Two New Phenolic Amides from the Seeds of *Pharbitis nil*

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Two new phenolic amides, pharnilatins A (1) and B (2), were isolated from the seeds of *Pharbitis nil*. These new compounds possess a *p*-coumaroyl unit with a structurally unique side chain, (2*S*,3*S*)-2,3-dihydroxyputrescine. The chemical structures and absolute stereochemistries of the new compounds were determined on the basis of spectroscopic analyses including 1D- and 2D-NMR experiments and chemical reactions. Compounds 1 and 2 exhibited cytotoxicity against A549, SK-OV-3, SK-MEL-2, and HCT-15 human tumor cells. However, none of the compounds inhibited nitric oxide (NO) production in lipopolysaccharide (LPS)-activated microglia cells.

Key words *Pharbitis nil*; Convolvulaceae; phenolic amide; pharnilatin A; pharnilatin B; cytotoxicity

The seeds of *Pharbitis nil* Choisy (Convolvulaceae), well-known as Pharbitidis Semen, have been traditionally used as a purgative drug in Korea, China, and Japan.[1)] Previous phytochemical investigations on the seeds and flowers of this plant have shown the presence of resin glycosides,[2,3)] gibberellins,[4,5)] flavonoids, chlorogenic acid derivatives,[6)] anthocyanins,[7,8)] and diterpenoids.[9,10)] Seeds of this plant were reported to exhibit antitumor and anti-fungal activities.[11,12)] In the course of our continuing search for biologically active compounds from natural Korean medicinal sources, we investigated the EtOH extract of the seeds of *P. nil*,[9,10)] which showed considerable cytotoxic activity using a sulforhodamine B (SRB) assay in screening procedures. Chemical investigation of the extract led to further isolation of two new phenolic amides, pharnilatins A (**1**) and B (**2**). These new compounds are structurally interesting and biogenetically novel, containing the unique side chain, (2*S*,3*S*)-2,3-dihydroxyputrescine. The structures of **1** and **2** were determined on the basis of spectroscopic analyses including 1D- and 2D-NMR experiments and chemical reactions. Herein, we describe the isolation, structural elucidation, and biological evaluation of the new compounds.

Results and Discussion

Compound **1** was obtained as a white amorphous powder. The molecular formula of **1** was deduced to be $C_{13}H_{18}N_2O_4$ by positive mode high resolution fast atom bombardment mass spectrometry (HR-FAB-MS) data at *m/z* 289.1157 $[M+Na]^+$ (Calcd for $C_{13}H_{18}N_2NaO_4$, 289.1164). The UV spectrum exhibited absorption maxima at 230 and 315 nm, suggesting the presence of an aromatic ring in the molecule. The IR spectrum showed absorption bands for hydroxyl (3356 cm^{-1}), amide (1666 cm^{-1}), and amine (3356, 1650 cm^{-1}) functionalities, as well as aromatic residue. The ^{1}H-NMR spectrum of **1** (Table 1) indicated the presence of a 1,4-disubstituted aromatic ring at δ_H 6.80 (2H, d, J=8.5 Hz, H-3/5) and 7.48 (2H, d, J=8.5 Hz, H-2/6), and two olefinic protons at δ_H 6.72 (1H, d, J=15.8 Hz, H-8) and 7.53 (1H, d, J=15.8 Hz, H-7), suggesting the presence of *trans*-*p*-coumaroyl moiety in **1** due to a characteristic J value of 15.8 Hz.[13)] As expected, the ^{13}C-NMR spectrum of **1** showed carbon signals that were classified as *trans*-*p*-coumaroyl unit at δ_C 116.0 (C-8), 116.9 (C-3/5), 127.9 (C-1), 131.1 (C-2/6), 143.9 (C-7), 160.9 (C-4), and 168.4 (C-9). In addition, the proton H-1′$_{a,b}$ at δ_H 3.59 (1H, dd, J=12.0, 2.5 Hz) and 3.71 (1H, dd, J=12.0, 5.5 Hz), H-4′$_{a,b}$ at δ_H 3.67 (1H, dd, J=12.0, 2.5 Hz) and 3.91 (1H, dd, J=12.0, 5.5 Hz), H-2′ at δ_H 4.11 (1H, m), and H-3′ at δ_H 4.18 (1H, m) were shown in the ^{1}H-NMR spectrum, respectively. These proton peaks were correlated with δ_C 53.1 (C-1′), 53.7 (C-4′), 74.9 (C-2′), and 76.5 (C-3′) by heteronuclear multiple quantum coherence (HMQC) spectrum. The spectra suggest that the compound contains unique side chain, 1,4-diamino-2,3-butanediol.[14)] The ^{1}H–^{1}H correlation spectroscopy (COSY) experiment showed the correlation system starting at 1′-H$_2$, continuing *via* 2′-H, 3′-H, and ending at 4′-H$_2$. Connectivity between the coumaroyl unit and side chain was further verified by heteronuclear multiple bond correlation (HMBC) spectra, which showed that the methylene proton (H-1′) was correlated to C-2′ (δ_C 74.9), C-3′ (δ_C 76.5), and C-9 (δ_C 168.4), indicating that the side chain was connected to C-9 (Fig. 2).

