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Nonpeptide small molecules from the insect *Aspongopus chinensis* and their neural stem cell proliferation stimulating properties

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Four pairs of enantiomeric hypoxanthine analogues (**1**, **2**, **5** and **6**), two pairs of enantiomeric adenine analogues (**3** and **4**), two pyrazines, aspongpyrazines A (**7**) and B (**8**), and a pair of glycerides ((+)-**9** and (–)-**9**), along with twenty-eight known substances were isolated from the insect *Aspongopus chinensis*. The structures of the new natural products were assigned by using spectroscopic and computational methods. Chiral HPLC was used to separate the (–)- and (+)-antipodes of **1**–**6**, and **9**. Several of the new, *A. chinensis* derived natural products were found to promote proliferation of neural stem cells (NSCs) at a concentration of 10 μM.

1. Introduction

Insects account for a majority of animal species on earth. In the past, much attention has been given to the isolation and identification of the peptides and proteins present in the insects. However, much less is known about insect derived small molecule natural products and their biological properties. *Aspongopus chinensis* Dallas (Pentatomidae), widely distributed in the major provinces of China such as Guizhou, Sichuan, and Yunnan Provinces, is a traditional Chinese medicine commonly used for the treatment of erectile dysfunction, pain, stomach disease, and cancer.[1] In addition, this insect is consumed as a multi-regional food in China not only because of its delicious taste but also as a consequence of its health-care value. The results of pharmacological studies showed that the extract of *A. chinensis* possesses pronounced antibacterial, anticancer, analgesic, antifibrotic, and angiogenesis promoting activities.[1,2] Besides, the results of an earlier chemical investigation showed that *N*-acetyldopamine derivatives are present in this insect.[3,4] However, thus far, still little is known about the chemical and biological profiles of *A. chinensis* in spite of its wide applications in clinical practice. This deficit prompted us to conduct an investigation, which led to the isolation and structural identification of 9 new natural products together with 28 known small molecules from *A. chinensis* (Fig. 1). In addition, the results of a biological evaluation showed that several of the new substances promote neural stem cell proliferation at a concentration of 10 μM. The results of this effort are described below.

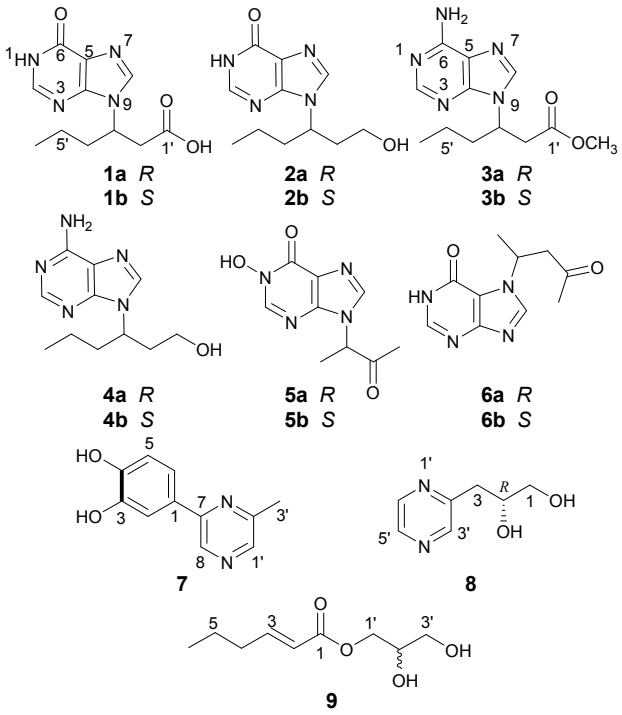

Fig. 1 The chemical structures of compounds 1–9.

2. Results and discussion

2.1. Structure elucidation

A. chinensis (powder, 20 kg) was subjected to successive extraction with petroleum ether, acetone and 50% aqueous MeOH, the latter of which was concentrated to obtain a crude extract which was suspended in water followed by extraction with *n*-BuOH. The *n*-BuOH extract was separated using a MCI gel CHP 20P column to produce portions that were subjected to chromatographic separation

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Electronic Supplementary Information (ESI) available: Detailed isolation procedure; Calculation of ECD of **1b**, **3b**, **5b**, **6b**, and **8**; Spectra of the new compounds **1**–**9**.

using various column chromatography to give the new and known natural products **1–9** and **10–37**, respectively. Structural assignments to **1–9** were made by using the spectroscopic and computational methods described below.

