg secretion (4.6%) was much lower.

Based on the analysis of the anti-HBV potencies and the structural characteristics of these tested alkaloids from

Table 3. Inhibitory activity of compounds **1**–**11** against HBsAg and HBeAg secretion in HepG2 2.2.15 cell line

Compound	Concentration (μmol/mL)	HBsAg (inhibition %)	HBeAg (inhibition %)
1[a]	0.2	39.7	17.1
	0.1	35.3	15.8
2[a]	0.2	30.9	2.7
	0.1	8.9	0
3[a]	0.2	31.6	19.5
	0.1	18.1	13.3
4[a]	0.2	27.7	10.8
	0.1	25.1	9.6
5[a]	0.2	48.3	24.6
	0.1	39.9	22.4
6[a]	0.2	57.2	34.6
	0.1	21.6	31.4
7[a]	0.2	24.5	5.7
	0.1	0	0
8[a]	0.2	40.8	4.6
	0.1	28.0	0
9[a]	0.2	52.6	25.4
	0.1	25.1	22.6
10[b]	0.8	79.3	27.6
	0.4	78.2	22.4
	0.2	63.4	6.8
	0.1	17.9	0
11[c]	0.4	33.1	0
	0.2	19.8	0
3TC[d]	1.0	29.6	35.4

[a] These compounds showed cytotoxicity against HepG2 2.2.15 cell line at the concentration of 0.4 μmol/mL. Cell damage was assessed using MTT assay, and cell growth inhibition against HepG2 2.2.15 cell line ⩾25% was considered as cytotoxic.

[b] This compound showed cytotoxicity against HepG2 2.2.15 cell line at the concentration of 1.6 μmol/mL.

[c] This compound showed cytotoxicity against HepG2 2.2.15 cell line at the concentration of 0.8 μmol/mL.

[d] Positive control.

S. flavescens, it was found that the alkaloids with double bond in ring D, while without hydroxyl group, were the most potent. Introduction of an N→O group (**5**) or an α-hydroxyl group (**7** and **8**) into sophocarpine (**6**) (with unsaturated D ring) negatively influenced the anti-HBV activity, especially the HBeAg inhibitory potency. Interestingly, as to the matrine derivatives (with saturated D ring), this influence was dramatically opposite, that is, the presence of N→O group (**1**) and α-substituted hydroxyl group (**3**) in (+)-matrine (**2**) markedly enhanced the anti-HBV potency. Comparison of the structure and potency of **7** with those of **3** revealed that introduction of an unsaturated C-13–C-14 bond into ring D of the hydroxylated matrine-derivatives might be unfavourable to anti-HBV activity. In addition, the influence of stereochemistry on HBsAg inhibitory activity is opposite to that on HBeAg inhibitory effect, for example, compound **2** or **10** exhibited a stronger anti-HBsAg activity, while weaker anti-HBeAg potency than compound **4** (C-6 epimer of **2**) or **6** (C-5 epimer of **10**). Thus, in order to fully elucidate the structural determi-

nants for anti-HBV activity of the quinolizidine alkaloids, further investigation is necessary to be carried out.

