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Linear Peptides Containing D-Leucine with Neuroprotective Activities from the Leech *Whitmania pigra* Whitman

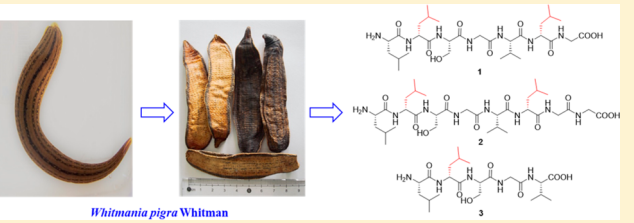
Zi-Yue Zhang,[†,‡] Nan Ma,[†,‡] Li-Jun Tao,[†,‡] Xue-Ying Gong,[†,‡] Wen-Cai Ye,*[,†,‡] and Lei Wang*[,†,‡]

[†]Institute of Traditional Chinese Medicine & Natural Products, College of Pharmacy, Jinan University, Guangzhou 510632, People's Republic of China

[‡]Guangdong Province Key Laboratory of Pharmacodynamic Constituents of TCM and New Drugs Research, Jinan University, Guangzhou 510632, People's Republic of China

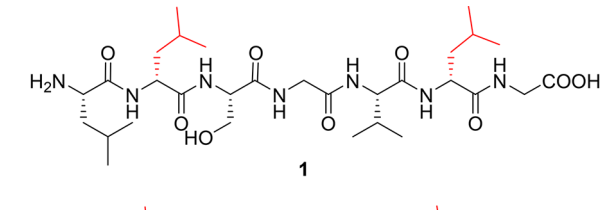
Supporting Information

ABSTRACT: Three new linear peptides containing D-leucine, named whitmantides A−C (**1**−**3**), were isolated from the dried whole bodies of *Whitmania pigra* Whitman. Their structures with absolute configurations were elucidated by Edman degradation, mass spectrometry, Marfey's analysis, and solid-phase synthesis. It is the first time that peptides containing D-amino acid in leeches were discovered. Compounds **1**−**3** displayed neuroprotective activities against oxygen−glucose deprivation/reperfusion injury on Neuro-2a cells. In addition, ex vivo serum stability tests showed that **1**−**3** were resistant to protease degradation.



It had been assumed for many years that in higher animals proteins and peptides were composed of amino acids in the L-form exclusively. However, in 1981, the first D-amino acid-containing peptide (DAACP), dermorphin, was isolated from the tree frog *Phyllomedusa sauvagei*, generating attention from both the chemical and biological communities.[1−5] To date, more than 30 DAACPs have been discovered both from invertebrates and vertebrates, such as arthropods, molluscs, and mammals.[6,7] The L- to D-amino acid residue isomerization within a peptide is a subtle change, but leads to structural and functional diversification.[8] For example, dermorphin containing D-Ala targeted μ-opioid receptors, while the L-form peptide was inactive. This is also the case for NdWFa^{i}, a cardioactive peptide with D-Trp isolated from the sea hare *Aplysia kurodai*. In addition, DAACPs can resist digestion by peptidases, thus increasing the effective lifetime of peptides.[1] Therefore, it is of great interest to search for new bioactive DAACPs.

The leech *Whitmania pigra* Whitman (Haemopidae) is widely farmed in Asia as an important medicinal resource.[9] It is traditionally used to promote blood circulation, relieve stasis, and activate energy pathways.[10] Some preparations made from *W. pigra*, such as "Shu-Xue-Tong" injection and "Nao-Xue-Kang" tablets, have been clinically used to treat blood clots and paralytic stroke in China. Previous chemical studies of this animal led to the isolation of only several furanones, pteridines, and peptides.[11−16] The medicinal importance and our interest in animal chemistry prompted us to investigate the chemical constituents of *W. pigra*. As a result, three new linear peptides with D-leucine, whitmantides A−C (**1**−**3**), were isolated from the dried whole bodies of *W. pigra*. Compounds **1**−**3** significantly increased cell viability following oxygen−glucose deprivation/reperfusion (OGD/R) injury and exhibited sustained serum stability compared with their all-L-form isomers.