The structure of compound **1**, having the molecule formula $C_{11}H_{14}N_4O_3$, was assigned by using a combination of HRESIMS, ^{13}C NMR and DEPT spectroscopic methods. The ^1H-^1H COSY spectrum exhibits correlations of H-2′/H-3′/H-4′/H-5′/H-6′. The HMBC spectrum displays a correlation of H-2′ with a carboxylic group at δ_C 172.8, indicating the presence of a hexanoic acid residue. In addition, other NMR signals demonstrate the presence in 3 of a 2-deaminoguanine moiety, which is connected to C-3′ via N-9. This conclusion is supported by the existence of HMBC (Fig. 2) correlations of H-3′/C-4, C-8. This substance is not enantiomerically pure which was further purified by chiral HPLC to afford (+)-**1** (**1a**) and (–)-**1** (**1b**) with a ratio of 60:40. Determination of absolute configurations at the stereogenic centers in each antipode was performed by using computational methods (Fig. S1). Consequently, the structure of **1**, named asponguanine A, are those shown in Fig. 1.

Compound **2** has the molecular formula $C_{11}H_{16}N_4O_2$, as deduced by analysis of its HREIMS (*m/z* 236.1273 $[M]^+$, calcd for $C_{11}H_{16}N_4O_2$, 236.1273). Careful comparison of the NMR data of **2** with those of **1** reveals that they have similar structures, except for the presence of a hydroxymethylene (δ_C 59.2) in **2** in place of a carbonyl group (δ_C 172.8) in **1**. This proposal gains support from the analysis of the ^1H-^1H COSY spectrum that exhibits correlations of H-1′/H-2′/H-3′/H-4/H-5/H-6. **2** is also not enantiomerically pure, further purification by chiral HPLC to afford (+)-**2** (**2a**) and (–)-**2** (**2b**) with a ratio of 56:44. Because **1** and **2** contain similar subunits at their key stereogenic centers and similar ECD spectra, they must have the same configuration at their respective stereogenic centers. Therefore, the structure of **2**, named asponguanine B, is that shown in Fig. 1.

The molecular formula of compound **3** was determined to be $C_{12}H_{19}N_5O$ based on its HREIMS, ^{13}C NMR and DEPT spectroscopic data. The ^1H-^1H COSY spectrum exhibits correlations of H-2/H-3/H-4/H-5/H-6, which when considered along with the chemical shift of C-1' at δ_C 172.4, demonstrate the presence of a hexanoic acid residue. Apart from those associated with this fragment, the remaining NMR signals agree well with those in an adenine unit. That this moiety is connected to C-3 via *N*-9 is indicated by the observation of HMBC correlations of H-3/C-4, C-8. The HMBC correlation of CH_3/C-1' indicates the position of the CH_3O group in **3**. The enantiomerically impure **3** was finally purified by chiral HPLC to give (+)-**3** (**3a**) and (–)-**3** (**3b**) with a ratio of 55:45. Computational methods enabled assignment of the absolute configuration at the stereogenic center in each antipode of **3** (Fig. S2). As a result, the structure of **3**, named aspongadenine A, is deduced to be that shown in Fig. 1.

The molecule formula of **4** was assigned as $C_{11}H_{17}N_5O$ by

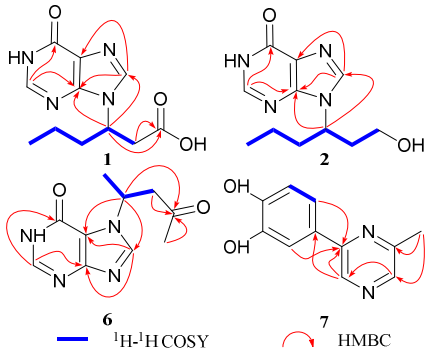

Fig. 2 Key ^1H-^1H COSY and HMBC correlations of **1**, **2**, **6** and **7**.

Table 1
NMR spectroscopic data for **1**[a], **2**[a], and **4**[a]

position	**1**[b]		**2**[c]		**4**[c]	
	δ_H[b] (*J* in Hz)	δ_C[b]	δ_H[c] (*J* in Hz)	δ_C[c]	δ_H[c] (*J* in Hz)	δ_C[c]
2	7.97 s	144.9, CH	8.02 s	146.4, CH	8.18 s	153.4, CH
4		148.4, qC		150.4, qC		150.7, qC
5		124.0, qC		125.3, qC		120.2, qC
6		156.9, qC		159.5, qC		157.4, qC
8	8.06 s	139.8, CH	8.12 s	141.3, CH	8.19 s	141.9, CH
1′a		172.8, qC	3.50 dt (11.2, 5.9)	59.2, CH_2	3.48 m	59.2, CH_2
1′b			3.31 overlap		3.31 overlap	
2′a	2.50 overlap	43.4, CH_2	2.27 m	38.4, CH_2	2.27 m	38.4, CH_2
2′b			2.14 m		2.14 m	
3′	4.78 m	54.2, CH	4.74 m	55.0, CH	4.72 m	54.6, CH
4′a	1.87 m	36.4, CH_2	2.11 m	37.9J, CH_2	2.11 m	37.8, CH_2
4′b	1.80 m		1.89 m		1.90 m	
5′a	1.05 m	19.0, CH_2	1.23 m	20.4, CH_2	1.22 m	20.4, CH_2
5′b	0.96 m		1.11 m		1.11 m	
6′	0.78 t (7.0)	13.6, CH_3	0.90 t (7.4)	13.8, CH_3	0.90 t (7.4)	13.9, CH_3

