JOURNAL OF NATURAL PRODUCTS

Note

pubs.acs.org/jnp

Pharbinilic Acid, an Allogibberic Acid from Morning Glory (*Pharbitis nil*)

Ki Hyun Kim,[†,‡] Sang Un Choi,[§] Mi Won Son,[⊥] Sang Zin Choi,[⊥] Jon Clardy,[†] and Kang Ro Lee*[,‡]

[†]Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, Massachusetts 02115, United States

[‡]Natural Products Laboratory, School of Pharmacy, Sungkyunkwan University, Suwon 440-746, Korea

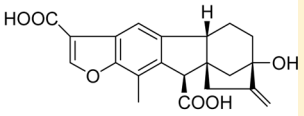
[§]Bio-organic Science Division, Pharmacology Research Center, Korea Research Institute of Chemical Technology, Teajeon 305-600, Korea

[⊥]Dong-A Pharm Institute, Kiheung, Youngin 449-905, Korea

Supporting Information

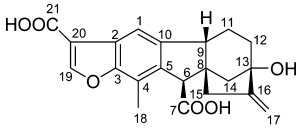
ABSTRACT: Pharbinilic acid (**1**), the first naturally occurring allogibberic acid, was isolated from ethanol extracts of morning glory (*Pharbitis nil*) seeds. Its absolute configuration was determined by NOESY NMR and ECD experiments. Compound **1** showed weak cytotoxicity against A549, SK-OV-3, SK-MEL-2, and HCT-15 cells and weakly inhibited nitric oxide production in lipopolysaccharide-activated BV-2 microglia cells.

The Seeds of *Pharbitis nil*



Pharbinilic acid

The gibberellic acids (GAs, gibberellins) are a large group of diterpenoids that are best known as phytohormones that regulate growth and developmental processes—seed germination, stem elongation, flowering, and fruit setting—in higher plants.[1−3] Gibberellins were originally discovered from the fungal pathogen responsible for "crazy seedling" disease in rice, where they dysregulated plant growth to produce abnormally elongated seedlings. GAs are also produced by bacteria. Pharbitidis Semen, the seeds of *Pharbitis nil* (Convolvulaceae), are well known to be a rich source of GAs.[4−7] Our previous reports focused on *ent*-kaurane diterpenoids, the biosynthetic precursors of GAs,[8] from these seeds.[9,10] A single GA aldehyde analogue was also isolated from these seeds,[10] and its occurrence prompted us to conduct additional studies. Pharbitidis Semen is both a food and a medicinal agent that has traditionally been used as a purgative in Korea, China, and Japan.[11] A recent investigation of bioactive and structurally unusual GAs from the seeds of morning glory (*P. nil*) led to the identification of pharbinilic acid (**1**), a new allogibberic acid, isolated through bioassay-guided fractionation from the most active EtOAc-soluble fraction. Here we report the isolation and structure elucidation of **1**. Compound **1** was evaluated for antiproliferative activity against four human cancer cell lines (A549, SK-OV-3, SK-MEL-2, and HCT-15) and for inhibitory activity of nitric oxide (NO) production in lipopolysaccharide (LPS)-activated BV-2 cells, a microglial cell line.



1

Dried seeds (2 kg) of *P. nil* were extracted at room temperature with 50% EtOH (3 × 1 L, × 3 days). The extract was successively partitioned with *n*-hexane, $CHCl_3$, EtOAc, and *n*-BuOH. Each extract was evaluated for cytotoxic and anti-inflammatory activities, and a bioactivity-guided fractionation method was used for isolation. The EtOAc-soluble fraction (2 g) was the most active in the bioassay, and further fractionation on a silica gel column ($CHCl_3$/MeOH), an RP-C_{18} silica gel column (H_2O/MeOH), and a reversed-phase HPLC column (Econosil RP-18 10 μm; 250 × 10 mm, 33% MeCN, Shodex refractive index detector) yielded pure compound **1** (6.0 mg, 0.0003%).

