Identification and Evaluation of Apoptosis-Inducing Activity of Ipomone from *Ipomoea nil*: A Novel, Unusual Bicyclo-[3.2.1] Octanone Containing Gibberic Acid Diterpenoid

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ABSTRACT: Ipomone (1), a novel diterpenoid along with seven known compounds (2–8), was isolated for the first time from the acidified hydroalcoholic extract of *Ipomoea nil* seeds. The structures of the isolated compounds were elucidated via comprehensive NMR spectroscopic data. The absolute configuration of 1 was ascertained through NOESY, NMR, and ECD analyses. Compound 1 was found to contain an unusual bicyclo-[3.2.1] octanone, which appeared first time in any natural product that might be an artifact resulting from the acid-catalyzed 1,2 alkyl shift/rearrangement. The novel compound was screened for cytotoxic activity against a panel of 12 human cancer cell lines and exhibited weak cytotoxicity with IC₅₀ values in the range of 34–86 μM (except for HEK-293 cells). Microscopic studies revealed that compound 1 induced apoptosis and autophagy in A549 cells. To further explore the signaling pathway involved, immunoblot analysis was performed that confirmed inhibition of apoptotic proteins PARP-1 and caspase-3 expression and upregulation of LC3B expression by compound 1. The compound was further subjected to molecular docking studies to evaluate its binding affinity with p110α, PARP-1, and caspase-3 proteins.

1. INTRODUCTION

*Ipomoea nil* (L.) Roth (*Pharbitis nil* (L.) Choisy, Convolvulaceae) is a flowering plant, commonly known as morning glory.1 Its seeds are traditionally used as purgative in the Chinese system of medicines.2 An array of diverse chemical constituents have been isolated from the *Ipomoea nil* seeds viz. gibberellins and their glycosides (from the immature seeds),3 allogibberic acid,4 monoterpen glycosides,5 phenylethanoid glycosides, and phenylpropanoids,6 ent-kaurane diterpenoids and their glycosides,7 ent-gibbane diterpene glycoside,7a resin glycosides,8 phenolic amides,9 lignans,6,10 neolignans,10 triterpenoid saponins,11 fatty acid derivatives,12 and spermidine alkaloid.13 The plant has been reported to have diverse pharmacological activities including antibacterial,14 antifungal,15 anti-inflammatory,5,7c antioxidant,7c cytotoxic,7ab,9,16 α-glucosidase inhibitory,17 hepatoprotective,18 and multidrug-resistance reversal activities.8b *Ipomoea nil* has shown its anticancer potential in many *in vitro* studies. The root extract of the plant is found to trigger apoptotic cell death of gastric cancer cells in a dose- and time-dependent manner.16b The ent-kaurane diterpene glycosides isolated from *I. nil* seeds displayed moderate *in vitro* cytotoxicity against five human tumor cell lines.7a Two phenolic amides, pharnilatins A and B, possessed cytotoxicity against human tumor cell lines.9 Lignans displayed anti-inflammatory activity and cytotoxicity against cancer cells.6,10 Resin glycosides (pharbatins) reversed multidrug resistance in KB/VCR cells and also showed synergistic effects with vincristine.8b Further, the seeds were shown to induce autophagy and apoptosis in lung cancer cells.18 Also, DA-9701 (Motilitone), a herbal formulation consisting of *Corydalis yanhusuo* (Papaveraceae) roots and *Pharbitis nil* seeds, has been used in the treatment of functional dyspepsia in Korea.19 DA-9701 had completed six clinical trials (*clinicalTrials.gov*) for its global use.

In the continuing search for anticancer phytoconstituents from *I. nil*, a new gibberic acid diterpene ipomone (1), along with seven known compounds, have been isolated from the acid-treated hydroalcoholic (H₂O:Methanol, 2:8) extract of the seeds. Compound 1 exhibits structural similarity to pharbinic acid, an allogibberic acid.4 Both compounds are unusually different due to the presence of ketone functionality at position 8 that suggests the possibility of rearrangement. The novel compound was screened for cytotoxic activity against a panel of 12 human cancer cell lines and exhibited weak cytotoxicity with IC₅₀ values in the range of 34–86 μM (except for HEK-293 cells). Microscopic studies revealed that compound 1 induced apoptosis and autophagy in A549 cells. To further explore the signaling pathway involved, immunoblot analysis was performed that confirmed inhibition of apoptotic proteins PARP-1 and caspase-3 expression and upregulation of LC3B expression by compound 1. The compound was further subjected to molecular docking studies to evaluate its binding affinity with p110α, PARP-1, and caspase-3 proteins.