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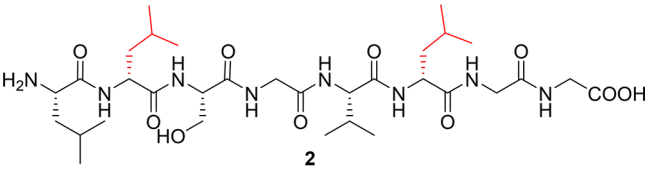

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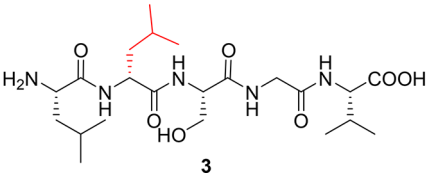

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The dried whole bodies of *W. pigra* were extracted with saline to give an extract. The extract was ultrafiltered and separated by column chromatography to yield whitmantides A−C (**1**−**3**).

Whitmantide A (**1**) was isolated as an amorphous, white powder. The molecular formula of **1** was deduced as

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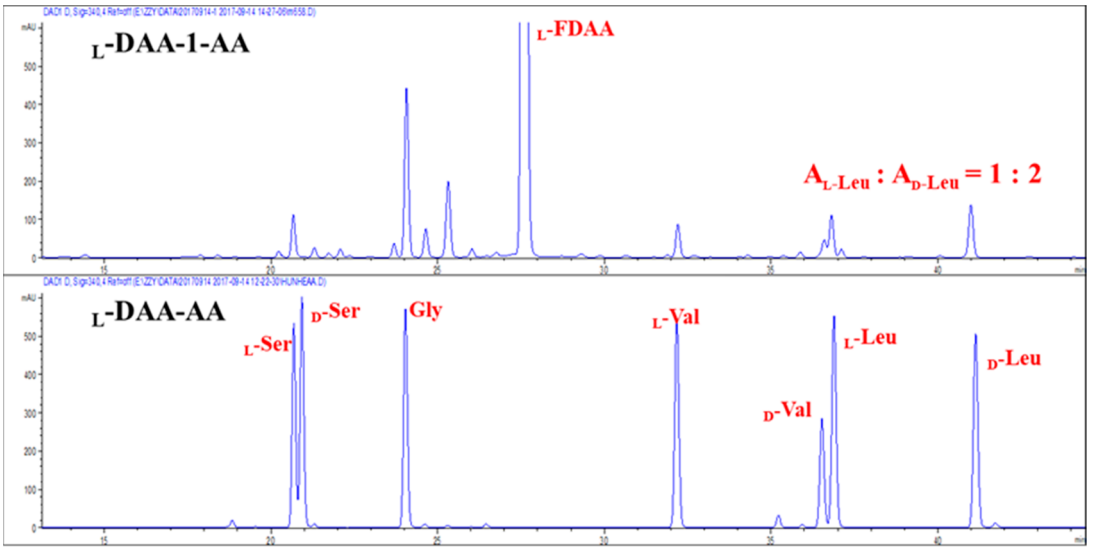

Figure 1. Marfey's analysis result of **1**.