The stereochemistry of the side chain, 1,4-diamino-2,3-butanediol was inferred from application of *trans* or *cis* configuration of 1,3,5,7-tetraheterodecalin system by condensation of compound **1** with aldehyde (see Chart 1).[14,15)] The reaction of **1** with formaldehyde gave the bi-cyclic *cis*-1,5-dioxa-3-aza-7-*cis*-*p*-coumaroyl-decalin product (**1a**) formed

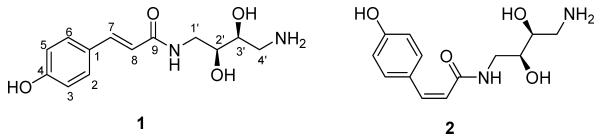

Fig. 1. Chemical Structures of **1** and **2**

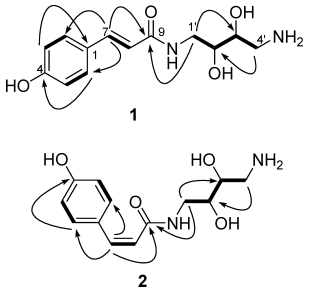

Fig. 2. Key ^{1}H–^{1}H COSY (Bold) and HMBCs (→) of **1** and **2**

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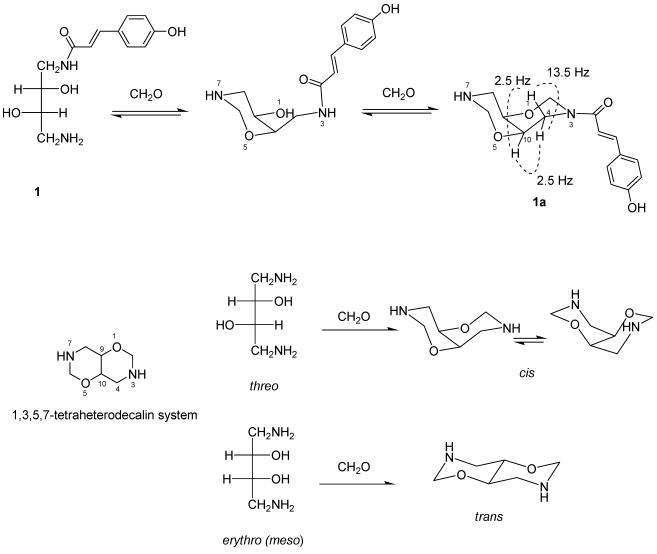

Chart 1. Synthesis of **1a** and the 1,3,5,7-Tetraheterodecalin System in All Its Diastereomeric Forms