[a] 600 MHz for ^1H, 150 MHz for ^{13}C. [b] In DMSO-d_6. [c] In methanol-d_4.

analysis of its HREIMS, ^{13}C NMR and DEPT spectral data. The ^1H-^1H COSY spectrum exhibits correlations of H-1′/H-2′/H-3′/H-4′/H-5′/H-6′, which when considered in light of the chemical shift of C-1′ at δ_C 59.2, reveal the presence of a hexanol residue. Apart from those corresponding to this fragment, the rest of the NMR signals are in good agreement with the presence of an adenine unit. The observation of HMBC correlations of H-3′/C-4, C-8 show that the adenine unit is connected to C-3′ via *N*-9. The lack of an optical rotation indicates that **4** is a racemate. Chiral HPLC separation afforded (+)-**4** (**4a**) and (–)-**4** (**4b**) whose ratio is 62:38. Because no Cotton effect was observed in the ECD spectrum of each enantiomer of **4** and based on the fact that **4** and **3** have similar structures, the absolute configurations at the stereogenic centers in the enantiomers of **4** were tentatively assigned by comparison of optical rotations with those of the enantiomers of **3**. Consequently, the structure of **4**, named aspongadenine B, was assigned to be that shown in Figure 1.

Compound **5** has the molecular formula $C_9H_{10}N_4O_3$ as determined by analysis of its HREIMS (*m/z* 222.0751 $[M]^+$; calcd for $C_9H_{10}N_4O_3$, 222.0753). The ^{13}C NMR spectra of **5** contain resonances for nine carbons, including four quaternary, three methine and two methyl carbons. Based on ^{13}C NMR data, the structure of **5** is similar to that of **1**, except for a difference in the side chain. The ^1H-^1H COSY spectrum exhibits correlation of H-3′/H-4′. The HMBC spectrum shows correlations of H-1′, H-3′, and H-4′ with a ketone group at δ_C 207.3, indicating the presence of a 2-butanone side chain, which is connected to C-3′ via *N*-9 as suggested by the HMBC correlations of H-3′/C-4, C-8. The existence of the *N*-OH in this substance came from analysis of its HREIMS. The lack of an optical rotation indicates that **5** is a racemate. Chiral HPLC separation afforded (+)-**5** (**5a**) and (–)-**5** (**5b**) with a ratio of 57:43, whose absolute configurations were determined using computational methods (Fig. S3). Therefore, compound **5**, named asponguanine C, was determined to have the structure shown in Fig. 1.

Compound **6**, having the molecule formula $C_{10}H_{12}N_4O_2$ deduced from its HRESIMS (*m/z* 243.0855 $[M+Na]^+$; calcd for $C_{10}H_{12}N_4O_2Na$, 243.0858), has ^{13}C NMR data that are similar to those of **1**. The ^1H-^1H COSY spectrum exhibits correlations of H-3′/H-4′/H-5′ and the HMBC (Fig. 2) results show correlations of H-1′, H-3′, and H-4′ with a ketone group at δ_C 207.7. The latter observation indicates the presence of a 2-butanone side chain, which is connected to C-3′ via *N*-7. This proposal is supported by the HMBC correlations of H-3′/C-5, C-8. Compound **6** was isolated as a

Table 2
NMR spectroscopic data for **3**[a], **5**[a], and **6**[a]