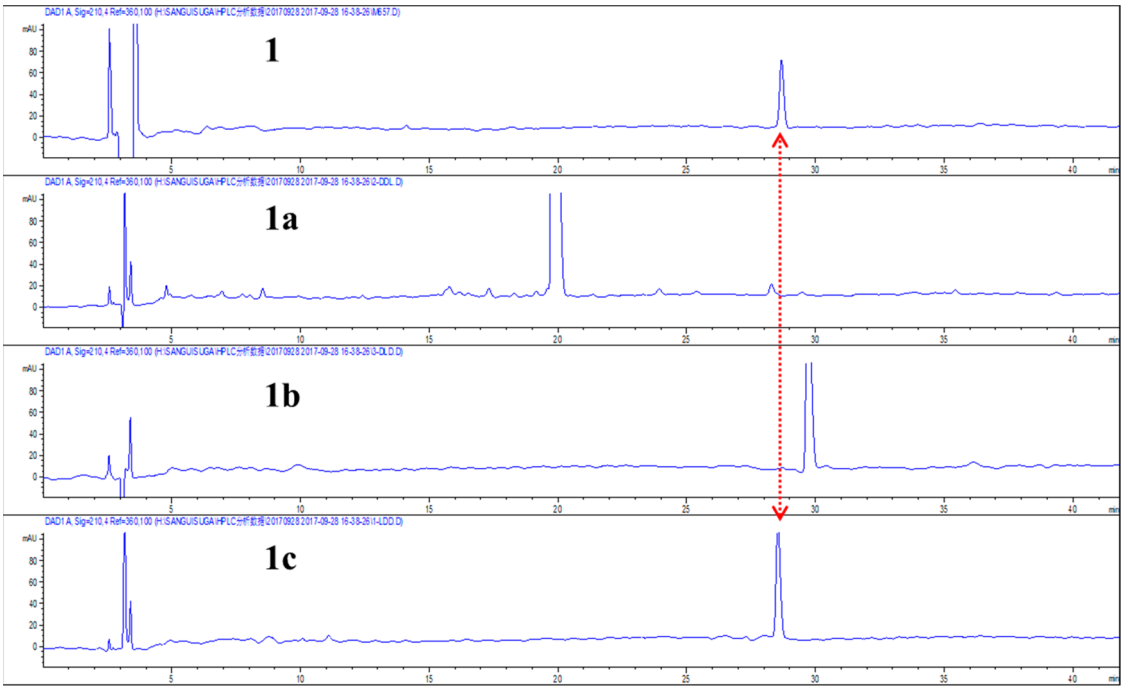

Figure 2. HPLC chromatograms for **1**, **1a**, **1b**, and **1c**.

$C_{30}H_{55}N_7O_9$ based on its HRESIMS data (*m*/*z* 658.4155 [M + H]$^+$). The amino acid sequence of **1** was determined by automated Edman degradation. The signals of the phenylthiohydantoin (PTH) derivatives of the amino acids were high and clear enough to determine the sequence as NH_2-L-L-S-G-V-L-G-COOH (Figure S1). The MS/MS fragment signals of 86.09(a_1), 227.17(b_2), 432.24(y_5), 314.20(b_3), 345.22(y_4), 371.22(b_4), 470.29(b_5), 189.12(y_2), and 583.37(b_6) further confirmed the sequential connectivity of **1** (Figure S5). According to the sequence of **1**, a synthetic peptide (**L-1**) with all L-amino acids was obtained using a solid-phase method. However, the synthetic **L-1** behaved chromatographically different from natural compound **1** (Figure S32). It had been reported that for oligopeptides converting a single L-amino acid to a D-form could cause significant differences in chromatographic and other properties.[17] Therefore, D-amino acids were speculated to exist in **1**. The absolute configurations of the amino acid residues in **1** were determined according to the Marfey's method.[18] The result showed both L-Leu and D-Leu, as well as L-Ser, L-Val, and Gly, were included in **1**. The ratio of D-Leu and L-Leu approached 2:1 by comparing the peak areas of L-DAA-D-Leu and L-DAA-L-Leu in the HPLC chromatogram (Figure 1). In light of this, there were three possible arrangements for **1** as follows: NH_2-D-L-D-L-S-G-V-L-G-COOH (**1a**), NH_2-D-L-L-S-G-V-D-L-G-COOH (**1b**), and NH_2-L-D-L-S-G-V-D-L-G-COOH (**1c**). Finally, the structure of **1** was verified by comparison of HPLC retention time between natural and synthetic peptides (Figure 2, Figure S34). As a result, the structure of **1** was elucidated as NH_2-L-D-L-S-G-V-D-L-G-COOH (**1c**).