via the corresponding monocyclic intermediate (see Chart 1). Additional corresponding bi-(oxazolidinyl), aminal and/or acetal by-products are possible,[14,15] but were not detected. Because these are pH dependent reactions and the nitrogen atoms are the most reactive centers in this system, the thermodynamic product **1a** could be obtained at higher temperature. The stereochemistry was further assigned by coupling constant value ($^3J_{H,H}$). In the ^{1}H-NMR spectrum of **1a**, the characteristic proton (H-4) appeared at δ_H 3.40 (dd, ^2J=13.5, ^3J=2.5 Hz, H-4$_{eq}$), 2.76 (dd, ^2J=13.5, ^3J=2.5 Hz, H-4$_{ax}$). It has been observed that $^3J_{H\text{-}4ax,H\text{-}10}$ is small (2.4 Hz) in *cis*-type of the above system while $^3J_{H\text{-}4ax,H\text{-}10}$ is large (10.3 Hz) in *trans*-type of the above system.[14] These data indicated that the side chain of **1** is *threo*-2,3-dihydroxyputrescine (*threo*-1,4-diamino-2,3-butanediol), NMR data of which were in agreement with those of related synthetic compounds.[14] The absolute configuration of the side chain of **1** was confirmed by acidic hydrolysis[16] that afforded *threo*-1,4-diamino-2,3-butanediol (**1b**).[14] In order to identify its absolute configuration, **1b** was converted to 2,2-dimethyl-4,5-bis(aminomethyl)-1,3-dioxolane (**1c**) by treatment with 2,2-dimethoxypropane and *p*-toluenesulfonic acid (PTSA) in MeOH (see Chart 2),[14,17] which was identical to (4*S*,5*S*)-2,2-dimethyl-4,5-bis(aminomethyl)-1,3-dioxolane.[18] The negative optical rotation value ($[\alpha]_D^{25}$ −73.1 in acetone) of **1c** confirmed that absolute configuration of **1b** is 2*S* and 3*S*.[17] Therefore, compound **1** was assigned as 9-[(2′*S*,3′*S*)-1′,4′-diamino-2′,3′-butanediol]-(*E*)-*p*-coumarate.

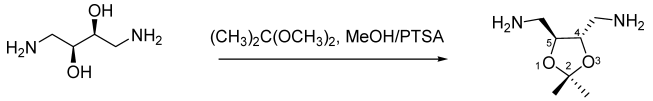

Chart 2. Synthesis of **1c**

Compound **2** was isolated as a white amorphous powder, whose molecular formula was determined to be $C_{13}H_{18}N_2O_4$ from the $[M+Na]^+$ peak at *m*/*z* 289.1167 (Calcd for $C_{13}H_{18}N_2NaO_4$, 289.1164) in the positive-ion HR-FAB-MS. This compound showed UV maxima at 229 and 315 nm and IR bands for hydroxyl (3355 cm^{-1}), amide (1667 cm^{-1}), and amine (3355, 1654 cm^{-1}) groups. The ^{1}H- and ^{13}C-NMR peaks and ^{1}H–^{1}H COSY, HMBC correlations of compound **2** were very similar to those of **1**, except for the aromatic region (Table 1). The ^{1}H-NMR spectrum of **2** showed a 1,4-disubstituted aromatic ring at δ_H 6.73 (2H, d, *J*=8.5 Hz, H-3/5) and 7.28 (2H, d, *J*=8.5 Hz, H-2/6), and two olefinic protons at δ_H 5.91 (1H, d, *J*=12.5 Hz, H-8) and 6.62 (1H, d, *J*=12.5 Hz, H-7). The coupling constant (12.5 Hz) of two olefinic protons at δ_H 5.91 (H-8) and 6.62 (H-7) on the ^{1}H-NMR proved to be *cis*-configurated protons in particular.[13] The absolute configuration of **2** was deduced to be the same as **1** from acidic hydrolysis of **2** that yielded (2*S*,3*S*)-2,3-dihydroxyputrescine (**2a**). NMR data of **2a** were in good agreement with those of **1b** and comparison of the optical rotation values of **2a** ($[\alpha]_D^{25}$ −14.4 in H_2O) and **1b** ($[\alpha]_D^{25}$ −13.5 in H_2O) confirmed the 2*S*,3*S*-configuration of **2a**. Thus, compound **2** was determined to be 9-[(2′*S*,3′*S*)-1′,4′-diamino-2′,3′-butanediol]-(*Z*)-*p*-coumarate. Interestingly, although compounds **1** and **2** were isolated as a single compound at first, they became soon a mixture. Compounds **1** and **2** seem to be interconvertible to each other. The isomerization of the olefin unit from the *E* to *Z* forms was expected owing to exposure to sunlight, and similar phenomena were observed in previous studies.[19,20]

Previously, hydroxyputrescine, an unusual chiral polyamine, has been identified from several strains of *Pseudomonas*.[21] The unusual naturally occurring amino acid, hypusine ((2*S*,9*R*)-2,11-diamino-9-hydroxy-7-azaundecanoic acid), formally a conjugate between hydroxyputrescine and lysine, has been isolated from extracts of bovine brain[22] and abnormal metabolite, 2-hydroxyputrescine amide of ferulic acid has been detected in rust-infected wheat.[23] We are not certain about the biogenesis of **1** and **2** but these may possibly be derived from related metabolites similar to the afore mentioned compounds.