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resulting from acid-catalyzed pinacol-pinacolone type 1,2 shift rearrangement. The compound 1 was screened for cytotoxicity against a panel of 12 human cancer cell lines of different types of tissues (A549, HL-60, MOLT-4, MDB-MB-231, Hs578T, MCF-7, PC-3, OVCAR-3, HeLa, Mia PaCa2, HCT-116, and HEK293).

2. RESULTS AND DISCUSSION

A hydroalcoholic (H2O:Methanol, 2:8) extract of I. nil seeds was prepared under acidic conditions (pH 3 by HCl) and concentrated to evaporate methanol, which was subsequently fractionated with ethyl acetate to separate and enrich alkaloids and non-alkaloids in aqueous and ethyl acetate fraction, respectively. The initial cytotoxicity study against HL60 cells showed the ethyl acetate fraction (containing non-alkaloidal content) to be active. The active fraction was concentrated and resuspended in 1:9 methanol–water and allowed to pass through a column of polymeric HP20 resin. Adsorbed organic metabolites were then eluted by step gradient elution of increased content of methanol in water. Five fractions were pooled and further subjected to repeated silica gel and sephadex LH20-based size-exclusion chromatography using hexane-ethyletacetate and methanol, respectively (summary of isolation and purification is provided in Supporting Information). Eight compounds were isolated and characterized using 1D and 2D NMR spectroscopic and mass spectrometric analyses. Apart from the novel gibberic acid diterpene ipomone (1), other known compounds viz. behenic acid (2), docosanol (3, behenyl alcohol), ester of ferulic acid with linear chain fatty alcohol, i.e., tetracosyl ferulate (4), stigmasterol (5), β-sitosterol (6), 7,3′,S′-tri-O-methyltiricin (7), and apigenin (8) were identified by comparing the observed NMR spectra with the reported spectroscopic data (Supporting Information).

Compound 1 was isolated as an optically active, brown gummy mass, [α]D19 +19 (c 0.40, CHCl3), with molecular formula C19H20O3 based on the HRMS (observed m/z [M + H]+ 297.1487, calcd for C19H20O3, 297.1491) and detailed NMR data analyses (Table 1 and Supporting Information). The 1H NMR spectrum showed the presence of three aromatic proton resonances at δH 7.29, 7.21, and 7.07, one olefinic proton at δH 5.92, one methoxy group at δH 3.70, and one methyl group attached to the aromatic system at δH 2.24. The 13C NMR, DEPT-135, and HSQC spectra displayed two carbonyl groups at δC 219.66 and 172.22, eight olefinic carbons including four quaternary carbons between 148.83 and 113.97, and nine aliphatic carbons between δC 55.15 and 18.61 including one CH group at δC 55.15, one methoxy carbon at δC 51.95, three CH2 carbons at δC 54.79, 42.10, and 39.24, two methyl carbons at δC 20.66 and 18.61, and two quaternary carbons. The COSY, HSQC, and HMBC spectra exhibited the following correlations. The olefinic proton at δH 7.21 (δC 128.70, C-3) showed 1H–1H COSY correlations to protons at δH 7.29 (δC 113.97, C-5), which in turn possessed HMBC correlations to carbons at δC 129.70 (C-2), 135.15 (C-1), and 139.85 (C-10a), with C-2 and C-10a showing HMBC correlations to an olefinic proton at δH 7.29 (δC 113.97, C-4). This proton showed another HMBC correlation to δC 138.53, C-4a. C-10a resonance also showed an additional HMBC correlation with an aliphatic proton at δH 4.02 (δC 55.15, C-10), which in turn possessed HMBC correlations with a carbonyl carbon at δC 172.22 (C-12) and carbon resonances at δC 48.84 (C-9a) and 138.53 (C-4a). The C-12 resonance also showed a strong HMBC correlation to a methoxy group at δH 3.70 (δC 51.95, C-15), claiming the carbonyl group to be that of a methyl ester.