Whitmantide B (**2**) was purified as an amorphous, white powder. The high-resolution ESIMS spectrum showed a

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protonated molecule at m/z 715.4360 $[M + H]^+$, consistent with the molecular formula $C_{32}H_{58}N_8O_{10}$. Automated Edman degradation showed the presence of seven amino acids which were identical to that of **1**, but the last residue at the C terminal could not be detected (Figure S2). Comparison of the HRESIMS data of **1** and **2** indicated that **2** had one more glycine residue than **1**. The MS/MS fragments of 715.43 and 640.39 further confirmed the existence of a glycine residue at the C terminal (Figure S15). Similar to **1**, the Marfey's analysis of **2** indicated that both L- and D-Leu existed and the other chiral amino acids were in the L-configuration. The ratio of D-Leu and L-Leu in **2** was also 2:1 (Figure S40). Accordingly, three peptides with different arrangements were synthesized as NH_2-D-L-D-L-S-G-V-L-G-G-COOH (**2a**), NH_2-D-L-L-S-G-V-D-L-G-G-COOH (**2b**), and NH_2-L-D-L-S-G-V-D-L-G-G-COOH (**2c**). Compound **2c** was the only synthetic peptide that was identical to natural **2** on HPLC (Figures S35 and S36). Thus, the structure of **2** was determined as NH_2-L-D-L-S-G-V-D-L-G-G-COOH (**2c**).

Whitmantide C (**3**) was also isolated as an amorphous, white powder. The HRESIMS spectrum of **3** displayed a protonated molecule at m/z 488.3094 $[M + H]^+$, suggesting a molecular formula of $C_{22}H_{41}N_5O_7$. The complete amino acid sequence of **3** was suggested to be NH_2-L-L-S-G-V-COOH by the results of automated Edman sequencing (Figure S3). Furthermore, the sequence of **3** was confirmed by analyses of the ESIMS/MS data. The fragment ions at m/z 262.14, 175.10, and 118.08 were formed by eliminating Ser and Gly residues successively (Figure S25). The absolute configurations of the amino acid residues in **3** were definitively assigned using the Marfey's method. The signals for both L-Leu and D-Leu, as well as L-Ser, L-Val, and Gly, were exhibited. The ratio of D-Leu and L-Leu in **3** was determined to be 1:1 by HPLC analysis (Figure S49). Furthermore, using solid-phase synthesis, two peptides with different arrangements were obtained as NH_2-L-D-L-S-G-V-COOH (**3a**) and NH_2-D-L-L-S-G-V-COOH (**3b**). The retention time of **3a** at the HPLC chromatogram was in good accord with that of **3** (Figures S37–S38). Based on the above evidence, the structure of **3** was identified as NH_2-L-D-L-S-G-V-COOH (**3a**).

Compounds **1**–**3** are three new linear peptides containing D-leucine, representing the first discovery of DAACPs from leeches. Additionally, all of the reported DAACPs from animals have only one D-amino acid residue.[6,19] The appearance of two D-amino acid residues in one peptide chain like **1** and **2** is unusual. In order to confirm that the isolated DAACPs **1**–**3** are indeed natural products, the saline extract of fresh whole bodies of *W. pigra* was analyzed by UPLC-QTOF/MS (Figures S69–S75). The ion peaks in accord with those of **1**–**3** were detected, which confirmed the natural occurrence of these peptides.

Compounds **1**–**3** were tested for their neuroprotective effects with OGD/R injured Neuro-2a cells. The protective effect was evaluated on the basis of cell viability. Compared with the OGD control (DMSO, cell viability of 61 ± 5%), compounds **1** (6.25 μM), **2** (50 μM), and **3** (50 μM) exhibited significant neuroprotective effects with cell viabilities of 79 ± 2%, 82 ± 4%, and 86 ± 2%, respectively (Figure 3), while the positive control edaravone (50 μM) showed a cell viability of 90 ± 4%. Generally, native peptides have an advantage in terms of biocompatibility. However, a major problem limiting the application of peptides is their instability, which is attributed to the proteolysis.[20] It was reported that inclusion

