FULL PAPER

Chemical Constituents from the Fermented Mycelia of the Medicinal Fungus *Xylaria nigripes*

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Nineteen compounds mainly including pyrrole-containing alkaloids and phytosterols were isolated from the EtOH extract of the fermented mycelia of *Xylaria nigripes*, a precious medicinal fungus known as Wuling Shen in Chinese. On the basis of spectroscopic methods, the structures of the new naturally occurring compounds were determined to be (4*S*)-3,4-dihydro-4-(4-hydroxybenzyl)-3-oxo-1*H*-pyrrolo[2,1-*c*][1,4]oxazine-6-carbaldehyde (**1**), methyl (2*S*)-2-[2-formyl-5-(hydroxymethyl)-1*H*-pyrrol-1-yl]-3-(4-hydroxyphenyl)propanoate (**2**), and 3-{4-[(2*R*)-(2,3-dihydroxy-3-methylbutoxy]phenyl}-7-hydroxy-4*H*-chromen-4-one (**3**), respectively. The absolute configurations of **1** and **2** were deduced by the observed *Cotton* effects in their circular dichroism (CD) spectra, whereas that of the 1,2-diol moiety in **3** was determined using the *Snatzke*'s method. Their biological activities such as neuroprotective, anti-neuroinflammatory, and cytotoxic properties were also reported.

Introduction. – *Xylaria nigripes* (Koltz.) Sacc. (family Xylariaceae), under the folklore name of Wuling Shen in Chinese, is a precious medicinal fungus used in traditional Chinese medicine (TCM) as a nerve tonic for insomnia and depression [1]. The wild fungus is hard to acquire since it is usually growing around the abandoned termite (*Odontotermes formosanus*) nests [2]. Nevertheless, the mycelia of *X. nigripes* can be now largely manufactured through fermentation. An on-shelf drug, under the trade name of Wuling Capsule, is a single herbal formula made from the commercially available fermented product (Wuling Powder) [3]. Wuling Capsule was approved in 1999 by the China Food and Drug Administration (CFDA) as a First Grade New TCM, and has been used in clinic for more than 15 years, exerting significant benefits for patients with insomnia, anxiety, and depression [4]. However, studies on the secondary metabolites and their biological properties of this fungus remain limited. Chemically, only a few ubiquitous constituents, including steroids [5][6], chromanones [6], isoflavones [7], isocoumarins [7][8], and cerebrosides [5], were previously reported from this fungus. In our preceding work on the EtOH extract of the fermented mycelia of *X. nigripes*, four rare spiroketal pyrrole-derived alkaloids (*i.e.*, xylapyrrosides) have been isolated as the minor components [9]. Further investigation on this extract resulted in the isolation of three new (**1** – **3**) and sixteen known (**4** – **19**) naturally occurring compounds. Reported herein are their isolation and structure elucidation, along with their neuroprotective, anti-neuroinflammatory, and cytotoxic activities.

Results and Discussion. – From a 75% EtOH extract of the commercially fermented mycelia of *X. nigripes*, a number of natural products were isolated, including nine pyrrole-containing alkaloids (**1**, **2**, and **4** – **10**) and six steroids (**14** – **19**; *Fig. 1*). Comparing their spectroscopic data and physicochemical properties with those reported in literature, the known compounds were identified to be (4*S*)-4-benzyl-3,4-dihydro-3-oxo-1*H*-pyrrolo[2,1-*c*][1,4]oxazine-6-carbaldehyde (**4**) [10][11], (4*S*)-3,4-dihydro-3-oxo-4-(propan-2-yl)-1*H*-pyrrolo[2,1-*c*][1,4]oxazine-6-carbaldehyde (**5**) [12], (4*S*)-3,4-dihydro-4-(2-methylpropyl)-3-oxo-1*H*-pyrrolo[2,1-*c*][1,4]oxazine-6-carbaldehyde (**6**) [12], 4-[2-formyl-5-(hydroxymethyl)-1*H*-pyrrol-1-yl]butanoic acid (**7**) [13], methyl 4-[2-formyl-5-(methoxymethyl)-1*H*-pyrrol-1-yl]butanoate (**8**) [14], 4-[2-formyl-5-(methoxymethyl)-1*H*-pyrrol-1-yl]butanoic acid (**9**) [14], 5-(ethoxymethyl)-1*H*-pyrrole-2-carbaldehyde (**10**) [14], 5-(hydroxymethyl)furan-2-carbaldehyde (**11**) [15], curdione (**12**) [16], genistein (**13**) [17], (3*β*,22*E*)-ergosta-7,9(11),22-trien-3-ol (**14**; ergosterol D) [18], (3*β*,5*α*,8*α*,22*E*)-ergosta-6,22-diene-3,5,8-triol (**15**) [19], (3*β*,5*α*,6*β*,22*E*)-ergosta-7,22-diene-3,5,6-triol (**16**; cerevisterol) [20], (3*β*,5*α*,6*β*,22*E*)-6-methoxyergosta-7,22-diene-3,5-diol (**17**) [21], (3*β*,5*α*,6*β*)-stigmastane-3,5,6-triol (**18**) [21], and (3*β*)-stigmast-5-en-3-yl 6-*O*-[(9*Z*,12*Z*)-octadeca-9,12-dienoyl]-*β*-D-