Position	3		5		6	
	δ_H (J in Hz)	δ_C	δ_H (J in Hz)	δ_C	δ_H (J in Hz)	δ_C
2	8.18 s	153.5, qC	8.03 s	147.4, CH	8.01 s	146.1, CH
4		150.6, qC		149.9, qC		155.9, qC
5		120.2, qC		125.5, qC		116.0, qC
6		157.3, qC		160.7, qC		159.0, qC
8	8.19 s	142.2, CH	8.09 s	140.7, CH	8.29 s	144.8, CH
1′		172.4, qC	2.11 s	30.1, CH_3	2.09 s	30.1, CH_3
2′a	3.24 dd (16.5, 9.5)	39.6, CH_2		207.3, qC		207.7, qC
2′b	3.01 dd (16.5, 4.9)					
3′a	4.90 m	54.5, CH	5.08 q (7.0)	49.3, CH	3.49 dd (18.3, 8.0)	49.6, CH_2
3′b					3.17 dd (18.3, 5.6)	
4′a	2.17 m	37.0, CH_2	1.59 d (7.0)	20.8, CH_3	5.23 m	52.1, CH
4′b	1.90 m					
5′a	1.23 m	20.3, CH_2			1.62 d (6.9)	21.4, CH_3
5′b	1.09 m					
6′	0.90 d (7.3)	13.7, CH_3				
OCH_3	3.54, s	52.3, CH_3				

[a] 600 MHz for ^{1}H, 150 MHz for ^{13}C. In methanol-d_4.

racemate, which was resolved by using chiral HPLC to afford the antipodes **6a** and **6b** with a ratio of 53:47. The absolute configurations at the stereogenic centers in the enantiomers were assigned by using computational methods (Fig. S4). Thus, the structure of **6**, named asponguanine D, was determined to be that shown in Fig. 1.

Analysis of HREIMS, ^{13}C NMR and DEPT spectra of compound **7** showed that it has the molecular composition $C_{11}H_{10}N_2O_2$, suggesting 8 degrees of unsaturation. The ^{1}H NMR spectrum of **7** contains resonances for a typical ABX spin system (δ_H 7.48, d, J = 1.6 Hz, H-2; δ_H 6.88, d, J = 8.2 Hz, H-5; δ_H 7.38, dd, J = 8.2, 1.6 Hz, H-6), which in conjunction with the carbon chemical shifts suggest that the arene ring C-3 and C-4 carbons are oxygenated. The ^{13}C NMR and DEPT spectra contain signals for 10 carbons attributed to 1 CH_3, 5 CH (all aromatic), and 4 C. HMBC (Fig. 2) correlations of H-2, H-6/C-1, C-7, H-8/C-7, C-1, C-1′, in conjunction with the chemical shifts of C-8 and C-1. These observations indicate that **7** is a dopamine derivative. In addition, HMBC correlations of H-1′, H-3′/C-2′, H-1′/C-3′, and C-8 enable elucidation of the structure of the fragment. One benzene ring and two double bonds, consisting of C-7 and C-8, C-1′ and *N,* account for 6 of the 8 degrees of unsaturation. The chemical shift of C-2′ indicates the presence of additional double bond in **7** and the remaining degree of unsaturation is occupied by one ring. Importantly, C-7 and C-2′ are quaternary aromatic carbons having chemical shifts that suggest that they are connected via a *N*-atom. In this way, we conclude that a pyrazine ring is present in the architecture of **7**. The combined results show that the structure **7**, named aspongpyrazine A, is that shown in Fig. 1.

Compound **8** has the molecular formula $C_7H_{10}N_2O_2$ (4 degrees of unsaturation), as deduced from its HRESIMS, ^{13}C NMR and DEPT spectra. The ^{1}H NMR spectrum of **8** contains resonances for three aromatic protons (δ_H 8.54, m, 2H, H-3′, H-6′; δ_H 8.44, d, J = 2.4 Hz, 1H, H-5′). The ^{13}C NMR and DEPT spectra shows that this substance contains 7 carbons comprised of 2 CH_2 (one oxygenated), 4 CH (three aromatic, one oxygenated aliphatic), and 1 C (aromatic). The ^{1}H-^{1}H COSY spectrum of **8** exhibits the correlations of H-1/H-2/H-3 and H-5′/H-6′. The structure of **8** was assigned by analysis of its HMBC correlations. The HMBC correlations of H-2/C-2′, H-3/C-2′, and C-3′ reveal the partial structure from C-1 to C-3′. The observed HMBC correlations of H-6′/C-2′ and H-5′/C-3′ along with consideration of the chemical shifts of C-2′, C-3′, C-5′, and C-6′ and the molecular composition, strongly support the presence of a pyrazine ring in the structure. The absolute configuration at the stereogenic center in **8** was assigned by using computational methods (Fig. S5). Therefore, the structure of **8**, named aspongpyrazine B, is that shown in Fig. 1.