Acknowledgments

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9. Plant materials: the roots *of Sophora flavescens* Ait. were purchased from Huayu Materia Medica Co., Ltd., Shanghai, in February of 2001, and verified by Dr. Dao-Feng Chen. A voucher specimen (KS-SH-0102) is deposited in the Herbarium of Materia Medica, Department of Pharmacognosy, School of Pharmacy, Fudan University, Shanghai, People's Republic of China.
10. Extraction and isolation: the pulverized roots of *S. flavescens* (14 kg) were extracted with aqueous 1% (v/v) H_2SO_4 solution (4× 24 L) at room temperature. The filtered acid extract was basified to pH 10–11 with Na_2CO_3 and partitioned with $CHCl_3$ six times. The $CHCl_3$ extract was concentrated in vacuo to give the total alkaloids (250 g) in a yield of 1.79%. The total alkaloids were dissolved with $CHCl_3$ (200 mL), Et_2O (2 L) was then added to give a precipitate (70 g) of the mixture of **1** and **5**. Part of the precipitate (1.2 g) was eluted with $CHCl_3$/EtOAc/MeOH/$NH_3{\cdot}H_2O$ (10:2:1:0.1) on silica gel to give **1** (100 mg) and **5** (120 mg). The residue of the total alkaloids (150 g) was subjected to silica gel column chromatography eluting with $CHCl_3$/EtOAc/MeOH/$NH_3{\cdot}H_2O$ (10:2:0.3:0.05, 10:2:0.7:0.07, and 10:2:1.3:0.15) gradiently to give fractions 1–6. Fraction 1 (10.5 g) was subjected to silica gel column chromatography with Et_2O/MeOH/$NH_3{\cdot}H_2O$ (12:0.2:0.1) to give **2** (68 mg), **6** (20 mg), and **9** (40 mg). Fraction 2 (5.6 g) was eluted with Et_2O/MeOH/$NH_3{\cdot}H_2O$ (12:0.3:0.1) on silica gel to yield **11** (50 mg). Fraction 3 (3.8 g) was applied to silica gel column chromatography eluting with Et_2O/MeOH/$NH_3{\cdot}H_2O$ (10:0.8:0.1) twice to give **4** (15 mg). Fraction 4 (6.2 g) was chromatographed over silica gel eluting with Et_2O/MeOH/$NH_3{\cdot}H_2O$ (10:1:0.1), $CHCl_3$/EtOAc/MeOH/$NH_3{\cdot}H_2O$ (8:2:1:0.1), and Et_2O/MeOH/$NH_3{\cdot}H_2O$ (10:0.8:0.15) successively to give **7** (10 mg), the residue of which was then purified on silica gel with Et_2O/MeOH/$NH_3{\cdot}H_2O$ (10:0.8:0.15) to yield **3** (30 mg). Fraction 5 (8.6 g) was applied to silica gel column chromatography eluting with Et_2O/MeOH/$NH_3{\cdot}H_2O$ (10:0.5:0.1) to give fractions 5-A and 5-B, fraction 5-A (200 mg) was applied to prep. TLC developed with Et_2O/MeOH/$NH_3{\cdot}H_2O$ (9.5:0.5:0.4) to give **8** (15 mg), and fraction 5-B (3.5 g) was chromatographed over silica gel eluting with Et_2O/MeOH/$NH_3{\cdot}H_2O$ (10:0.5:0.1) to afford **10** (30 mg).
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20. (+)-12α-Hydroxysophocarpine (**8**): (7a*S*,13*S*,13a*S*,13b*S*,13c*S*)-2,3,6,7,7a,8,13,13a,13b,13c-decahydro-13-hydroxy-1*H*,5*H*,10*H*-dipyrido[2,1-*f*:3′,2′,1′-*ij*][1,6]naphthyridin-10-one. White plates (petroleum ether/acetone 5:1); mp 90–92 °C; $[\alpha]_D^{24}$ +137.3° (*c* 0.10, MeOH); UV (MeOH) λ_{max} nm (log ε): 206 (3.86) and 258 (3.37); IR (KBr) ν_{max} 3311, 2931, 2891, 2870, 2833, 2811, 2783, 1665, 1598, 1446, 1354, 1340, 1295, 1097, 1057 and 823 cm^{-1}; ^1H NMR ($CDCl_3$, 500 MHz) δ 6.74 (1H, dd, *J* = 9.6, 6.0 Hz, H-13), 5.99 (1H, d, *J* = 9.6 Hz, H-14), 4.17 (1H, m, H-12β), 3.98 (1H, dd, *J* = 13.2, 4.6 Hz, H-17α), 3.73 (1H, br d, *J* = 10.4 Hz, H-11β), 3.22 (1H, t, *J* = 13.2 Hz, H-17β), 2.84 (1H, br d, *J* = 10.8 Hz, H-2β), 2.80 (1H, br d, *J* = 10.9 Hz, H-10β), 2.29 (1H, br d, *J* = 11.0 Hz, H-7α), 2.13 (1H, br s, H-6α), 1.94–2.00 (3H, m, H-2α, 8β, 10α), 1.78 (3H, m, H-3β, 5α, 9β), 1.62 (3H, m, H-4α, 4β, 8α), 1.48 (2H, m, H-3α, 9α); ^{13}C NMR (Table 2); EIMS *m*/*z* (%): 262 $[M]^+$ (1), 246 (20), 245 (100), 244 (8), 243 (3), 177 (4), 150 (5), 148 (4), 136 (5), 122 (3), 96 (9), 68 (3), 55 (3), 41 (4); HRESIMS *m*/*z* 263.1761 $[M+H]^+$ (calcd for $C_{15}H_{23}N_2O_2$, 263.1760).
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22. Crystal data for **8**: $C_{15}H_{24}N_2O_3$, MW = 280. 36 ($C_{15}H_{22}N_2O_2{\cdot}H_2O$). Orthorhombic, $P2_12_12_1$, *a* = 7.936(3) Å, *b* = 12.128(4) Å, *c* = 14.834(5) Å, α = β = γ = 90°, *V* = 1427.7(8) $Å^3$, *Z* = 4, *Dc* = 1.304 mg/m^3, approximate crystal dimension of 0.15 × 0.10 × 0.04 mm^3. Mo Kα (λ = 0.71073 Å), *F*(000) = 608, *T* = 298(2) K, *R* = 0.0354, R_w = 0.0572, for 1853 unique $I > 2\sigma(I)$ (total = 2800). All the data were collected in the ω scan mode on a computer-controlled Bruker SMART APEX-CCD diffractometer, maximum θ values 2.17 ⩽ 26.00°. The structure was solved by direct methods (SHELXTL97) and refined by full-matrix least-squares on F^2. Crystallographic data for **8** have been deposited with the