C-4a resonance was also HMBC-correlated to an olefinic proton at δH 5.92 (δC 113.97, C-5), which was subsequently correlated to carbon resonances at δC 148.83 (C-4b) and 48.84 (C-9a), along with a 1H–1H COSY correlation to a methylene proton resonance at δH 2.29 (δC 39.24, C-6). These proton resonances showed HMBC correlations with carbon resonance at δC 49.65 (C-7) and another carbon at δC 219.66 (C-8). The C-7 carbon resonance was correlated to methylene proton resonance at δH 2.08 and 1.99 (δC 42.12, C-11), and a methyl proton resonance at δH 1.21 (δC 20.66, C-14). The carbonyl carbon (C-8) also showed HMBC correlation with methyl (C-14) protons and methylene protons at δH 2.45 (δC 54.80, C-9), with another HMBC correlation at δH 2.08 and 1.99 (δC 42.12, C-11) and 2.45 (δC 54.80, C-9) to C-9a were also observed. Based on these observations, the planar structure of compound 1 was determined as a bicyclo[3.2.1] octanone containing methyl 1,7-dimethyl-8-oxo-6,8,9,10-tetrahydro-7H-7,9a-methanobenzo[a]azulene-10-carboxylate.

The absolute configuration of 1 was determined by electronic circular dichroism (ECD) analysis and the NOESY spectrum. The ECD analysis has been used for the determination of the stereochemistry of C-10 carboxylic acid in
Based on this established absolute conformation of C-10, the NOESY spectrum revealed the conformations of C-7 and C-9a stereocenters. NOE interactions were observed between H-10/H-11, H-11/H-14, and H-16/H-14 that confirmed the 7R and 9aS conformations (Figure 2b). Therefore, the absolute configuration of 1 was 7R, 9aS, and 10R. Thus, the structure of compound 1 was elucidated as methyl (7R,9aS,10R)-1,7-dimethyl-8-oxo-6,8,9,10-tetrahydro-7H-7,9a-methanobenzof[a]azulene-10-carboxylate and was named as ipomone as shown in Figure 1.

The literature search revealed that a similar planar structure has been reported previously and was produced from gibberellic acid by a synthetic reaction.29 Ipomone is analogous to the allogibberic class of compounds, and earlier, an allogibberic compound, pharbinilic acid, was also obtained as a methyl ester, while previously reported allogibberic acids have free carboxylic acid groups. Thus, the esterification of acid with methanol, being an acid-catalyzed process, supports our hypothesis.

### 2.1. Cytotoxicity of Ipomone (1) against Cancer Cells.

The novelty of ipomone (1) prompted us to investigate it for anti-cancer activity. Compound 1 was evaluated for cytotoxicity against a panel of 12 human cancer cell lines, i.e., A549 (lung epithelial carcinoma), HL-60 (acute promyelocytic leukemia), MOLT-4 (acute lymphoblastic leukemia), MDA-MB-231 (breast adenocarcinoma), Hs579T (breast carcinoma), MCF-7 (breast carcinoma), PC-3 (prostate adenocarcinoma), OVCAR-3 (ovarian epithelial carcinoma), HeLa (cervical epithelial carcinoma), Mia PaCa2 (pancreatic epithelial cell carcinoma), HCT-116 (colorectal carcinoma), and HEK-293 (embryonic kidney), at the concentrations of 10, 20, 30, 40, 50, 60, 70, 80, 90, and 100 μM for 72 h, using MTT assay.31 It exhibited weak cytotoxicity against all cell lines, except HEK-293 cells, with IC₅₀ values in the range of 34–86 μM after 48 h (Table 2). The results revealed that A549 cells
were the most sensitive cancer cells among all (A549, IC₅₀ 34.1 μM).