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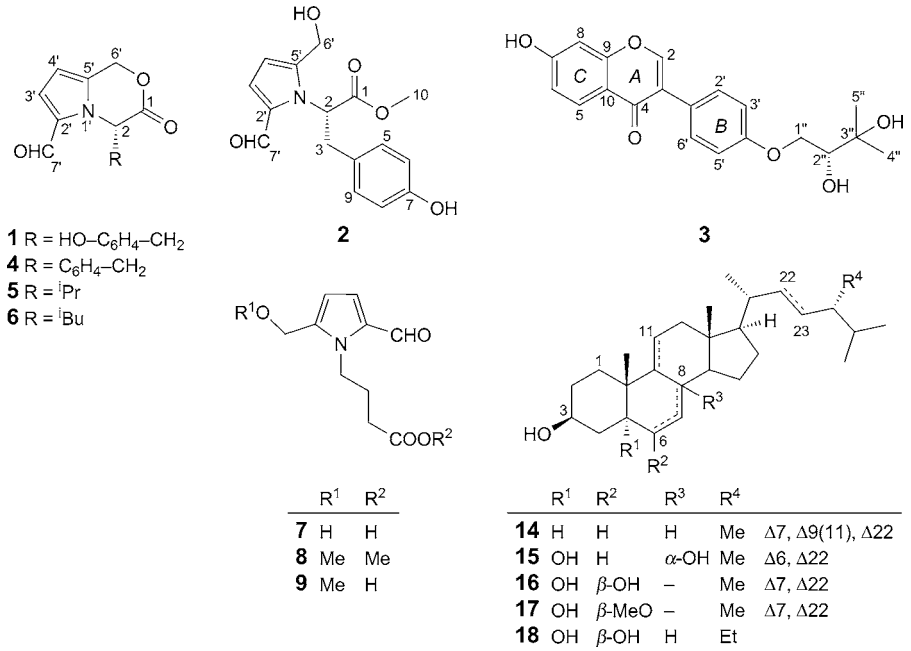

	R^1	R^2
7	H	H
8	Me	Me
9	Me	H

	R^1	R^2	R^3	R^4	
14	H	H	H	Me	Δ7, Δ9(11), Δ22
15	OH	H	α-OH	Me	Δ6, Δ22
16	OH	β-OH	–	Me	Δ7, Δ22
17	OH	β-MeO	–	Me	Δ7, Δ22
18	OH	β-OH	H	Et	

Fig. 1. *Structures of compounds* **1**–**9** *and* **14**–**18**

glucopyranoside (**19**) [22]. Interestingly, all the alkaloids (**1**, **2** and **4**–**10**, *Fig. 1*) possess a common 2-formyl-5-(hydroxymethyl)pyrrole skeleton, which could be also found in xylapyrrosides A and B, and other related spiro-alkaloids produced by this fungus [9]. To our knowledge, this class of 2-formyl-5-(hydroxymethyl)-pyrrole-containing alkaloids has never been reported from any mushrooms as the secondary metabolites until the present study. In addition, the above known compounds (except **13** and **16**) were identified for the first time from *X. nigripes*.

Compound **1** was obtained as a colorless gum and its molecular formula was determined to be $C_{15}H_{13}NO_4$ based on a *pseudo*-molecular ion $[M+H]^+$ at *m/z* 272.0917 (calc. 272.0928) in the HR-ESI-MS (positive-ion mode). The IR absorption bands suggested the presence of a lactone (1747 cm^{-1}) and an α,β-unsaturated CO (1647 cm^{-1}) group. An absorption band at 291 nm in the UV spectrum of **1** was indicative of a pyrrole-2-aldehyde moiety [23], and this was confirmed by the presence of a formyl H-atom at δ(H) 9.59 (*s*, H–C(7′)) and two mutually-coupling H-atoms at δ(H) 7.08 (*d*, $J=4.0$, H–C(3′)) and 6.00 (*d*, $J=4.0$, H–C(4′)) for a typical 2,5-disubstituted pyrrole ring [9][23] in the ^{1}H-NMR spectrum (*Table*). The ^{1}H-NMR spectrum of **1** also displayed signals for a *para*-substituted benzene ring (δ(H) 6.68, 6.62 (each 2 H, br. *d*, $J=8.8$)), an isolated –$CHCH_2$– group (δ(H) 5.99 (*dd*, $J=4.8$, 3.2, H–C(2)), 3.46 (*dd*, $J=14.4$, 3.2, H_a–C(3)), 3.35 (*dd*, $J=14.4$, 4.8, H_b–C(3))), and an O–CH_2 group with an *AB* system (δ(H) 4.89 and 3.65 (*AB*, $J=14.8$, CH_2(6′)); *Table*). The ^{13}C-NMR data of **1** (*Table*) exhibited 15 signals including two CO groups (δ(C) 179.2, 167.9), ten olefinic C-atoms (δ(C) 105.7–155.9) due to two aromatic rings, one sp^3 CH group (δ(C) 59.3) and two sp^3 CH_2 groups (δ(C) 63.7, 39.5). The above NMR data of **1** together with its ESI-MS data were all identical to those of a synthetic compound, 2-[5-(hydroxymethyl)-2-formyl-1*H*-pyrrol-1-yl]-3-(4-hydroxyphenyl)propionic acid lactone, which was synthesized by a roasting reaction between L-tyrosine and D-glucose [11], indicating a (2*S*) configuration for compound **1**. This was in agreement with the observation of two positive *Cotton* effects around 208 and 285 nm in the circular dichroism (CD) spectrum of **1**, which matched well with those of compounds **4**–**6** [24]. Thus, the absolute configuration of **1** was unambiguously determined as (*S*), and it was reported herein as a new naturally occurring pyrrole lactone. Indeed, the known congeners **4**–**6** have also been reported to be generated by reactions of D-glucose with a corresponding L-amino acid: L-phenylalanine for **4**, L-valine for compound **5**, and L-leucine for **6** [11][23][25]. In these reactions, the stereogenic center at the L-amino acid moieties (*i.e.*, C(2) in compounds **1** and **4**–**6**) are conserved. However, no data on absolute configuration, such as specific rotations and CD spectra, could be found in any literature. Until recently, their absolute configurations have been well-defined by the lactone sector rule, and by comparison of their experimental CD spectra with those predicted by time-dependent DFT (TDDFT) calculations [24].