Cambridge Crystallographic Data Centre as supplementary publication numbers CCDC281903. Copies of the data can be obtained, free of charge, on application to CCDC, 12 Union Road, Cambridge CB2 1EZ, UK [fax: +44 (0) 1223-336033 or e-mail: deposit@ccdc.cam.ac.uk].

23. Anti-HBV tests: drug stock solutions were prepared in DMSO and stored at −70 °C. Upon dilution into culture medium, the final DMSO concentration was ⩽1% DMSO (v/v), a concentration without effect on cell replication. Cell culture and other procedures were the same as those reported previously: Wu, T.; Huang, H.; Zhou, P. *Zhongguo Bing Du Xue* **1998**, *13*, 45. A HepG2-derived human hepatablastoma cell line, 2.2.15, was used in this study, which was transfected with cloned HBV DNA to produce HBV particles. All stock cultures were grown in T-25 flasks containing the DMEM supplemented with 10% (v/v) fetal bovine serum, 0.03% (v/v) L-glutamine, 100 μg/mL penicillin, 100 μg/mL streptomycin, and 380 μg/mL G418 at 37 °C in a humidified atmosphere containing 5% CO_2. After the HepG2 2.2.15 cell suspensions seeded in 24-well microtiter plates were cultured for 48 h, they were incubated at 37 °C for 9 days in the presence of various concentrations of drugs (1.6, 0.8, 0.4, 0.2, and 0.1 μmol/mL, respectively) from DMSO-diluted stock, and the medium was refreshed every 3 days. Then the culture supernatants were harvested to detect the HBsAg and HBeAg secretion using diagnostic kit for HBsAg and HBeAg (ELISA) (Shanghai SIIC KEHUA Biotech Co., Ltd) as described in the instruction of the kit. Each test was performed in three times, and the SEM (standard error of the mean) of inhibition values varied no more than 5%. Cell damage was assessed using MTT assay.
24. Ding, P. L.; Chen, D. F. *Zhongguo Lin Chuang Yao Xue Za Zhi* **2003**, *12*, 315.