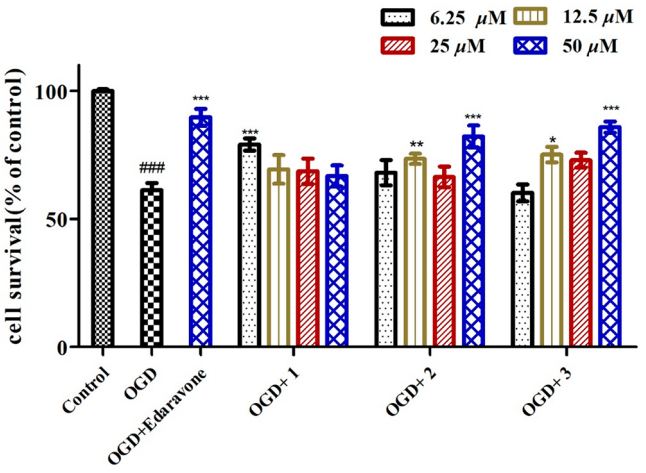

Figure 3. Evaluation of neuroprotective effects of peptides **1**–**3** in OGD/R injury in vitro. The results are expressed as mean ± SD (n = 4). $^{###}P < 0.001$ as compared with control (normal cells). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ as compared with the OGD alone.

of D-amino acids in proteins or peptides could result in resistance to enzymatic degradation.[1,21] Therefore, ex vivo serum stabilities of **1**–**3** were studied. The serum stabilities of **1**–**3** were compared with their all-L-amino acids isomers (**L-1**, **L-2**, and **L-3**). As shown in Figure 4, all-L-amino acid peptides were almost fully degraded within 2 h, while 65% or more of the original amounts of **1**–**3** remained after 24 h. Therefore, the serum stabilities of **1**–**3** suggest that they could have prolonged neuroprotective effects.

For DAACPs, several hypotheses have been proposed to explain the presence of a D-residue in the peptide chain. It may result from different mechanisms such as incorporation of a free D-amino acid during peptide elongation or enzymatic epimerization of an L-residue after peptide chain synthesis.[6,19] Modifications by isomerases have been successfully characterized in the spider *Agelenopsis aperta* and in *Bombinae*.[22,23] Whitmantides A–C (**1**–**3**) are the first DAACPs isolated from leeches. Therefore, the mystery of D-leucine formation and the incorporation of two D-leucines in one peptide chain in *W. pigra* are awaiting investigation.

EXPERIMENTAL SECTION

General Experimental Procedures. 1D and 2D NMR spectra were obtained on a Bruker AVANCE-400 NMR spectrometer. IR spectra were recorded on a JASCO FT/IR-480 Plus Fourier transform infrared spectrometer. UV spectra were conducted on a JASCO V-550 UV/vis spectrophotometer. Optical rotation values were measured on a JASCO P-1020 digital polarimeter. MS data were obtained on a Waters Xevo G2 QTOF-MS spectrometer. Analytical HPLC was carried on an Agilent 1260 liquid chromatograph equipped with a 1260 pump, a 1260 DAD detector, and a Cosmosil 5C_{18}-MS-II reversed-phase column (4.6 mm × 250 mm, 5 μm, Nacalai Tesque Inc.) or YMC-Pack ODS-A reversed-phase column (4.6 mm × 250 mm, 5 μm, YMC Co. Ltd). Semipreparative HPLC was performed on an Agilent 1200 liquid chromatograph (Agilent) equipped with a 1200 pump, a 1200 VWD detector, and a YMC-Pack ODS-AQ reversed-phase column (10 mm × 250 mm, 5 μm, YMC Co. Ltd). Column chromatography (CC) was performed on octadecyl silica (ODS) (40–64 μm, YMC) and Sephadex G25 or G10 (GE Healthcare Life Sciences). All solvents used in CC and HPLC were chromatographic grade (Fisher Scientific).