### 2.2. Ipomone (1) Causes A549 Cell Death by Apoptosis

A dose/time-dependent cytotoxicity study was also performed on lung cancer A549 cells (Figure 3). DAPI (4′,6-diamidino-2-phenylindole) staining displayed that compound 1 caused chromatin condensation and DNA fragmentation, resulting in the formation of apoptotic bodies as visualized via fluorescence microscopy (Figure 4). Hence, it proves that cytotoxicity is being mediated through induction of apoptosis and also proposes that this compound inhibits the growth of A549 cells by inducing apoptosis.

### 2.3. Ipomone (1) Induces Autophagy

To confirm the autophagy-inducing effect of compound 1, A549 cells were further examined in fluorescent micrographs after staining with acridine orange (AO) and propidium iodide (PI). As shown in Figure 5, cells treated with 10 and 30 μM of compound 1 exhibited significant evidence of autophagy induction. The induction of autophagy increased in a dose-dependent manner.

### 2.4. Immunoblot Analysis

To confirm the above hypothesis, immunoblot analysis was performed for the apoptosis and autophagy genes. It was analyzed that compound 1 can induce apoptosis/autophagy in A549 cells, and the expression levels of proteins involved viz. p110α, PARP-1, caspase-3, and LC-3 B were measured by western blot analysis after treatment with compound 1 at 0, 10, 30, and 50 μM for 24 h. Compound 1 displayed a dose-dependent induction of LC3B and decrease in the PARP-1 and caspase-3 expression patterns as shown in Figure 6.

### 2.5. Molecular Docking

Upon successful completion of the docking simulation, the best confirmation was selected with the best binding energy in the largest cluster of 2.0 Å. The binding energy for ipomone (ligand) was found to be −7.39 kcal/mol for caspase-3, −7.62 kcal/mol for p110α, and −7.92 kcal/mol for PARP-1. The ligand showed important interactions with the protein viz. H-bond, van der Waals, π-alkyl, π-π-stacked, and π-cation. The interactions of ipomone with caspase-3, p110α, and PARP-1 proteins are represented in Figure 7.

### 3. CONCLUSIONS

In conclusion, one new gibberic acid diterpenoid ipomone (1) along with seven known compounds were isolated from the acidified hydroalcoholic extract of seeds of *Ipomoea nil*. Compound 1 was characterized by extensive NMR and ECD analysis. The new compound appeared to be an artifact that might have resulted from acid-catalyzed rearrangement of allogibberic acid diterpenoids. A plausible mechanism, 1,2 alkyl shift, pinacol–pinacolone rearrangement, has been proposed to justify the conversion to the artifact. This compound was further screened for cytotoxic activity against a panel of 12 human cancer cell lines. Although it exhibited weak cytotoxicity against all cell lines tested, i.e., 34−86 μM (except HEK-293 cells), mechanistic investigation suggested it a lead that induces apoptosis and autophagy.

### 4. EXPERIMENTAL SECTION

#### 4.1. General Experimental Procedures

All chemicals were purchased from Sigma Aldrich and used as received. Optical rotation was measured on a JASCO P-2000 polarimeter. The 1D and 2D NMR spectra were recorded on a Bruker-Avance III HD 500 MHz NMR spectrometer using tetramethylsilane (TMS) as the internal standard and are referenced to the residual proton/carbon in the NMR solvent (CDCl₃, 7.26/77.1 ppm; DMSO-d₆, 2.50/39.5 ppm). The ECD spectrum was measured on a JASCO J-1500 spectropolarimeter. ESI-MS and HRMS spectra were recorded on Agilent 1100LC-QTOF and HRMS-6540-UHD spectrometers. The UV spectrum was recorded on a Cary 60 UV–vis spectrophotometer (Agilent Technologies). IR spectra were recorded on a Perkin-Elmer IR spectrophotometer. All chromatographic purifications were performed on silica gel (#60−120 or #100−200) obtained from Merck. The thin-layer
chromatography (TLC) was performed on pre-coated silica gel 60 GF254 aluminum sheets (Merck) and visualized under UV light (254 nm) and by spraying an anisaldehyde–sulfuric acid reagent followed by heating.