Compound **2**, purified as a colorless gum, has a molecular formula of $C_{16}H_{17}NO_5$ as deduced by HR-ESI-MS (*m/z* 326.0985 ($[M+Na]^+$)). The ^{1}H- and ^{13}C-NMR spectroscopic data (*Table*), as well as the UV spectrum, were quite similar to those of **1**, indicating **2** was also a conjugate of 2-formyl-5-(hydroxymethyl)pyrrole and tyrosine. The most distinct difference between these two compounds was that **2** possesses an additional MeO group (δ(H) 3.77 (*s*), δ(C) 52.7). Moreover, from the ESI-MS data, nine degrees of unsaturation were required for **2**, one

Table. *^1H-* (400 MHz, *J* values in Hz) *and ^{13}C-* (100 MHz) *NMR Data for* **1** – **3**

Position	**1**[a])		**2**[a])		Position	**3**[b])	
	δ(H)	δ(C)	δ(H)	δ(C)		δ(H)	δ(C)
1		167.9		169.7	2	8.34 (*s*)	153.6
2	5.99 (*dd*, *J* = 4.8, 3.2)	59.3	4.84 (br. *s*)	61.3	3		123.7
3	3.46 (*dd*, *J* = 14.4, 3.2), 3.35 (*dd*, *J* = 14.4, 4.8)	39.5	3.65 (*dd*, *J* = 14.4, 3.2), 3.25 (*dd*, *J* = 14.4, 10.4)	37.1	4		175.1
4		132.4		132.3	5	7.98 (*d*, *J* = 8.8)	127.8
5	6.68 (br. *d*, *J* = 8.8)	130.8	6.66 (br. *d*, *J* = 8.4)	130.3	6	6.95 (br. *d*, *J* = 8.8)	115.6
6	6.62 (br. *d*, *J* = 8.8)	115.9	6.61 (br. *d*, *J* = 8.4)	115.4	7		162.9
7		155.9		154.5	8	6.88 (br. *s*)	102.6
8	6.62 (br. *d*, *J* = 8.8)	115.9	6.61 (br. *d*, *J* = 8.4)	115.4	9		157.9
9	6.68 (br. *d*, *J* = 8.8)	130.8	6.66 (br. *d*, *J* = 8.4)	130.3	10		117.1
10			3.77 (*s*)	52.8	1′		124.5
2′		130.2		133.4	2′/6′	7.50 (br. *d*, *J* = 8.4)	130.5
3′	7.08 (*d*, *J* = 4.0)	126.0	7.00 (*d*, *J* = 3.2)	125.9	3′/5′	7.00 (br. *d*, *J* = 8.4)	114.7
4′	6.00 (*d*, *J* = 4.0)	105.7	6.10 (*d*, *J* = 3.2)	110.3	4′		159.1
					1″	4.27 (br. *d*, *J* = 9.6), 3.84 (*dd*, *J* = 9.6, 7.8)	70.3
5′		125.9		142.7			
6′	4.89 (*AB*, *J* = 14.8), 3.65 (*AB*, *J* = 14.8)	63.7	4.21 (*d*, *J* = 12.4) 3.84 (*d*, overlapped)	56.6	2″	3.56 (br. *d*, *J* = 7.8)	76.2
					3″		71.2
7′	9.59 (*s*)	179.2	9.49 (*s*)	179.1	4″	1.16 (*s*)	27.8
					5″	1.10 (*s*)	24.7

[a]) Recorded in $CDCl_3$. [b]) Recorded in (D_6)DMSO.