The cytotoxic activities of the isolated compounds **1** and **2** were evaluated by determining their inhibitory effects on human tumor cell lines (A549, SK-OV-3, SK-MEL-2, and HCT-15) using the sulforhodamine B (SRB) assay.[24] It was

Table 1. ^{1}H- (500 MHz) and ^{13}C-NMR (125 MHz) Spectral Data of **1** and **2** in CD_3OD (δ in ppm)

Position	**1**		**2**		**1**, **2**	
	δ_H	δ_C	δ_H	δ_C	HMBC (H→C)	COSY (H→H)
1		127.9		128.2		
2/6	7.48 (d, 8.5)	131.1	7.28 (d, 8.5)	131.4	C-4, 7	H-3, 5
3/5	6.80 (d, 8.5)	116.9	6.73 (d, 8.5)	116.4	C-1	H-2, 6
4		160.9		159.5		
7	7.53 (d, 15.8)	143.9	6.62 (d, 12.5)	136.1	C-1, 2, 6, 8, 9	H-8
8	6.72 (d, 15.8)	116.0	5.91 (d, 12.5)	120.9	C-7, 9	H-7
9		168.4		171.0		
1′	3.71 (dd, 12.0, 5.5)	53.1	3.66 (dd, 12.0, 5.5)	52.6	C-9, 2′, 3′	H-2′
	3.59 (dd, 12.0, 2.5)		3.51 (dd, 12.0, 2.5)			
2′	4.11 (m)	74.9	4.10 (m)	75.0	C-1′, 3′, 4′	H-1′, 3′
3′	4.18 (m)	76.5	4.01 (m)	76.1	C-1′, 2′, 4′	H-2′, 4′
4′	3.91 (dd, 12.0, 5.5)	53.7	3.53 (dd, 12.0, 5.5)	54.1	C-2′, 3′	H-3′
	3.67 (dd, 12.0, 2.5)		3.35 (dd, 12.0, 2.5)			

Assignments were based on 2D-NMR including COSY, HMQC and HMBC. Well-resolved couplings are expressed with coupling patterns and coupling constants in Hz in parentheses.

found that compounds **1** and **2** showed moderate cytotoxicity against A549, SK-OV-3, SK-MEL-2, and HCT-15 cell lines (IC_{50} (**1**): 24.3, 37.0, 19.7, and 25.8 μM, and IC_{50} (**2**): 25.5, 45.4, 13.9, and 25.1 μM, respectively).

The anti-neuroinflammatory effects of **1** and **2** were also evaluated using lipopolysaccharide (LPS)-activated BV-2 cells, a microglia cell line. However, none of the compounds inhibited nitric oxide (NO) production in LPS-activated microglia cells.

Experimental

General Experimental Procedures All melting points were determined on a Gallenkamp melting point apparatus and are uncorrected. Optical rotations were measured on a Jasco P-1020 polarimeter. IR spectra were recorded on a Bruker IFS-66/S FT-IR spectrometer. UV spectra were recorded with a Shimadzu UV-1601 UV–Visible spectrophotometer. FAB and HR-FAB mass spectra were obtained on a JEOL JMS700 mass spectrometer. NMR spectra, including ^{1}H–^{1}H COSY, HMQC, and HMBC experiments, were recorded on a Varian UNITY INOVA 500 NMR spectrometer operating at 500 MHz (^{1}H) and 125 MHz (^{13}C), with chemical shifts given in ppm (δ). Preparative high-performance liquid chromatography (HPLC) was performed using a Gilson 306 pump with a Shodex refractive index detector. Silica gel 60 (Merck, 230—400 mesh) and RP-C_{18} silica gel (Merck, 230—400 mesh) were used for column chromatography. Merck precoated Silica gel F_{254} plates and RP-18 F_{254s} plates were used for thin layer chromatography (TLC). Spots were detected on TLC under UV light or by heating after spraying with 10% H_2SO_4 in EtOH (v/v). The packing material for molecular sieve column chromatography was Sephadex LH-20 (Pharmacia Co., Sweden).