Pharbinilic acid (**1**) was isolated as an optically active, colorless gum, $[\alpha]_D^{25}$ −19.1 (*c* 0.30, MeOH). The molecular formula of **1** as $C_{21}H_{20}O_6$ was established from the HRESIMS, indicating an $[M + Na]^+$ ion peak at *m*/*z* 391.1154 (calcd for $C_{21}H_{20}NaO_6$, 391.1158) and ^1H and ^{13}C NMR data (Table 1). The ^1H NMR spectrum showed the presence of two protons in the aromatic region at δ_H 8.36 and 7.67, one exomethylene group at δ_H 5.19 and 5.08, and one typical aromatic methyl group at δ_H 2.46. The ^{13}C NMR and HSQC spectra displayed two carbonyl carbons at δ_C 175.8 and 167.3, 10 olefinic carbons between δ_C 156.4 and 107.2, one oxygen-bearing carbon at δ_C 79.1, one methyl carbon at δ_C 11.9, and seven aliphatic carbon signals between δ_C 57.2 and 21.9. In the UV spectrum of **1**, absorption maxima were observed at 288 nm, which indicated the presence of a conjugated aromatic ring.

Interpretation of the HSQC data allowed all single-bond proton and carbon correlations to be assigned. An analysis of

Received: April 22, 2013
Published: July 1, 2013

dx.doi.org/10.1021/np400326e | *J. Nat. Prod.* 2013, 76, 1376−1379

Table 1. ^{1}H (500 MHz) and ^{13}C NMR (125 HMz) Spectroscopic Data for Pharbinilic acid (1) (methanol-d_4)

no.	δ_H	mult (J in Hz)	δ_C	HMBC
1	7.67	s	113.5 d	C-3, 5, 9, 20
2			125.4 s	
3			155.5 s	
4			120.4 s	
5			139.7 s	
6	3.72	s	57.2 d	C-4, 7, 9, 10, 14, 15
7			175.8 s	
8			55.5 s	
9	3.54	br d (8.0)	48.9 d	C-1, 6, 8, 10, 12, 14, 15
10			144.3 s	
11	2.31	m	21.9 t	C-8, 10, 13
	2.06	m		
12	1.75	m	39.5 t	C-9, 11, 13, 14, 16
	1.72	m		
13			79.1 s	
14	1.67	d (11.0)	49.6 t	C-8, 9, 12, 13, 15, 16
	1.45	dd (11.0, 2.5)		
15	2.90	d (16.5)	39.9 t	C-8, 9, 13, 14, 16
	2.60	d (16.5)		
16	156.4 s			
17	5.19	s	107.2 t	C-13, 15
	5.08	s		
18	2.46	s	11.9 q	C-3, 4, 5
19	8.36	s	152.5 d	C-2, 3, 20, 21
20			116.7 s	
21			167.3 s	

the COSY and HMBC data for **1** led to two substructures (Figure 1). A methyl resonance at δ_H 2.46 (δ_C 11.9), typical for

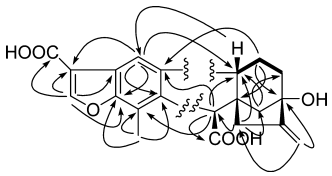


Figure 1. Substructures and ^{1}H–^{1}H COSY (bold lines) and key HMBC (arrows) correlations for pharbinilic acid (**1**).