less than that of compound **1**. Considering that two CO groups, a pyrrole, and a benzene ring accounted for the nine degrees of unsaturation, compound **2** was hence assumed to be a lactone ring-opened product of **1**, with the COOH group esterified (C(1): δ(C) 169.7). This was further confirmed by ^{1}H,^{1}H-COSY and HMBC experiments (see *Supporting Information*[1])). Especially, HMB correlations from the MeO group to C(1), from CH_2(3) (δ(H) 3.65, 3.25) to C(1), C(2) (δ(C) 61.3) and C(5)/C(9) (δ(C) 130.3), and from H–C(5)/H–C(9) (δ(H) 6.66) to C(3) (δ(C) 37.1) were observed. It is worth mentioning that the H-atom at C(2) in **2** resonated as a broad *singlet* instead of a *doublet* of *doublet*s in compound **1**. This phenomenon is probably due to the steric hindrance of the Ph ring, which has also been observed for two similar compounds, methyl 2-[2-formyl-5-(methoxymethyl)-1*H*-pyrrol-1-yl]-3-(4-hydroxyphenyl)propanoate [26] and methyl 2-[2-formyl-5-(methoxymethyl)-1*H*-pyrrol-1-yl]-3-phenylpropanoate [27]. Similar *Cotton* effects observed for **2** ($\Delta\varepsilon_{207}+8.00$, $\Delta\varepsilon_{286}+7.64$) and **1** ($\Delta\varepsilon_{208}+3.42$, $\Delta\varepsilon_{285}+3.94$) in their CD spectra indicated that they shared the same (2*S*) absolute configuration. Accordingly, the structure of **2** was characterized as methyl (2*S*)-2-[2-formyl-5-(hydroxymethyl)-1*H*-pyrrol-1-yl]-3-(4-hydroxyphenyl)propanoate.

However, in contrast to the negative optical rotations observed for those amino acid-derived pyrrole alkaloids (**4**: $[\alpha]_D^{20}=-46$, **5**: $[\alpha]_D^{20}=-88$, **6**: $[\alpha]_D^{20}=-32$, in acetone) recently reported by *Zhu et al.* [24], positive values were obtained for compounds **1**, **2**, and **4** – **6** (**1**: $[\alpha]_D^{22}=+52.0$, **2**: $[\alpha]_D^{22}=+36.7$, **4**: $[\alpha]_D^{22}=+72.3$, **5**: $[\alpha]_D^{22}=+46.8$, **6**: $[\alpha]_D^{22}=+55.4$, in acetone). Similar values were also acquired, when CH_2Cl_2 was used as the solvent (see *Exper. Part*). Actually, such conflicts could be always found for those synthetic analogues [28]. This could be explained by a significant conformational rearrangement under different measurement conditions that results in a distinct change of optical activity, which has been fully discussed for α-amino acids; *e.g.*, see [29].

Compound **3** was isolated as white amorphous powder, which was assigned the molecular formula $C_{20}H_{20}O_6$ by the HR-ESI-MS (*m*/*z* 379.1144 ($[M+Na]^+$)). The IR spectrum exhibited absorption bands at 3354 and 1624 cm^{-1} attributed to OH and conjugated CO functionalities, respectively. The UV absorption maximum at 249 nm was typical of an isoflavone [30], which was supported by the presence of characteristic NMR resonances at δ(H) 8.34 (*s*, H–C(2)) and δ(C) 153.6 (C(2)) [31]. Also, the aromatic region of the ^{1}H-NMR spectrum (*Table*) of **3** displayed signals of a typical *ABX* spin system (δ(H) 7.98 (*d*, *J* = 8.8, H–C(5)), 6.95 (br. *d*, *J* = 8.8, H–C(6)), 6.88 (br. *s*, H–C(8))) assignable to the three aromatic H-atoms on ring *C*, and signals of a *para*-substituted ring *B* with an *AA′BB′* spin system (δ(H) 7.50 (br. *d*, *J* = 8.4, H–C(2′) and H–C(6′)), 7.00 (br. *d*, *J* = 8.4, H–C(3′) and H–C(5′))). The above data were superimposable on those of daidzein [17], indicating that compound **3** was a daidzein derivative. The remaining signals shown in the ^{1}H-NMR spectrum (*Table*) included an O–CH_2 group (δ(H) 4.27 (br. *d*, *J* = 9.6, H_a–C(1″)), 3.84 (*dd*, *J* = 9.6, 7.8, H_b–C(1″))), an O-bearing CH group (δ(H) 3.56 (br. *d*, *J* = 7.8, H–C(2″))), and two Me *singlet*s (δ(H) 1.16 (*s*, Me(4″)), 1.10 (*s*, Me(5″))). These signals could be easily assigned to a 1,2,3-trihydroxylated

[1]) Supporting Information is available upon request from the authors.

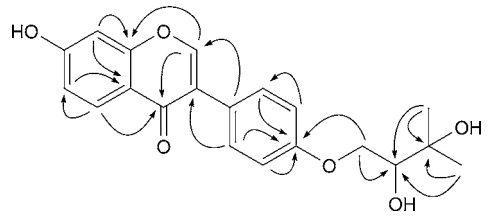

Fig. 2. *Observed key HMB correlations of compound* **3**

isopentyl unit. Correspondingly, three O-bearing C-atoms at δ(C) 70.3 (CH_2, C(1″)), 76.2 (CH, C(2″)), and 71.2 (qC, C(3″)), together with two Me groups at δ(C) 27.8 (Me, C(4″)) and 24.7 (Me, C(5″)), were observed in the high field region of the ^{13}C-NMR spectrum (*Table*) of **3**. Furthermore, the linkage position between the isoflavone skeleton and the isopentyl chain was determined by the observation of a clear ^3J correlation from H–C(1″) to C(4′) (δ(C) 159.1) in the HMBC spectrum of **3** (*Fig. 2*). Additionally, HMB correlations from H–C(5) to C(4) (δ(C) 175.1) and from H–C(6) to C(10) (δ(C) 117.1) confirmed that the OH group at the *C*-ring should be located at C(7), but not C(6).