Table 3
NMR spectroscopic data for **7**[a], **8**[a], and **9**[a]

position	7		8		9	
	δ_H (J in Hz)	δ_C	δ_H (J in Hz)	δ_C	δ_H (J in Hz)	δ_C
1a		129.1, qC	3.57 dd (10.3, 4.8)	66.9, CH_2		168.2, qC
1b			3.55 dd (10.3, 4.1)			
2	7.48 d (1.6)	114.6, CH	4.03 m	72.7, CH	5.89 d (15.6)	122.3, CH
3a		147.0, qC	3.06 dd (14.0, 4.1)	40.2, CH_2	7.01 dt (15.6, 7.0)	151.2, CH
3b			2.89 dd (14.0, 8.9)			
4		148.6, qC			2.22 q (7.2)	35.2, CH_2
5	6.88 d (8.2)	116.7, CH			1.52 m	22.4, CH_2
6	7.38 dd (8.2, 1.6)	119.7, CH			0.96 t (7.4)	14.0, CH
7		151.9, qC				
8	8.81 s	141.0, CH				
1′a	8.48 s	144.9, CH			4.20 dd (11.4, 4.2)	66.5, CH_2
1′b					4.11 dd (11.4, 6.3)	
2′		151.7, qC		156.9	3.85 m	71.2, CH
3′a	2.55 s	20.7, CH_3	8.54 m	146.7, CH	3.58 dd (11.3, 5.5)	64.4, CH_2
3′b					3.55 dd (11.3, 5.7)	
5′			8.44 d (2.4)	143.3, CH		
6′			8.54 m	145.3, CH		

[a] 600 MHz for ^{1}H, 150 MHz for ^{13}C. In methanol-d_4.

The molecular composition of **9** was determined to be $C_9H_{16}O_4$ by analysis of its HREIMS, ^{13}C NMR and DEPT spectra. The ^{1}H-^{1}H COSY spectrum of this substance show correlations of H-1′/H-2′/H-3′, which along with a consideration of carbon chemical shifts indicate the presence of a glycerol residue. ^{1}H-^{1}H COSY correlations H-2 (δ_H 5.89, d, J = 15.6 Hz)/H-3 (δ_H 7.01, dt, J = 15.6, 7.0 Hz)/H-4/H-5/H-6 and HMBC correlations of H-2, H-3/C-1 (δ_C 168.2) suggest that a hex-2-enoic acid residue connected to the glycerol residue via an ester bond exists in the structure. This conclusion is supported by the HMBC correlation of H-1′/C-1. The geometry of double bond in **9** is assigned as *E* as a consequence of the large coupling constant of involving H-2 (J = 15.6 Hz). The lack of an optical rotation indicates that **9** is racemic. The (+)-**9** and (–)-**9**

enantiomers were separated by using chiral HPLC. However, their absolute configurations remain unsolved due to the limit of ECD calculation. Further vibrational circular dichroism calculation might be useful to clarify this question. Thus the structure of **9**, named aspongester A, was deduced to be that shown in Fig. 1.

In addition to the new substances described above, 28 known compounds were also isolated from the insect extract. By comparison of their spectroscopic data to those of the previously reported substances, these natural products were shown to be aspongopusin (**10**),[2] nicodine (**11**),[6] 2-quinolinol (**12**),[7] *N*-(2-hydroxyethyl)succinimide (**13**),[8] 6-(hydroxymethyl)pyridin-3-ol (**14**),[9] 2-pyridone (**15**),[10] valerolactam (**16**),[11] 2-pyrrolidinone (**17**),[11] choline (**18**),[12] indole-3-aldehyde (**19**),[13] indole-3-carboxylic acid (**20**),[14] transtorine (**21**),[15] *L*-phenylalanine (**22**),[16] nicotinamide (**23**),[17] 4-hydroxyisobenzofuran-1(3*H*)-one (**24**),[18] 4-(3,4-dihydroxy-phenyl)-but-3-en-2-one (**25**),[19] vanillic acid (**26**),[20] 3,4-dihydroxyphenylacetic acid (**27**),[21] 2-hydroxy-3-methylbutanoic acid (**28**),[22] 3,4,5-trimethoxyphenol-β-D-glucopyranoside (**29**),[23] adenine (**30**),[24] thymine (**31**),[25] cyclo(*L*-Val-*L*-Ala) (**32**),[26] 2′-*O*-methyluridine (**33**),[27] cordysinin B (**34**),[28] adenosine (**35**),[25] deoxyadenosine (**36**),[29] and thymidine (**37**).[25] It is notable that all the other compounds are nonpeptide small molecules with the exception of compounds **22** and **32**.