an aromatic methyl group, showed HMBC correlations to carbon resonances at δ_C 155.5, 139.7, and 120.4, the carbon resonances at δ_C 155.5 and 139.7 of which also showed HMBC correlations with an olefinic proton at δ_H 7.67 (δ_C 113.5). This proton resonance additionally showed an HMBC correlation to a carbon resonance at δ_C 116.7, which also correlated with the olefinic proton at δ_H 8.36. This olefinic proton was attached to the carbon with a carbon resonance at δ_C 152.5, which in turn had HMBC correlations to a carbonyl carbon at δ_C 167.3 and carbon resonances at δ_C 155.5 and 125.4. These HMBC correlations defined the left-hand portion of the substructures, as illustrated in Figure 1. Two olefinic resonances at δ_H 5.19 and 5.08 (δ_C 107.2), typical for an exomethylene group, showed HMBC correlations to carbon resonances at δ_C 79.1 and 39.9. The carbon resonance at δ_C 39.9 was correlated to proton resonances at δ_H 2.90 and 2.60 in the HSQC, which in turn had HMBC correlations to carbon resonances at δ_C 57.2, 49.6, and 48.9. An HMBC correlation between the proton resonance at δ_H 3.72 and carbon resonance at δ_C 175.8 indicated that the other carbonyl carbon was linked to the carbon at C-6. A methylene resonance at δ_H 2.31 and 2.06 (δ_C 21.9) showed HMBC correlations to carbon resonances at δ_C 79.1 and 55.5, together with COSY correlations to proton resonances at δ_H 3.54, 1.75, and 1.72. The proton resonances at δ_H 1.75 and 1.72 were correlated with a carbon resonance at δ_C 39.5, and the proton resonance at δ_H 3.54 was correlated to the carbon resonance at δ_C 48.9 in the HSQC, both of which in turn showed HMBC correlation to the carbon resonance at δ_C 49.6, respectively. On the basis of these observations, the right-side part of the substructures was elucidated (Figure 1). Finally, HMBC correlations from the proton at δ_H 3.72 to the carbons at δ_C 144.3 and 120.4, from the proton at δ_H 3.54 to the carbon at δ_C 113.5, from the protons at δ_H 2.31 and 2.06 to the carbon at δ_C 144.3, and from the proton at δ_H 7.67 to the carbon at δ_C 48.9 allowed the combination of the two substructures of **1**, as shown in Figure 1.

The absolute configuration of **1** was clarified by an electronic circular dichroism (ECD) study in combination with the NOESY spectrum. The configuration of C-6 carboxylic acids in gibberic acids with an aromatic A ring can be determined by an application of ECD data, since the sign of the Cotton effect in the 230 nm region corresponded to the configuration of the C-6 carboxy group independent of structural changes in the C/D and B/C ring junctions.[12] The ECD spectrum of **1** showed a similar ECD curve to those of the known corresponding compounds (Supporting Information), which defined the orientation of the C-6 carboxy group as β, which then defines the 6*R* absolute configuration.[12] The orientations of the C-8, C-9, and C-13 stereogenic centers were determined by a NOESY experiment combined with molecular modeling based on the established configuration of C-6. A NOESY correlation was observed between H-6 (δ_H 3.72) and H-14 (δ_H 1.67 and 1.45), but not between H-6 (δ_H 3.72) and H-9 (δ_H 3.54), whereas a NOESY correlation was observed between H-9 (δ_H 3.54) and H-15 (δ_H 2.90 and 2.60). All of the observed NOESY correlations were consistent with the conformation of the 6*R*, 8*S*, 9*R*, 13*S* isomers in molecular modeling (Figure 2).

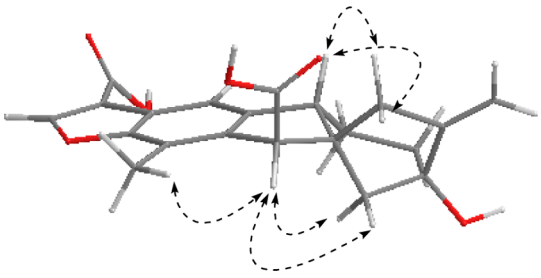


Figure 2. Key NOESY (dashed arrow) correlations for pharbinilic acid (**1**).

Moreover, the chemical shift of H-9 (δ_H 3.54) for **1** agreed well with the corresponding value for 9-epiallogibberic acid with 9*R* (the corresponding value for allogibberic acid with 9*S* is δ_H 2.78).[13] Therefore, the absolute configuration of **1** is 6*R*, 8*S*, 9*R*, and 13*S*.