To determine the absolute configuration of 2″,3″-diol moiety in compound **3**, an induced circular dichroism (ICD) spectrum which involved the *in situ* complexation of a 1,2-diol with dimolybdenum tetraacetate [$Mo_2(AcO)_4$] in DMSO solution (*Snatzke*'s method) was applied [32]. According to the empirical rule proposed by *Snatzke* and *Flerek* [32], the sign of the *Cotton* effect at around 310 nm in the ICD spectrum originates from the chirality of the *vic*-diol expressed by the O–C–C–O torsion angle in the favored conformation. As shown in *Fig. 3*, the metal complex of compound **3** in DMSO gave a significant ICD spectrum, in which the negative *Cotton* effect observed at 306 nm permitted the assignment of the (*R*)-configuration at C(2″). Therefore, the structure of **3** was established as (2″*R*)-4′-(2,3-dihydroxy-4-methylbutoxyl)daidzein. But, it should be noted that the fungus (*X. nigripes*) itself seems not to produce the substituted daidzein but might transform the ingredients (daidzein and genistein (**13**)) of the soy meal medium into the compound **3** [33].

Considering the traditional application of *X. nigripes* and Wuling Capsule in treating central nervous system (CNS) associated diseases, *e.g.*, anxiety disorders and depression, the compounds isolated in suitable amounts (**1** – **4**, **8**, **9**, **11**, **12**, and **14** – **18**), were evaluated for their therapeutic potential against CNS-related disorders. Several well-established cell-based models have been utilized, including neuroprotection [34][35], anti-neuroinflammation [35], and acetylcholine esterase (AChE) inhibition [34]. As shown in *Fig. 4*, (3β,5α,6β,22*E*)-6-methoxyergosta-7,22-diene-3,5-diol (**17**), at the concentration of 10 μM, exhibited a significant neuroprotective effect by attenuating β-amyloid$_{25\text{-}35}$ (A$\beta_{25\text{-}35}$)-induced cell damage in human SH-SY5Y neuroblastoma cells [34] (20.9% of increase in cell viability, *Fig. 4*). Meanwhile, in the anti-neuroinflammatory test, the above mentioned isolates were evaluated

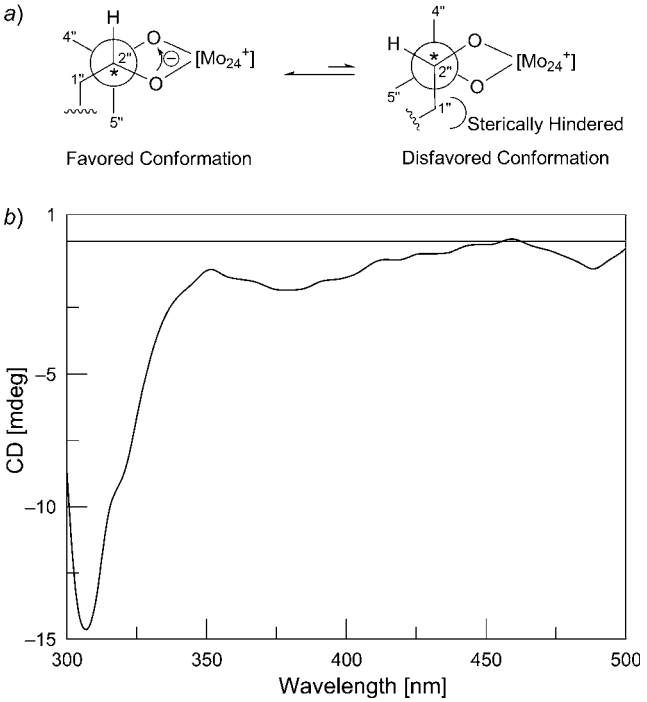

Fig. 3. *a*) *Conformation of the* Mo_2^{4+} *complex of* **3**; *b*) *ICD spectrum of* **3** *in DMSO containing* $Mo_2(OAc)_4$ *with the inherent CD spectrum subtracted*

for their inhibitory effects against the lipopolysaccharide (LPS)-induced nitric oxide (NO) production in BV-2 microglial cells [35]. As a result, only (3β,5α,8α,22*E*)-ergosta-6,22-diene-3,5,8-triol (**15**) could significantly decrease NO production with an IC_{50} value of 27.6 μM, which was comparable to that of the positive control, N^G-monomethyl-L-arginine (L-NMMA, $IC_{50} = 14.4$ μM). Additionally, none of the tested compounds showed significant inhibitory effects against AChE [34] (inhibition rate < 50% at 40 μM). Taken together, the above findings provide new clues on the mechanism of the therapeutic effect of Wuling Capsule on improving CNS associated diseases. Undoubtedly, other related bioactivities of the pyrrole-containing alkaloids (**1**, **2**, **4** – **10**) need to be evaluated in a further study.