4.2. Plant Material. The seeds of Ipomoea nil were obtained from the local market of Varanasi, India, in March 2019, and authenticated by Prof. Nawal Kishore Dubey, Centre of Advanced Study in Botany, Institute of science, Banaras Hindu University, Varanasi. A specimen sample (voucher number, Convolvula, 2020/1) was preserved in the herbarium.

4.3. Extraction and Isolation. The seeds of I. nil (1 kg) were coarsely powdered and extracted with acidified methanol–water (80:20, 1.5 L × 2, each for 48 h) at room temperature. The hydroalcoholic extract was concentrated to evaporate alcohol, and the remaining aqueous portion was portioned with ethyl acetate. The ethyl acetate fraction was concentrated to get a 6.4 gm fraction that was again resuspended in 9:1 water–methanol (200 mL) and loaded to HP20 resin (10 mL, 5% v/v) in a glass column. A pre-activated HP-20 resin (soaked in methanol overnight) was loaded in a glass column and equilibrated with distilled water. The extract was loaded to the resin column and allowed to settle down for some time. The adsorbed material was eluted with methanol in a gradient manner increasing from 100% distilled water to 100% methanol. A total of 5 fractions were collected (each 50 mL except Fr 1 at 200 mL) at the gradient elution of increasing methanol in water; Fr 1: 100% water, Fr 2: 75% water, Fr 3: 50% water, Fr 4: 25% water, and Fr 5: 100% methanol. Fr 1 and 2 were loaded with highly polar primary metabolites like sugar. Fr 3 was subjected to repeated sephadex LH-20-based size exclusion chromatography in methanol, and compounds 7 and 8 were obtained as pure compounds and characterized as tri-O-methyltricetin and apigenin, respectively. Fr 4 yielded compound 1 after repeated sephadex LH-20. Fr 5 contained mainly lipophilic metabolites and compounds 2–6 were purified after repeated silica gel column chromatography in the hexane–ethyl acetate gradient. Novel metabolite 1 was identified as Ipomone based on detailed 2D NMR, and compounds 2–7 were identified by comparing the observed NMR spectra with the reported spectroscopic data (Supporting Information).

4.3.1. Ipomone (1). The compound is a light brown gummy solid; 15 mg; [α]D20 +19 (c 0.40, CHCl3); UV (MeOH) λmax (log ε) 205 (4.11), 259 (3.33), 270 (3.27), 288 (3.02), 300

Figure 4. Fluorescence micrographs (DAPI staining) of A549 cells untreated and treated with 1 for 24 h. Ipomone (1)-induced apoptosis in A549 cells.

Figure 5. Morphology of untreated A549 cells (control) and those treated with 10, 30, and 50 μM of ipomone (1). Cells were stained by acridine orange (AO)/propidium iodide (PI) to study the autophagy-inducing potential of compound 1.

Figure 6. Immunoblot analysis using antibodies against the p110α, PARP-1, caspase-3, and LC 3B proteins in A549 cells after treatment with compound 1 (0–50 μM) for 24 h.
(2.96) nm; ECD (MeOH) λ_{max} 234, 269 nm; IR (KBr) ν_{max} 2345, 2130, 1638, 1618, 1384, 1352, 618 cm⁻¹; ¹H NMR (500 MHz, CDCl₃) and ¹³C NMR (125 MHz, CDCl₃) data, see Table 1; ESIMS m/z 297.30 [M + H]⁺; HRMS m/z 297.1487 [M + H]⁺ (calcd for C₁₉H₂₁O₃, 297.1491).

4.4. Cell Line and Cell Culture. All the cell lines were procured from NCI-Bethesda, USA, and cultured as per the protocol provided. Cells were grown in a CO₂ incubator (Esco) at 37 °C with a 98% humidity and 5% CO₂ gas environment.