Animal Material. Dried whole bodies of *Whitmania pigra* were collected from Funing, Jiangsu Province of P. R. China, in February

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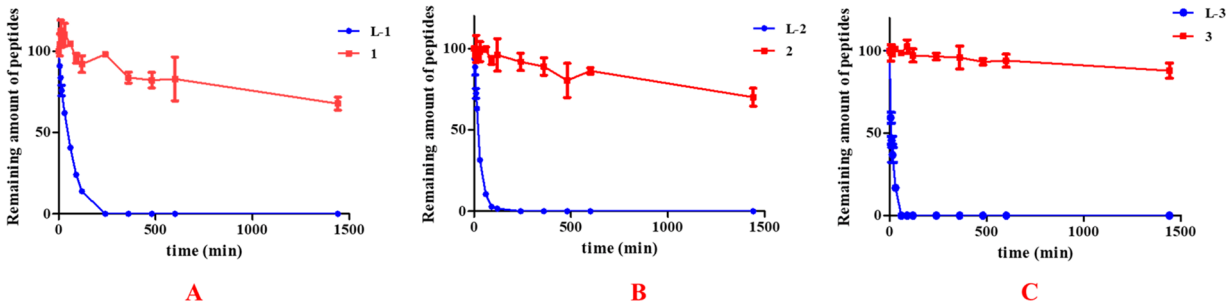

Figure 4. Degradation of peptides **1**−**3** in 50% rat serum. (A) Degradation of **1** and **L-1**. (B) Degradation of **2** and **L-2**. (C) Degradation of **3** and **L-3**. The peptides were incubated with 50% rat serum at 37 °C for different time periods, and the remaining amounts were determined by HPLC. Points represent the mean ± SD of three replicates.

2017. The material was authenticated by Prof. Guang-Xiong Zhou (Institute of Traditional Chinese Medicine and Natural Products, Jinan University, Guangzhou, P. R. China). A voucher specimen of dried material (No. 20170224) was deposited in the Institute of Traditional Chinese Medicine and Natural Products, College of Pharmacy, Jinan University, Guangzhou, P. R. China.

Extraction and Isolation. The dried whole bodies (1.0 kg) were soaked and cleaned in normal saline at room temperature until the wrinkled bodies fully stretched. The cleaned leeches were soaked in normal saline again at 0−4 °C for 30 h. Then, the leeches were separated from the soaking solution and homogenized. The homogenate was stored at −20 °C for 24 h and thawed at 4 °C, which was repeated three times. The resulting mixture was centrifuged at a speed of 3600*g* for 15 min; then the supernatant was subjected to ultrafiltration columns with 50 and 10 kDa molecular mass cutoffs successively. After being concentrated and desalted, the flow-through with molecular mass <10 kDa was lyophilized and yielded a brown residue (6 g). The lyophilized material was dissolved in H_2O and subjected to a G25 column (0.03 m × 1.8 m) using H_2O as the eluent to afford seven major fractions (Fr.1−Fr.7). Fr.1 (3 g) was chromatographed on an ODS column eluting with CH_3CN−H_2O mixtures (1:10 → 1:0, v/v) to provide five subfractions (Fr.1A to Fr.1E). Fr.1E (180 mg) was separated with a YMC C_{18} semi-preparative HPLC column eluting with a gradient of 10−30% CH_3CN from 0 to 30 min and an isocratic step of 30% CH_3CN from 30 to 40 min, which afforded 10 fractions (Fr.1E-1 to Fr.1E-10). Fr.1E-10 was further purified on a Cosmosil C_{18} analytical column with an isocratic step of 22% CH_3CN with 0.1% trifluoroacetic acid (TFA) to yield **1** (about 0.2 mg) and **2** (about 0.2 mg). Fr. 1E-7 was further purified by HPLC using an isocratic step of 20% CH_3CN (0.1% TFA) to yield **3** (about 0.5 mg).