Pharbinilic acid (**1**) is a member of the relatively rare class of allogibberic acids, which are GA decomposition products. GAs decompose thermally in aqueous solution to gibberellenic acid, which can undergo further thermal or photochemical decomposition to yield allogibberic acid or 9-epiallogibberic acid.[14−16] To date, several allogibberic acids have been reported, but none have been isolated from natural sources;

dx.doi.org/10.1021/np400326e | J. Nat. Prod. 2013, 76, 1376−1379

they have been produced artificially from gibberellic acid under laboratory conditions.[14−16] The apparent uniqueness of compound **1** suggested that it might be an artifact generated during extraction and isolation. To check this possibility, a second specimen of the morning glory seeds was extracted by the same procedure, and this minimally handled extract was used in a separate experiment in which HPLC and LC-MS were used to detect the component by comparison with the isolated sample of compound **1**. Pharbilinic acid (**1**) was observed in both HPLC and LC-MS analyses, which indicates that it is not an isolation artifact but a genuine natural product. This is the first allogibberic acid isolated from natural sources as a genuine metabolite, representing a unique example of an allogibberic acid possessing a furan ring adjacent to the A ring. A literature survey revealed that several similar compounds with the benzofuran moiety have been reported as hydrolysis products of the fungal metabolite wortmannin.[17,18]

Compound **1** was evaluated for relevant potentially anticancer cytotoxicity against A549 (non-small-cell lung carcinoma), SK-OV-3 (ovary malignant ascites), SK-MEL-2 (skin melanoma), and HCT15 (colon cancer) cell lines using the sulforhodamine B (SRB) bioassay.[19] Compound **1** exhibited weak cytotoxicity against all of the cell lines tested, with IC_{50} values of 15.72 ± 0.74, 4.83 ± 0.31, 8.36 ± 0.52, and 6.79 ± 1.13 μM for the A549, SK-OV-3, SK-MEL-2, and HCT-15 cell lines, respectively. On the basis of the understanding that cancer progression is associated with inflammatory responses, the anti-inflammatory activity of compound **1** was also evaluated by examining its effect on lipopolysaccharide-induced nitric oxide production in murine microglia BV-2 cells. As a result, compound **1** weakly inhibited NO production, with an IC_{50} value of 32.9 μM without cell toxicity, compared to 16.8 μM of the positive control L-NMMA.

The discovery of pharbinilic acid, the first allogibberic acid isolated from natural sources, provides crucial evidence that allogibberic acids indeed exist as a genuine natural product.

EXPERIMENTAL SECTION

General Experimental Procedures. Optical rotations were measured on a Jasco P-1020 polarimeter (Jasco, Easton, MD, USA). The IR spectra were recorded on a Bruker IFS-66/S FT-IR spectrometer (Bruker, Karlsruhe, Germany). The ECD spectra were measured on a Jasco J-715 spectropolarimeter (Jasco, Easton, MD, USA). The UV spectra were recorded with a Shimadzu UV-1601 UV−visible spectrophotometer (Shimadzu, Tokyo, Japan). HR-ESI mass spectra were recorded on an SI-2/LCQ DecaXP liquid chromatography (LC)-mass spectrometer (Thermo Scientific, West Palm Beach, PL, USA). NMR spectra were recorded on a Varian UNITY INOVA 500 NMR spectrometer (Varian, Palo Alto, CA, USA) operating at 500 MHz (^{1}H) and 125 MHz (^{13}C), with chemical shifts given in ppm (δ). Semipreparative HPLC used a Gilson 306 pump (Gilson, Middleton, WI, USA) with a Shodex refractive index detector (Shodex, New York, NY, USA). Column chromatography was performed with silica gel 60 (Merck, 70−230 mesh and 230−400 mesh) and RP-C_{18} silica gel (Merck, 230−400 mesh). Merck precoated silica gel F_{254} plates and reversed-phase (RP)-18 F_{254s} plates (Merck, Darmstadt, Germany) were used for TLC. Spots were detected on TLC under UV light or by heating after spraying with 10% H_2SO_4 in EtOH (v/v).