4.5. Cell Viability Assay. The MTT colorimetric assay (an assay which measures color changes) was used for measuring the activity of enzymes that reduce MTT to formazan, giving a purple color. Yellow MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a tetrazole) is reduced to purple formazan in living cells. A solubilization solution (usually either dimethyl sulfoxide, an acidified ethanol solution, or a solution of the detergent sodium dodecyl sulfate in diluted hydrochloric acid) was added to dissolve the insoluble purple formazan product into a colored solution. The absorbance of this colored solution was quantified by measuring at a certain wavelength (usually between 500 and 600 nm) by a spectrophotometer. The absorption maximum is dependent on the solvent employed.¹¹

4.6. Fluorescence Microscopy. A549 cells were treated with indicated concentrations of ipomone for 48 h. After treatment, cells were collected, washed with PBS twice, and fixed in 400 μL cold acetic acid-methanol (1:3, v/v) by leaving overnight at 4 °C. The next day, cells were washed and dispensed in 50 μL of fixing solution. The cells were then spread on a clean slide and dried overnight at room temperature. Cells were stained with DAPI (5 μg/mL in 0.01 M citric acid and 0.45 M disodium phosphate containing 0.05% Tween 20) for 30 min at room temperature, and subsequently, the slides were washed with distilled water followed by washing with PBS. While wet, 40 μL of mounting fluid (PBS: glycerol, 1:1) was poured over the slide and covered with a glass coverslip and sealed. Cells were observed under a microscope for any nuclear morphological changes that occur during apoptosis. For phase-contrast microscopy, cells were simply photographed using a microscope after treatment.³¹

4.7. Acridine Orange Staining. The induction of autophagy was analyzed by staining cells with acridine orange (AO) as described earlier.³² Briefly, 0.5 × 10⁶ cells were seeded in a six-well plate and treated with ipomone for 48 h. Cells were incubated with 1 mg/mL AO for 15 min before

Figure 7. Protein–ligand interaction diagrams of ipomone (1) with caspase-3 (PDB id. 3KJF) (A), p110α (PDB id. 5DXT) (B), and PARP-1 (PDB id. 6I8T) (C).
termination of the experiment and were washed with PBS before analyzing on a fluorescence microscope.

**4.8. Preparation of Whole-Cell Lysates for Immunoblotting.** Cells (2 × 10^6/well) were seeded and treated with different concentrations of ipomone. A549 cells were treated with 10, 30, and 50 μg/mL concentrations of ipomone for 48 h. After 48 h treatment, the cells were harvested and washed with cold PBS. The PBS was decanted, and the pellets resuspended in appropriate volumes of cold RIPA lysis buffer, freshly supplemented with a 1% (v/v) eukaryotic protease inhibitor cocktail for 45 min and vortexed at 10 min intervals while maintaining on ice. The cell lysate was cleared by centrifugation at 14,000 g at 4 °C for 15 min. The supernatant was transferred to a new tube and used as whole-cell lysates for western blot analysis for the expression status of various proteins.

**4.9. Molecular Docking.** Molecular docking studies were performed using AutoDock 4.2 to deepen the understanding of the molecular interaction between ipomone and proteins viz. caspase-3 (PDB id. 3KJF), p110α (PDB id. SDXT), and PARP-1 (PDB id. 6I8T).33 The crystal structures were obtained from the protein data bank (https://www.rcsb.org/). The correct protonation state to the residues was assigned using the pdb2pqr web server. All the water molecules, ligands, and ions were removed. Non-polar hydrogen atoms were removed, and Gasteiger charges were added using M.G.L Tools 1.5.6. AutoDock employs Autogrid4 to compute maps. The active site of the protein was determined using the PLIP web server (https://projects.biotec.tu-dresden.de/plip-web/plip).34 The docking study was performed using Lamarckian Genetic Algorithm (LGA). The docking was performed with 100 runs, 150 population size, 27,000 number of generations, and 2,500,000 number of energy evaluation. It employs a “semiempirical free energy force field” to evaluate conformations at the time of docking simulation. The docked pose was visualized by Maestro for studying interactions.

**ASSOCIATED CONTENT**

**Supporting Information**

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.0c06304. Isolation scheme and spectroscopic data of compounds (1–8) (PDF)

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**Notes**

The authors declare no competing financial interest.

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