In addition, steroids **14**, **17**, and **18** exhibited remarkable cytotoxic effects against human U2OS lung osteosarcoma cells, with IC_{50} values of 0.93, 6.0, and 13.2 μM, respectively. Compound **17** also showed cytotoxicity against A549 human lung carcinoma cell line ($IC_{50} = 11.9$ μM). Staurosporine was used as a positive control, with IC_{50} values of 0.01 and 0.003 μM, respectively.

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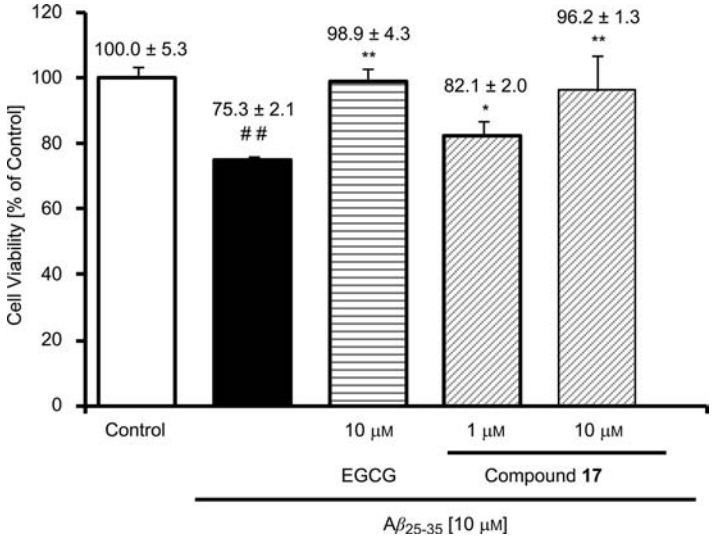

Fig. 4. *Neuroprotective effect of compound* **17** *against* $A\beta_{25\text{-}35}$*-induced cell viability decrease in SH-SY5Y cells.* Three independent experiments were carried out in triplicate. The data were means ± SD expressed as percentage of control value. $^{\#\#}P < 0.01$ *vs.* control group, $^{*}P < 0.05$, $^{**}P < 0.01$ *vs.* $A\beta_{25\text{-}35}$-group. EGCG (epigallocatechin-3-gallate) was used as a positive control.

Experimental Part

General. SiO_2-precoated plates (*GF254*, 0.25 mm, *Kang-Bi-Nuo Silysia Chemical Ltd.*, Yantai, P. R. China) were used for TLC. Spots were visualized using UV light (254 and/or 366 nm) and by spraying with 5% (*v*/*v*) H_2SO_4/EtOH or 15% $CeSO_4$/EtOH followed by heating to 120°. Column chromatography (CC) was performed using SiO_2 (200 – 300 mesh, *Ji-Yi-Da Silysia Chemical Ltd.*, Qingdao, P. R. China), *Sephadex LH-20* (*GE Healthcare Bio-Sciences AB*, Uppsala, Sweden), and *ODS* (*50*, *YMC Co. Ltd.*, Kyoto, Japan). Semi-prep. HPLC: *Waters e2695* system coupled to a *Waters 2998 Photodiode Array Detector* (PAD) and a *Waters 2424 Evaporative Light-scattering Detector* (ELSD); a *Sunfire ODS* column (5 μm, 250 mm × 10 mm) was utilized. Optical rotations: *JASCO P-1020* polarimeter. UV and IR spectra: *Shimadzu UV-2550* and *Nicolet AVATAR 360* FT-IR spectrophotometer, resp. CD Spectra: *Jasco 810* spectrometer. NMR Spectra: *Varian Mercury Plus 400* or *Bruker Advance II 400* spectrometer; chemical shifts were given in δ [ppm] with tetramethylsilane (TMS) as an internal standard. ESI-MS: *Agilent 1100 Series* mass spectrometer, and high-resolution mass spectra (HR-ESI-MS) were recorded on a *Bruker Daltonics micrOTOF-QII* mass spectrometer.

Fungi Material. The commercially fermented mycelia of *X. nigripes*, also called Wuling Powder, were produced by *Zhejiang Jolly Pharmaceutical Company* (Deqing County in Zhejiang Province, P. R. China). In September 2008, the crude samples were dried on site and then shipped to the laboratory, where they were frozen upon arrival.