2.2. Biological evaluation

A study was conducted to determine if the new, insect derived natural products stimulate proliferation of neural stem cells (NSCs). For this purpose, NSCs were obtained from P7 (7 d postnatal) mouse dentate gyrus of hippocampus, and SRB assay was performed. The result of a sulforhodamine B (SRB) colorimetric assay showed that all the new natural products except **3a**, **3b**, **9a** and **9b** cause an increase in NSCs proliferation compared with the basal medium condition with 10 ng/mL growth factor bFGF (Fig. 3A). Among the active group, **2a** is the most potent and, as a result, it was subjected to further study. The results show that **2a** acts synergistically with 20 ng/mL bFGF and 20 ng/mL EGF to promote NSCs proliferation (Fig. 3A). The results of a neurosphere clonal assay, demonstrated that larger spheres are generated when **2a** is present compared to when DMSO is present (Fig. 3B). To test whether **2a** increases cell cycle progression, a BrdU incorporation assay was carried out. The results demonstrate that about 1.5-fold more BrdU-positive cells in DAPI-positive are formed when **2a** is present compared to when DMSO is present. The data indicate that **2a** enhances the self-renewal ability of NSCs *in vitro* (Fig. 3C and 3D).

It is noted that **1**–**6** are nucleoside and neucleobase analogues, which typically possess anti-viral activities. However, owing to the small quantities of these compounds we have on hand, we were unable at the current time to evaluate this property of the *A. chinensis* natural products.

3. Experimental section

3.1. General procedure

Column chromatography (CC) was performed on silica gel (200–300 mesh; Qingdao Marine Chemical Inc., P. R. China), on C-18 silica gel (40–60 μm; Daiso Co., Japan), MCI gel CHP 20P (75–150 μm, Mitsubishi Chemical Industries, Tokyo, Japan) and on Sephadex LH-20 (Amersham Pharmacia, Sweden). Optical rotations were recorded on a Horiba SEPA-300 polarimeter. UV spectra were recorded on a Shimadzu UV-2401PC spectrometer. CD spectra were measured on a Chirascan instrument. Semi-preparative or analytical HPLC was carried out using an Agilent 1200 liquid chromatograph (250 mm × 9.4 mm, i.d. column containing 5 μm, Zorbax SB-C_{18} or a 250 mm × 4.6 mm, i.d., 5 μm column containing Daicel Chiralpak (IC for **3**–**6** and **9**, and AD-H for **1** and **2**). NMR spectra were recorded on a Bruker AV-600 spectrometer, with TMS as an internal standard. EIMS and HREIMS were collected by using an AutoSpec Premier P776 spectrometer. ESIMS and HRESIMS data were collected by API QSTAR Pulsar 1 spectrometer.

3.2. Insect material

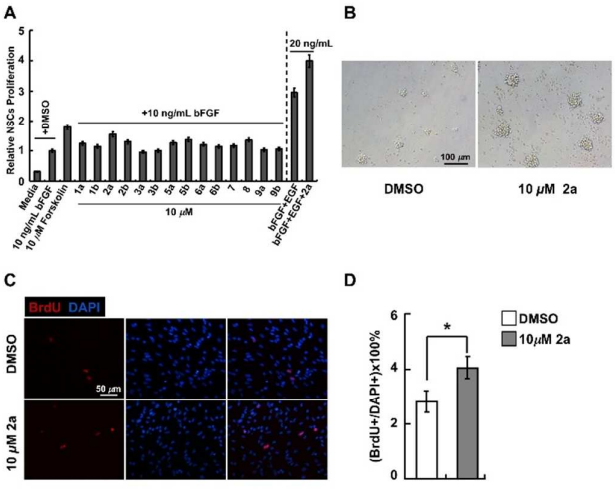

Fig. 3 Analyzing functions of compounds on NSCs proliferation. (A) SRB assay was performed in NSCs treated with various compounds or reagents. The relative proliferation ratios were normalized to basal medium condition with10 ng/mL bFGF, which was set to 1. One positive control was 10 μM forskolin; the other positive control was 20 ng/mL bFGF+20 ng/mL EGF. (B) NSCs neurosphere assay incubated with **2a** or DMSO. Scale bar: 100 μm. (C) Using BrdU incorporation assay to examine NSCs proliferation rate treated with **2a** or DMSO. (D) Quantification data for (C). The ratio of BrdU positive cells in DAPI positive cells treated by **2a** and DMSO. Scale bar: 50 μm, $p \leq 0.05$. Each experiment was repeated at least 3 times.

A. chinensis Dallas was purchased from Hunan Corporation of Chinese Materia Medica (Changsha) People's Republic of China, in September 2010. The material was identified by Prof. Xiao-Jiang Zhou at Hunan University of Chinese Medicine, and a voucher specimen (CHYX-0633) was deposited at the State Key Laboratory of Photochemistry and Plant Resources in West China, Kunming Institute of Botany, Chinese Academy of Sciences, People's Republic of China.