*Whitmantide A (**1**) (synthetic):* amorphous, white powder, $[\alpha]^{25}_D$ −86.7 (*c* 0.1, H_2O); UV (H_2O) λ_{max} (log ε) 195 (3.20) nm; IR (KBr) ν_{max} 3275, 2960, 1657, 1541 cm^{-1}; ^1H and ^{13}C NMR data, Table S1 (Supporting Information); HRESIMS *m*/*z* 658.4155 $[M + H]^+$ (calcd for $C_{30}H_{56}N_7O_9$: 658.4140).

*Whitmantide B (**2**) (synthetic):* amorphous, white powder, $[\alpha]^{25}_D$ −83.7 (*c* 0.1, H_2O); UV (H_2O) λ_{max} (log ε) 196 (3.18) nm; IR (KBr) ν_{max} 3275, 2957, 1640, 1544, 1132 cm^{-1}; ^1H and ^{13}C NMR data, Table S2 (Supporting Information); HRESIMS *m*/*z* 715.4331 $[M + H]^+$ (calcd for $C_{32}H_{59}N_8O_{10}$: 715.4354).

*Whitmantide C (**3**) (synthetic):* amorphous, white powder, $[\alpha]^{25}_D$ −84.2 (*c* 0.1, H_2O); UV (H_2O) λ_{max} (log ε) 194 (3.26) nm; IR (KBr) ν_{max} 3288, 2963, 1654, 1529, 1398 cm^{-1}; ^1H and ^{13}C NMR data, Table S3 (Supporting Information); HRESIMS *m*/*z* 488.3094 $[M + H]^+$ (calcd for $C_{22}H_{42}N_5O_7$: 488.3084).

Peptide Sequencing. Automated Edman degradation, with online HPLC analyses of the PTH amino acids released at each cycle, was performed on a PPSQ-31A sequenator and PPSQ-51A sequenator.

Marfey's Analysis of 1−3. Compounds **1** (about 0.05 mg), **2** (about 0.05 mg), and **3** (about 0.2 mg) were treated with 6 M HCl (500 μL) at 115 °C for 18 h, respectively. The hydrolyzed products were concentrated to dryness under a stream of N_2 and dissolved in H_2O (50 μL). Then the hydrolysate solution was suspended in $NaHCO_3$ (1.0 M, 50 μL), mixed with a solution of L-FDAA (1 mg/mL, 100 μL), and heated (40 °C, 60 min). The mixture was neutralized with HCl (2.0 M, 25 μL) to quench the reaction. The amino acid standards were derivatized with L-FDAA under the same conditions. An aliquot (30 μL) was analyzed by HPLC on a Cosmosil 5C_{18}-MS-II reversed-phase column with a gradient elution using TFA (0.1%) in H_2O (mobile phase A) and CH_3CN (mobile phase B). The gradient elution program was 10−60% B from 0 to 50 min, 60−10% B from 50 to 55 min, and 10% B from 55 to 65 min. The elution profile was monitored at 340 nm.[18] The measured retention times (t_R, min) of the amino acid standard L-DAA derivatives for assignment of the L-DAA-AAs in **1** and **2** were as follows (min): L-DAA-L-Ser (20.9), L-DAA-D-Ser (21.1), L-DAA-Gly (24.2), L-DAA-L-Val (32.0), L-DAA-D-Val (36.2), L-DAA-L-Leu (36.6), and L-DAA-D-Leu (40.6) (Figures S42−S48). The measured retention times (t_R, min) of the amino acid standard L-DAA derivatives for assignment of the L-DAA-AAs in **3** were as follows (min): L-DAA-L-Ser (21.9), L-DAA-D-Ser (22.1), L-DAA-Gly (25.4), L-DAA-L-Val (33.4), L-DAA-D-Val (37.6), L-DAA-L-Leu (38.0), and L-DAA-D-Leu (42.1) (Figures S51−S57).