Extraction and Isolation. A 20 kg aliquot was extracted with 75% EtOH (3 × 20 l) at r.t. to afford a brown residue (1.3 kg), which was suspended in H_2O (2.0 l) and then successively extracted with petroleum ether (PE, 3 × 1.5 l), AcOEt (3 × 1.5 l), and BuOH (3 × 1.5 l). The AcOEt-soluble fraction (281.6 g) was subjected to SiO_2 CC (PE/AcOEt 20 : 1 → 2 : 1, *v*/*v*; CH_2Cl_2/MeOH 20 : 1 → 2 : 1, *v*/*v*) to give 15 fractions (*Frs. 1 – 15*). *Fr. 3* (27.7 g) was repeatedly chromatographed over SiO_2 with PE/AcOEt (from 50 : 1 to 20 : 1, *v*/*v*) to afford compounds **12** (24.1 mg) and **19** (7.0 mg). *Fr. 4* (52.8 g) was subjected to CC over SiO_2 (PE/AcOEt 15 : 1 → 5 : 1, *v*/*v*) followed by gel permeation chromatography (GPC) on *Sephadex LH-20* (CH_2Cl_2/MeOH 2 : 1, *v*/*v*) to furnish compounds **14** (65.7 mg) and **15** (19.4 mg). *Fr. 5* (10.0 g) was applied to a SiO_2 column (PE/Acetone 20 : 1 → 5 : 1, *v*/*v*) followed by semi-prep. HPLC (MeOH/H_2O 40 : 60, *v*/*v*; flow rate, 3.0 ml/min) to afford compounds **5** (2.2 mg, t_R = 26.1 min) and **10** (3.5 mg, t_R = 18.5 min). Compounds **1** (9.0 mg) and **2** (1.5 mg) were isolated from *Fr. 8* (1.5 g) by eluting over SiO_2 CC with PE/acetone (5 : 1, *v*/*v*). *Fr. 10* (35.0 g) was chromatographed over a SiO_2 column eluted with CH_2Cl_2/MeOH (50 : 1 → 10 : 1, *v*/*v*), and six subfractions (*Frs. 10a – 10f*) were collected. Compounds **8** (8.2 mg) and **9** (30.0 mg) were isolated from *Fr. 10b* (2.5 g) by SiO_2 CC with CH_2Cl_2/AcOEt (10 : 1 → 2 : 1, *v*/*v*), and each was finally refined by GPC over *Sephadex LH-20* (MeOH). *Fr. 10c* (2.1 g) was subjected to *Sephadex LH-20* (MeOH), and *C18* HPLC (MeOH/H_2O 20 : 80, *v*/*v*; flow rate, 3.0 ml/min) to yield compound **11** (18.5 mg, t_R = 5.3 min). Compound **13** (50.8 mg) was crystallized from *Fr. 10d*, and compound **7** (10.0 mg) was purified from *Fr. 10f* (1.5 g) by size-exclusion chromatography over *Sephadex LH-20* (MeOH). *Fr. 11* (3.95 g) was fractionated by SiO_2 CC (CH_2Cl_2/MeOH 50 : 1 → 30 : 1, *v*/*v*), and six subfractions (*Frs. 11a – 11f*) were obtained. *Fr. 11a* (174.0 mg) was chromatographed over SiO_2 with PE/AcOEt (20 : 1 → 2 : 1, *v*/*v*) and then *Sephadex LH-20* (CH_2Cl_2/MeOH 2 : 1, *v*/*v*) to give compounds **4** (1.0 mg) and **6** (4.0 mg). Compound **3** (24.3 mg) was isolated from *Fr. 11e* (350 mg) by GPC on *Sephadex LH-20* (MeOH) followed by flash CC over SiO_2 (CH_2Cl_2/MeOH 25 : 1, *v*/*v*). Purification of the *Frs. 11b – 11d* led to the isolation of four spirocyclic pyrrole alkaloids as described previously [9]. *Fr. 12* (11.6 g) was loaded on a SiO_2 column with a CH_2Cl_2/MeOH gradient (50 : 1 → 10 : 1, *v*/*v*) to yield five subfractions (*Frs. 12a – 12e*). Subsequent separation of *Fr. 12b* (102.5 mg) by SiO_2 CC (PE/AcOEt 10 : 1 → 2 : 1, *v*/*v*) led to the isolation of compound **17** (9.1 mg). Compound **16** (78.3 mg) was crystallized from *Fr. 12c* (250.0 mg), while compound **18** (6.5 mg) was obtained from *Fr. 12d* (0.8 g) by *ODS* CC (from MeOH/H_2O 9 : 1 to MeOH neat, *v*/*v*) and further purification by GPC on *Sephadex LH-20* with MeOH.

*(4*S*)-3,4-Dihydro-4-(4-hydroxybenzyl)-3-oxo-1*H*-pyrrolo[2,1-c][1,4]oxazine-6-carbaldehyde* (**1**). Colorless gum. $[\alpha]_D^{22} = +52.0$ (c = 0.1, acetone); $[\alpha]_D^{22} = +36.0$ (c = 0.1, CH_2Cl_2). UV (MeOH): 291 (2.95). CD ($c = 1.84 \times 10^{-3}$ M, MeOH): 285 (+3.94), 227 (−1.00), 208 (+3.42). IR: 3419, 1747, 1647, 1516, 1454, 1237, 1043. ^{1}H- and ^{13}C- NMR ((D_6)DMSO): see [11]; ^{1}H- and ^{13}C-NMR ($CDCl_3$): see *Table 1*. ESI-MS: 272 ($[M+H]^+$). HR-ESI-MS: 272.0917 ($[M+H]^+$, $C_{15}H_{13}NO_4^+$, calc. 272.0928).