3.3. Extraction and isolation

An air-dried powder of *A. chinensis* (20 kg) was extracted successively with petroleum ether (3 × 80 L), acetone (3 × 80 L) and 50% aqueous MeOH (3 × 80 L) at room temperature. The 50% aqueous MeOH extract was concentrated to obtain a crude extract (1 kg), which was suspended in water followed by extracted with *n*-BuOH to afford an *n*-BuOH soluble extract (100 g). The *n*-BuOH extract was separated by using a MCI gel CHP 20P column eluted with gradient aqueous MeOH (10:90–100:0) to provide five portions (Fr.A–Fr.E). For the remaining detailed isolation procedures, see Supporting Information.

3.4. Spectral data of the new compounds

3.4.1. *Asponguanine A* ***(1)***. White solid; $\{[\alpha]_D^{23}$ + 6.4 (*c* 0.27, MeOH); CD (MeOH) $\Delta\varepsilon_{210}$ + 2.37; **1a**$\}$; $\{[\alpha]_D^{23}$ – 12.9 (*c* 0.11, MeOH); CD (MeOH) $\Delta\varepsilon_{210}$ – 2.43; **1b**$\}$; UV (MeOH) λ_{max} (logε) 252 (3.76), 205 (4.11) nm; ESIMS *m/z* 273 $[M + Na]^+$; HRESIMS *m/z* 273.0942 $[M + Na]^+$ (calcd for $C_{11}H_{14}N_4NaO_3$, 274.0964); ^{1}H and ^{13}C NMR data, see Table **1**.

3.4.2. *Asponguanine B* ***(2)***. White solid; $\{[\alpha]_D^{23}$ + 6.7 (*c* 0.09, MeOH); CD (MeOH) $\Delta\varepsilon_{207}$ + 0.57; **2a**$\}$; $\{[\alpha]_D^{23}$ – 14.7 (*c* 0.08, MeOH); CD (MeOH) $\Delta\varepsilon_{207}$ – 0.72; **2b**$\}$; UV (MeOH) λ_{max} (logε) 250

(3.64), 204 (3.95) nm; EIMS *m/z* 236 $[M]^+$; HREIMS *m/z* 236.1273 $[M]^+$ (calcd for $C_{11}H_{16}N_4O_2$, 236.1273); ^1H and ^{13}C NMR data, see Table **1**.

3.4.3. *Aspongadenine A* ***(3)***. White solid; {$[\alpha]_D^{25}$ + 8.6 (*c* 0.23, MeOH); CD (MeOH) $\Delta\varepsilon_{217}$ + 1.27; **3a**}; {$[\alpha]_D^{25}$ – 13.7 (*c* 0.20, MeOH); CD (MeOH) $\Delta\varepsilon_{217}$ – 1.78; **3b**}; UV (MeOH) λ_{max} (logε) 261 (4.16), 207 (4.31) nm; EIMS *m/z* 263 $[M]^+$; HREIMS *m/z* 263.1384 $[M]^+$ (calcd for $C_{12}H_{17}N_5O_2$, 263.1382); ^1H and ^{13}C NMR data, see Table **2**.

3.4.4. *Aspongadenine B* ***(4)***. White solid; $[\alpha]_D^{26}$ + 13.9 (*c* 0.06, MeOH); **4a**}; {$[\alpha]_D^{26}$ – 8.5 (*c* 0.06, MeOH); **4b**}; UV (MeOH) λ_{max} (logε) 261 (4.00), 207 (4.19) nm; EIMS *m/z* 235 $[M]^+$; HREIMS *m/z* 235.1419 $[M]^+$ (calcd for $C_{11}H_{17}N_5O$, 235.1420); ^1H and ^{13}C NMR data, see Table **1**.

3.4.5. *Asponguanine C* ***(5)***. White solid; {$[\alpha]_D^{23}$ – 14.3 (*c* 0.07, MeOH); CD (MeOH) $\Delta\varepsilon_{210}$ – 2.45; **5a**}; {$[\alpha]_D^{23}$ + 11.4 (*c* 0.08, MeOH); CD (MeOH) $\Delta\varepsilon_{210}$ + 8.49; **5b**}; UV (MeOH) λ_{max} (logε) 250 (4.25), 203 (4.51) nm; EIMS *m/z* 222 $[M]^+$; HREIMS *m/z* 222.0751 $[M]^+$ (calcd for $C_9H_{10}N_4O_3$, 222.0753); ^1H and ^{13}C NMR data, see Table **2**.