Plant Materials The seeds of *P. nil* were purchased at Kyungdong herbal market, Seoul, Korea, in July 2006, and were identified by one of the authors (K.R.L.). A voucher specimen (SKKU 2006-7) was deposited in the herbarium of the School of Pharmacy, Sungkyunkwan University, Suwon, Korea.

Extraction and Isolation The dried seeds of *P. nil* (10 kg) were extracted with 50% EtOH (3×4 l, each 3 d) at room temperature and filtered. The filtrate was evaporated *in vacuo* to obtain EtOH extract (1.4 kg), which was suspended in distilled H_2O (7.2 l) and then successively partitioned with *n*-hexane, $CHCl_3$, EtOAc, and *n*-BuOH, yielding 10, 7, 10, and 550 g, respectively. The EtOAc-soluble fraction (10 g) was chromatographed on a silica gel (230—400 mesh, 300 g) column and eluted with $CHCl_3$–MeOH (10 : 1→1 : 1, gradient system) to yield five fractions (A—E). Fraction E (3.0 g) was chromatographed further on an RP-C_{18} silica gel (230—400 mesh, 150 g) column and eluted with MeOH–H_2O (1 : 1→1 : 0, gradient system) to give 11 subfractions (E1—E11). Fraction E1 (250 mg) was subjected to Sephadex LH-20 column chromatography (MeOH–H_2O, 4 : 1) and further purified by preparative reversed-phase HPLC (MeCN–H_2O, 1 : 4) to yield two new compounds **1** (5 mg) and **2** (5 mg).

Pharnilatin A (**1**): White amorphous powder; mp 221—223 °C; $[\alpha]_D^{25}$ −6.4 (c=0.12, MeOH); UV (MeOH) λ_{max} (log ε) 230 (3.9) 315 (4.7) nm; IR (KBr) ν_{max} 3356, 2945, 2834, 1666, 1650, 1452, 1028, 676 cm^{-1}; ^{1}H- (500 MHz) and ^{13}C- (125 MHz) NMR data, see Table 1; FAB-MS (positive mode) *m/z*: 289 $[M+Na]^+$; HR-FAB-MS *m/z*: 289.1157 $[M+Na]^+$ (Calcd for $C_{13}H_{18}N_2NaO_4$, 289.1164).

Pharnilatin B (**2**): White amorphous powder; mp 217—220 °C; $[\alpha]_D^{25}$ −7.7 (c=0.23, MeOH); UV (MeOH) λ_{max} (log ε) 229 (3.9) 315 (4.6) nm; IR (KBr) ν_{max} 3355, 2945, 2833, 1667, 1651, 1452, 1028, 676 cm^{-1}; ^{1}H- (500 MHz) and ^{13}C- (125 MHz) NMR data, see Table 1; FAB-MS (positive mode) *m/z*: 289 $[M+Na]^+$; HR-FAB-MS *m/z*: 289.1167 $[M+Na]^+$ (Calcd for $C_{13}H_{18}N_2NaO_4$, 289.1164).

Preparation of *cis*-1,5-Dioxa-3-aza-7-*cis*-*p*-coumaroyl-decalin (1a) Formaldehyde (paraformaldehyde, 2 mM) was added to an aqueous solution of **1** (1 mM) and the solution was heated in an ultrasonic bath at 60 °C for 2 h. The reaction solution was extracted with $CHCl_3$ to give **1a** (1.0 mg).

1a: White amorphous powder; ^{1}H-NMR ($CDCl_3$, 500 MHz) δ: 7.30 (1H, d, J=15.5 Hz, H-7′), 7.10 (2H, d, J=8.5 Hz, H-2′/6′), 6.80 (2H, d, J=8.5 Hz, H-3′/5′), 6.68 (1H, d, J=15.5 Hz, H-8′), 4.22 (1H, dd, ^{2}J=9.5, ^{4}J=2.0 Hz, H-2_{eq}), 4.17 (1H, dd, ^{2}J=9.5, ^{4}J=2.0 Hz, H-6_{eq}), 4.09 (1H, d, ^{2}J=9.5 Hz, H-2_{ax}), 3.94 (1H, d, ^{2}J=9.5 Hz, H-6_{ax}), 3.54 (1H, m, H-9), 3.51 (1H, m, H-10), 3.40 (1H, dd, ^{2}J=13.5, ^{3}J=2.5 Hz, H-4_{eq}), 3.35 (1H, m, ^{2}J=13.5 Hz, H-8_{eq}), 2.76 (1H, dd, ^{2}J=13.5, ^{3}J=2.5 Hz, H-4_{ax}) 2.70 (1H, dd, ^{2}J=13.5, ^{3}J=2.5 Hz, H-8_{ax}); FAB-MS (positive mode) *m/z*: 291 $[M+H]^+$.