*Methyl (2*S*)-2-[2-Formyl-5-(hydroxymethyl)-1*H*-pyrrol-1-yl]-3-(4-hydroxyphenyl)propanoate* (**2**). Colorless gum. $[\alpha]_D^{22} = +36.7$ (c = 0.05, acetone); $[\alpha]_D^{22} = +25.0$ (c = 0.1, CH_2Cl_2). UV (MeOH): 291 (2.44). CD ($c = 1.65 \times 10^{-3}$ M, MeOH): 286 (+7.64), 242 (−0.88), 207 (+8.00). IR: 3463, 1735, 1645, 1515, 1449, 1243. ^{1}H- and ^{13}C- NMR: see *Table 1*.

ESI-MS: 326 ($[M+Na]^+$). HR-ESI-MS: 326.0985 ($[M+Na]^+$, $C_{16}H_{17}NNaO_5^+$, calc. 326.0999).

*3-{4-[(2*R*)-2,3-Dihydroxy-3-methylbutoxy]phenyl}-7-hydroxy-4*H*-chromen-4-one* (**3**). White amorphous powder. $[\alpha]_D^{27} = +40.6$ ($c = 0.03$, MeOH). UV (MeOH): 249 (2.73). IR: 3354, 2916, 1624, 1512. ^{1}H- and ^{13}C-NMR: see *Table 1*. ESI-MS: 379 ($[M+Na]^+$), 355 ($[M-H]^-$). HR-ESI-MS: 379.1144 ($[M+Na]^+$, $C_{20}H_{20}NaO_6^+$, calc. 379.1152).

Determination of the Absolute Configuration at C(2) in Compound **3**. Dimolybdenum tetracetate (CAS No. 14221 – 06 – 8) and DMSO (spectroscopy grade) were purchased from *Alfa*, and the latter was dried with 4 Å molecular sieves. According to the published procedure [32], a mixture of compound **3** (1.0 mg) and $Mo_2(OAc)_4$ (1.2 mg) in anh. DMSO (1.6 ml) was prepared for CD measurement. The mixture was kept for 30 min to form a stable chiral metal complex, after which the CD spectrum was recorded. The inherent CD spectrum was subtracted. The sign of the diagnostic band at around 310 nm (band IV, according to *Snatzke*'s nomenclature) in the ICD spectrum is correlated to the absolute configuration of C(2″) in **3**.

Neuroprotective Activity Assay. The neuroprotective activity was tested against A$\beta_{25\text{-}35}$-induced injury in SH-SY5Y neuroblastma cells using the protocol described earlier [34][35]. The cells were high passages from the ATCC maintained at 37° in a humidified atmosphere containing 5% CO_2. Cells were seeded into 96-well plates at a density of 2.5×10^5 cells/ml in MEM/F12 medium supplemented with 10% (*v*/*v*) FBS. After 24 h, the serum-free MEM/F12 medium was used to substitute the original medium. The test compounds and the positive control (epigallocatechin-3-gallate, EGCG) were dissolved in DMSO to prepare 10^{-2} M stock solns., and then diluted to the corresponding concentrations with the cell culture medium. Cells were incubated with test compounds (1 or 10 μM) or EGCG (10 μM, *Sigma*, purity > 98%) for 2 h prior to treatment with 10 μM A$\beta_{25\text{-}35}$ for another 24 h without changing the culture medium. Ten μl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT, 5 mg/ml, *Sigma*, purity: 98%) was then added to each well and incubated at 37° for 3 h. The cells were finally lysed with 100 μl of DMSO, and the amount of MTT formazan was measured at 490 nm using a microplate reader (*M200*, *TECAN*, *Austria GmbH*, Austria). The data were evaluated for statistical significance with a one-way ANOVA followed by the Least Significant Difference (LSD) test using a computerized statistical package.

Anti-Neuroinflammatory Activity Assay. The mouse microglia BV-2 cell line was obtained from the *American Type Culture Collection* (ATCC; Manassas, VA, USA), and maintained in *Dulbecco*'s modified *Eagle*'s medium containing 1800 mg/l $NaHCO_3$, supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicilin, and 100 μg/ml streptomycin at 37° in a humidified atmosphere with 5% CO_2. The anti-neuroinflammatory activity in BV-2 cells was evaluated according to the reported protocol [35]. N^G-Monomethyl-L-arginine (L-NMMA; *Beyotime*, purity ≥ 99%), a well-known NO synthase inhibitor, was used as the positive control.

Cytotoxicity Assay. The U2OS human osteosarcoma and A549 human lung carcinoma cell lines were purchased from the cell bank of Shanghai Institute of Cell Biology (Shanghai, P. R. China). The U2OS cells were maintained in *Dulbecco*'s modified *Eagle*'s medium (DMEM), while the A549 cell lines were cultured in *Roswell Park Memorial Institute-1640* (*RPMI-1640*) medium. All media were supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin, and 100 units/ml streptomycin (*Invitrogen*). The cells were maintained at 37° in a humidified environment with 5% CO_2. The cell viability was determined by MTT assay as described earlier [36]. Staurosporine (*Sigma–Aldrich*, catalog No. S6942-200UL) was used as a positive control.

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