3.4.6. *Asponguanine D* ***(6)***. White solid; {$[\alpha]_D^{23}$ unstable; CD (MeOH) $\Delta\varepsilon_{270}$ – 0.56; **6a**}; {$[\alpha]_D^{23}$ unstable; CD (MeOH) $\Delta\varepsilon_{270}$ + 0.76; **6b**}; UV (MeOH) λ_{max} (logε) 255 (3.53), 204 (3.93) nm; ESIMS *m/z* 243 $[M + Na]^+$; HRESIMS *m/z* 243.0855 $[M + Na]^+$ (calcd for $C_{10}H_{12}N_4NaO_2$, 243.0858); ^1H and ^{13}C NMR data, see Table **2**.

3.4.7. *Aspongpyrazine A* ***(7)***. Colorless gum; UV (MeOH) λ_{max} (logε) 325 (3.90), 269 (4.02), 213 (4.23), 202 (4.23) nm; EIMS *m/z* 202 $[M]^+$; HREIMS *m/z* 202.0746 $[M]^+$ (calcd for $C_{11}H_{10}N_2O_2$, 202.0742); ^1H NMR and ^{13}C NMR data, see Table **3**.

3.4.8. *Aspongpyrazine B* ***(8)***. Colorless gum; $[\alpha]_D^{23}$ –34.6 (*c* 0.35, MeOH); UV (MeOH) λ_{max} (logε) 267 (3.77), 202 (3.79) nm; CD (MeOH) $\Delta\varepsilon_{266}$ – 0.90; ESIMS *m/z* 177 $[M + Na]^+$; HRESIMS *m/z* 177.0629 $[M + Na]^+$ (calcd for $C_7H_{10}N_2NaO_2$, 177.0640); ^1H and ^{13}C NMR data, see Table **3**.

3.4.9. *Aspongester A* ***(9)***. Colorless gum; {$[\alpha]_D^{26}$ + 5.1; (+)-**9**}; {$[\alpha]_D^{26}$ – 10.2; (–)-**9**}; UV (MeOH) λ_{max} (logε) 208 (4.04) nm; EIMS *m/z* 188 $[M]^+$; HREIMS *m/z* 188.1047 $[M]^+$ (calcd for $C_9H_{16}O_4$, 188.1049); ^1H and ^{13}C NMR data, see Table **3**.

3.5. Computational study

Geometry optimizations of all new compounds was carried out at the DFT/B3LYP/6-31G (d) level by means of a Gaussian 09 program. The calculated ECD spectra were determined by using Gaussian 09 software employing the TDDFT-B3LYP functional and the 6-311++G (2d, 2p) basis sets. The calculated CD spectra in $\Delta\varepsilon$ were obtained by using overlapping Gaussian functions.[31]

3.6. Cell culture and sulforhodamine B colorimetric (SRB) assay

Adult hippocampal neural stem cells (about 20,000) were evenly seeded into 96 well plates in growth medium with 10 ng/mL fibroblast growth factor (FGF), 1% penicillin/streptavidin, 1% N2 supplement (Gibco), 1 × B27 (Gibco) and 10 mg/mL heparin in a manner previously described.[32] After 24 h, to the cells were added DMSO or the new compounds at a concentration of 10 μM and then incubated for 48 h. SRB assays were performed using the standard protocol[33] with basal indicated media + basic fibroblast growth factor (10 ng/mL bFGF) (positive control 1 contained forskolin (10 μM) and positive control 2 contained media+EGF (20 ng/mL) + bFGF (20 ng/mL)). The value obtained using the basal was set to 1 and the other values were normalized accordingly.

For the sulforhodamine B colorimetric assay, NSCs were fixed with 16% (w/v) TCA for 1 h at 4 ^oC, stained with 0.4% (w/v) 100 μL of sulforhodamine B solution for 10 min and then washed with 1% acetic acid. The bounded dye was resolved by using 10 mM Tris base, and the absorbance of each well at 515 nm was determined using an automated plate reader. Assays for each compound were carried out in triplicate and each experiment was repeated at least 3 times.

3.7. Immunostaining

NSCs were pulsed with 10 M 5-bromo-2-deoxyuridine (BrdU) in NSCs growth medium (described above) for 15–20 min. Cells were fixed with PFA and washed, stained with BrdU antibody (Abcam 1:300) and mixed with a fluorescence conjugated secondary antibody (goat-anti-rat, 1:500). The ratio of BrdU-positive cells among DAPI-positive cells was quantified manually in a blind manner using Image J software. Each experiment was repeated at least 3 times.

4. Conclusions

In conclusion, we have isolated and characterized 9 new and 28 known small molecules from the insect *A. chinensis*. Several of them were isolated as enantiomerically impure. Chiral HPLC was used to separate the (–) - and (+)-antipodes. Several of the new compounds exhibited proliferation stimulating effect on neural stem cells. This contribution will add new facets for insect-derived nonpeptide small compounds.

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Notes and references

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