Peptide Synthesis. Peptides were synthesized in an automated solid-phase synthesizer (CSBio. Co) based on the Wang-Resin. The amino acid materials were *N*-(9-fluorenyl)methoxycarbonyl (*N*-Fmoc) derivatives. *N*,*N*-Dimethylformamide (DMF) and CH_2Cl_2 were chosen as washing solvents. The deprotection reagent was 20% piperidine in DMF or 2% 1,8-diazabicyclo[5.4.0]undec-7-ene and 2% piperidine in DMF.[24] Coupling was carried out in the presence of *O*-(benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HBTU) and *N*-ethyldiisopropylamine (DIEA). The mole ratio of the above reagents (resin: *N*-Fmoc-amino acids:HBTU:DIEA) was 1:4:4:8. Fifteen minutes was set for the deprotection step and 40 min for the coupling step. After completion of the synthesis, the peptides were cleaved from the resin with a mixture of TFA/triisopropylsilane/H_2O (95/2.5/2.5, v/v/v) for 2 h at room temperature. Soluble crude peptides were precipitated with methyl-*tert*-butyl ether and centrifuged. The pellet was dissolved in H_2O and lyophilized. The synthetic peptides were purified on a YMC C_{18} semipreparative column with a gradient of 10−30% CH_3CN in H_2O. The purified peptides were collected, lyophilized, and stored at −80 °C. The final yields of peptides were from 60% to 85%. All of the structures of the synthetic peptides were confirmed by MS/MS.

Cell Viability Assay. The neuro-2a cell line (mouse neuroblastoma cell line, ATCC-CCL131) was obtained from the American Type Culture Collection (ATCC). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 100 μg/mL streptomycin, and 100 U/mL penicillin in a humidified atmosphere with 5% CO_2 at 37 °C. OGD was performed as previously reported.[25] Briefly, the cell cultures were washed twice with phosphate-buffered saline (PBS) and incubated in the glucose-free DMEM media. The nerve cell cultures were placed in a hypoxia chamber (12 L vol) and equilibrated for 5 min with a

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continuous flux of gas (95% N_2/5% CO_2). The chamber was sealed and placed in an incubator at 37 °C for 4 h OGD. To terminate the oxygen−glucose deprivation, the cells were returned to the normal culture media in a normoxic incubator for 24 h of reoxygenation. Compounds were added 4 h prior to the induction of OGD. Control cell cultures that were not deprived of oxygen and glucose were placed in norm-oxygenated DMEM containing glucose. Cell viability was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay.

Ex Vivo Stability Studies of Peptides in Diluted Rat Serum. The stabilities of synthetic **1**−**3** and their all L-amino acids isomers were studied in commercially available pooled rat serum diluted to 50% with distilled H_2O. Each peptide (1.5 mg) was dissolved in 2 mL of pooled sterile rat serum. Peptides were incubated at 37 °C. After incubation periods of 0, 5, 10, 15, and 30 min and 1, 1.5, 2, 4, 6, 8, 10, and 24 h, 100 μL serum medium was taken out and added with a 500 μL mixture of MeOH and CH_3CN (1:2, v/v) to precipitate the serum proteins. After being stored at 4 °C for 30 min, the mixture was centrifuged at 13 000 r/min for 10 min. The remaining amounts of peptides were determined using HPLC [flow rate: 1 mL/min; detection wavelength: 214 nm; mobile phase: 10−35% CH_3CN−H_2O (0.1% TFA) from 0 to 30 min]. All experiments were carried out three times.[26]

Statistical Analysis. All data were expressed as mean ± SD (standard deviation). Statistical significance was determined by one-way analysis of variance (ANOVA).

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jnatprod.9b00322.

The amino acid sequence and Marfey's analysis data of **1**−**3**; UV, IR, HRESIMS, MS/MS spectra; NMR spectra and data; purity assay of **1**−**3** and their isomers; the UPLC-QTOF/MS analysis of the saline extract of the fresh bodies of *Whitmania pigra* Whitman (PDF)

■ AUTHOR INFORMATION

Corresponding Authors

*E-mail: chywc@aliyun.com.

*E-mail: cpuwanglei@126.com.

ORCID

Wen-Cai Ye: 0000-0002-2810-1001

Lei Wang: 0000-0001-9242-1109

Notes

The authors declare no competing financial interest.

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