Plant Material. 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, Korea.

Extraction and Isolation. The dried seeds (2 kg) of *P. nil* were extracted with 50% EtOH (3 × 1 L, × 3 days) at room temperature and filtered. The filtrate was evaporated *in vacuo* to obtain the EtOH extract (250 g), which was suspended in distilled H_2O (3.2 L) and then successively partitioned with *n*-hexane, $CHCl_3$, EtOAc, and *n*-BuOH, to yield 2, 1.5, 2, and 108 g of dried organic extracts, respectively. Each extract was tested for cytotoxic and anti-inflammatory activities, and a bioactivity-guided fractionation method was used for isolation work. The EtOAc-soluble fraction showed cytotoxicity against A549, SK-OV-3, SK-MEL-2, and HCT-15 cell lines and weakly reduced the production of LPS-induced pro-inflammatory factor, NO, in BV-2 cells. On the basis of the bioactivity of the extracts, the most active EtOAc-soluble fraction (2 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 six fractions (A−F). Fraction E (600 mg) was applied to an RP-C_{18} silica gel column (50% MeOH/100% MeOH, gradient system) to yield 11 subfractions (E1−E11). Fraction E5 (20 mg) was further purified by semipreparative reversed-phase HPLC (33% MeCN), using an Econosil RP-18 column (250 mm × 10 mm i.d., 10 μm, Alltech, Nicholasville, KY, USA), to give pure pharbinilic acid (**1**) (6 mg, t_R = 16.5 min).

Pharbinilic acid (1): colorless gum; $[\alpha]_D^{25}$ −19.1 (*c* 0.30, MeOH); UV (MeOH) λ_{max} (log ε) 288 (1.3), 227 (2.2), 210 (3.8) nm; ECD (MeOH) λ_{max} ($\Delta\varepsilon$) 233 (+12.5), 261 (−4.2) nm; IR (KBr) ν_{max} 3358, 2947, 2833, 2511, 2072, 1661, 1450, 1120, 1031, 674 cm^{-1}; ^{1}H (500 MHz) and ^{13}C (125 MHz) NMR data, see Table 1; ESIMS (positive-ion mode) *m*/*z* 391 $[M + Na]^+$; HRESIMS (positive-ion mode) *m*/*z* 391.1154 $[M + Na]^+$ (calcd for $C_{21}H_{20}O_6Na$, 391.1158).

Cytotoxicity Testing. The cell lines used were A549 (non-small-cell lung adenocarcinoma), SK-OV-3 (ovary malignant ascites), SK-MEL-2 (skin melanoma), and HCT-15 (colon adenocarcinoma). These cancer cell lines were provided by the National Cancer Institute (NCI). An SRB bioassay was used to determine the cytotoxicity of each compound against the cell lines.[19] The assays were performed at the Korea Research Institute of Chemical Technology. Doxorubicin was used as a positive control. Doxorubicin cytotoxicity against the A549, SK-OV-3, SK-MEL-2, and HCT-15 cell lines was IC_{50} 0.02, 0.01, 0.01, and 0.13 μM, respectively.

Measurement of NO Production and Cell Viability. Murine microglia BV-2 cells were plated into a 96-well plate (3 × 10^4 cells/well). After 24 h, cells were pretreated with samples for 30 min and then stimulated with 100 ng/mL of LPS for another 24 h. Nitrite, a soluble oxidation product of NO, was measured in the culture media using the Griess reaction. The supernatant (50 μL) was harvested and mixed with an equal volume of Griess reagent (1% sulfanilamide, 0.1% *N*-1-naphthylethylenediamine dihydrochloride in 5% phosphoric acid). Absorbance at 540 nm was measured after 10 min using a microplate reader. Sodium nitrite was used as the standard to calculate the NO_2^- concentration. Cell viability was assessed by a 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay. N^G-Monomethyl-L-arginine (L-NMMA, Sigma, USA) was tested as a positive control. L-NMMA is a nonspecific NO synthase inhibitor.