Acidic Hydrolysis of Compounds 1 and 2 Compounds **1** (2 mg) and **2** (1.5 mg) were individually hydrolyzed with 6 N HCl under reflux for 24 h. After cooling, each reaction mixture was diluted with H_2O and extracted with $CHCl_3$. The aqueous layer was neutralized by passage through an Amberlite IRA-67 column and was repeatedly evaporated to give each fraction. The fraction was individually chromatographed by silica gel Waters Sep-Pak Vac 6 cc ($CHCl_3$–MeOH, 10 : 1) to give the side chain, **1b** (1 mg) from **1** and **2a** (0.7 mg) from **2**.

1b: White powder; $[\alpha]_D^{25}$ −13.5 (c=0.05, H_2O); ^{1}H-NMR (D_2O, 500 MHz) δ: 3.90 (4H, dd, J=7.0, 4.0 Hz, H-1/4), 3.12 (2H, m, H-2/3); FAB-MS (positive mode) *m/z*: 143 $[M+Na]^+$. Compound **2a** was identical to **1b** in terms of ^{1}H-NMR and TLC and optical rotation of **2a** was $[\alpha]_D^{25}$ −14.4 (c=0.03, H_2O).

Synthesis of 1c Compound **1b** (1 mg) was dissolved in 2,2-dimethoxypropane (1 ml) and methanol (0.3 ml), and *p*-toluenesulfonic acid (2 mg) was added. The reaction was allowed to proceed for 12 h at room temperature and then quenched with 5% aqueous $NaHCO_3$, extracted 3 times with CH_2Cl_2. The CH_2Cl_2 solution was dried (anhydrous $MgSO_4$), the solvent was removed under reduced pressure, and the residue was purified by silica gel Waters Sep-Pak Vac 6 cc ($CHCl_3$–MeOH, 30 : 1) to provide compound **1c** (0.5 mg).

1c: Oil; $[\alpha]_D^{25}$ −73.1 (c=0.02, acetone); ^{1}H-NMR ($CDCl_3$, 500 MHz) δ: 3.75 (2H, m, –OC<u>H</u>CH_2–), 2.88 (2H, dd, J=13.5, 4.0 Hz, –C<u>H</u>$_2$$NH_2$), 2.80 (2H, dd, J=13.5, 6.0 Hz, –C<u>H</u>$_2$$NH_2$), 1.40 (6H, s, –CC<u>H</u>$_3$); FAB-MS (positive mode) *m/z*: 161 $[M+H]^+$.

Cytotoxicity Assay A sulforhodamine B (SRB) bioassay was used to determine the cytotoxicity of each compound isolated against four cultured human tumor cell lines.[24] The assays were performed at the Korea Research

Institute of Chemical Technology. The cell lines used were A549 (non-small cell lung carcinoma), SK-OV-3 (ovary malignant ascites), SK-MEL-2 (skin melanoma), and HCT-15 (colon adenocarcinoma). Doxorubicin was used as a positive control. The cytotoxicities of doxorubicin against the A549, SK-OV-3, SK-MEL-2, and HCT-15 cell lines were IC_{50} 0.16, 0.38, 0.04, and 0.82 μM, respectively.

Measurement of Nitric Oxide (NO) Production and Cell Viability Inhibition of NO production was evaluated in lipopolysaccharide (LPS)-activated murine microglia BV-2 cells. Cells were stimulated with 100 ng/ml of LPS in the presence or absence of samples for 24 h. Nitrite in the culture media, a soluble oxidation product of NO, was determined using the Griess reaction.[25] Cell viability was measured using a 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay.[26] N^G-monomethyl-L-arginine (L-NMMA, Sigma), a nitric oxide synthase (NOS) inhibitor, was tested as a positive control.

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