ASSOCIATED CONTENT

Supporting Information

1D (^{1}H NMR and ^{13}C NMR) and 2D NMR data (^{1}H−^{1}H COSY, HSQC, HMBC, NOESY) and ECD spectrum for pharbinilic acid (**1**) are available free of charge via the Internet at http://pubs.acs.org.

AUTHOR INFORMATION

Corresponding Author

*Tel: 82-31-290-7710. Fax: 82-31-290-7730. E-mail: krlee@skku.ac.kr.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This research was supported by the Global Leading Technology Program of the Office of Strategic R&D Planning (OSP) funded by the Ministry of Knowledge Economy, Republic of Korea (10039303). We are thankful to the Korea Basic Science Institute (KBSI) for the measurements of NMR and MS spectra.

■ REFERENCES

(1) Hedden, P.; Kamiya, Y. *Plant Mol. Biol.* **1997**, *48*, 431−460.
(2) Bomke, C.; Tudzynski, B. *Phytochemistry* **2009**, *70*, 1876−1893.
(3) Hedden, P.; Thomas, S. G. *Biochem. J.* **2012**, *444*, 11−25.
(4) Yokota, T.; Murofushi, N.; Takahashi, N.; Katsumi, M. *Phytochemistry* **1971**, *10*, 2943−2949.
(5) Yokota, T.; Takahasi, N.; Murofushi, N.; Tamura, S. *Tetrahedron Lett.* **1969**, *25*, 2081−2084.
(6) Murofushi, N.; Takahashi, N.; Yokota, T.; Tamura, S. *Agric. Biol. Chem.* **1968**, *32*, 1239−1245.
(7) Yokota, T.; Murofushi, N.; Takahashi, N.; Tamura, S. *Agric. Biol. Chem.* **1971**, *35*, 573−582.
(8) Hedden, P.; Kamiya, Y. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **1997**, *48*, 431−460.
(9) Kim, K. H.; Jin, M. R.; Choi, S. Z.; Son, M. W.; Lee, K. R. *Heterocycles* **2008**, *75*, 1447−1455.
(10) Kim, K. H.; Choi, S. U.; Lee, K. R. *J. Nat. Prod.* **2009**, *72*, 1121−1127.
(11) Bensky, D.; Gamble, A. *Chinese Herbal Medicine*, revised ed.; Materia Medica, Eastland Press: Seattle, 1993; p 121.
(12) Meguro, H.; Hachiya, K.; Tuzimura, K.; Mori, K.; Matsui, M. *Agric. Biol. Chem.* **1973**, *37*, 1035−1040.
(13) Salman, S. R.; Derwish, G. A. W.; Al-Salih, S. S. *Spectrochim. Acta, Part A* **1986**, *42*, 405−408.
(14) Al-Ekabi, H. K.; Derwish, G. A. W. *Can. J. Chem.* **1984**, *62*, 1996−1998.
(15) Pryce, R. J. *J. Chem. Soc., Perkin Trans. 1* **1974**, 1179−1184.
(16) Brian, P. W.; Grove, J. F.; Hemming, H. G.; Mulholland, T. P. C.; Radley, M. *Plant Physiol.* **1958**, *33*, 329−333.
(17) Dodge, J. D.; Bryant, H. U.; Kim, J.; Matter, W. F.; Norman, B. H.; Srinivasan, U.; Vlahos, C. J.; Sato, M. *Bioorg. Med. Chem. Lett.* **1995**, *5*, 1713−1718.
(18) MacMillan, J.; Vanstone, A. E.; Yeboah, S. K. *J. Chem. Soc., Perkin Trans. 1* **1972**, 2898−2903.
(19) Skehan, P.; Storeng, R.; Scudiero, D.; Monks, A.; MaMahon, J.; Vistica, D.; Warren, J. T.; Bokesch, H.; Kenney, S.; Boyd, M. R. *J. Natl. Cancer Inst.* **1990**, *82*, 1107−1112.