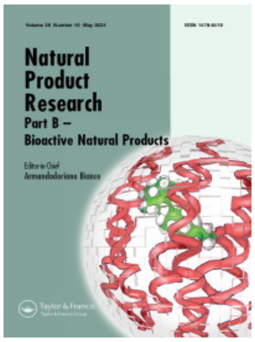

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A new benzoquinone and a new stilbenoid from *Paphiopedilum exul* (Ridl.) Rolfe

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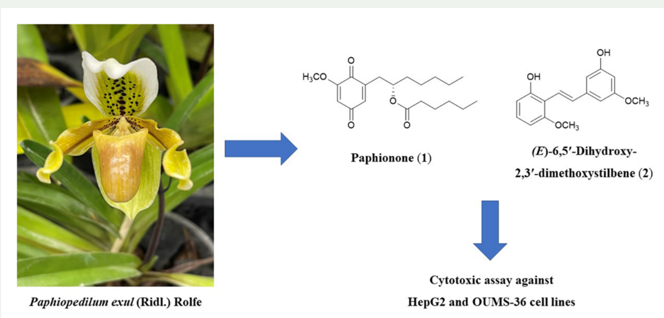
ABSTRACT

One new alkyl benzoquinone, paphionone (**1**), one new *trans*-stilbenoid, (*E*)-6,5′-dihydroxy-2,3′-dimethoxystilbene (**2**), and eight known stilbenoids and flavonoids (**3**-**10**) were isolated from the leaves and roots of *Paphiopedilum exul* (Orchidaceae). Their chemical structures were determined based on IR, ECD, MS and NMR analyses. Cytotoxicity of all isolated compounds towards human hepatocellular carcinoma (HepG2) cell line was examined *in vitro* by MTT assay. The *para*-hydroxybenzyl substituted stilbene **10** was potently cytotoxic to the cancer cells, with an IC_{50} value of $4.80 \pm 1.10\,\mu M$ (selectivity index = 20.83). All compounds were non-toxic to normal human embryo fibroblast (OUMS-36) cell line.



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1. Introduction

Lady slipper's orchids, in which slipper-shaped labellum of the flower is a distinct characteristic, belong to five genera within subfamily Cypripedioideae of the family Orchidaceae. Many of these orchids and their hybrids are cultivated as ornamental plants. *Paphiopedilum* is an Asian genus of slipper orchids which is distributed from southern India and China to New Guinea and the Solomon Islands (Pedersen et al. 2011). Although medicinal use of a *Paphiopedilum* species, *P. insigne*, to treat amoebic dysentery has been documented (Hossain 2011) and many orchid species were shown to contain potential anticancer chemicals (Śliwiński et al. 2022), investigations on the chemical constituents of *Paphiopedilum* orchids and their biological activities have only recently been conducted. Several stilbenoid and flavonoid constituents of these orchids displayed cytotoxicity towards cancer cell lines tested (Lertnitikul et al. 2016, 2022; Naphatsawan et al. 2016; Nwe et al. 2020). Interestingly, a stilbene from *P. dianthum*, pinosylvin monomethyl ether, was able to inhibit drug efflux transporters and sensitise drug-resistant breast cancer cells to chemotherapy (Sein et al. 2023).

Paphiopedilum exul (Ridl.) Rolfe, or excluded paphiopedilum, is an endemic terrestrial orchid that is found on limestone cliffs in the Phuket-Krabi area of southern Thailand peninsular and in Perlis state on the northwestern coast of Malaysia (Pedersen et al. 2011; Besi et al. 2022). The specific epithet "*exul*" in its scientific name reflects its restricted geographical distribution. A previous phytochemical study of *P. exul* roots reported the presence of five *trans*-stilbenes (Naphatsawan et al. 2016); one of them, i.e. (*E*)-3-methoxy-2-(4-hydroxybenzyl)-5,2′-dihydroxystilbene, firstly isolated from a *Phragmipedium* orchid (Garo et al. 2007), was able to induce both apoptotic and autophagic death of lung cancer cells (Tungsukruthai et al. 2021). In our continuing study of *Paphiopedilum* species, we report herein the isolation of one new alkyl benzoquinone (**1**), one new *trans*-stilbene (**2**), four known stilbenes (**3**-**4**, **8**, **10**) and four known flavonoids (**5**-**7**, **9**) from the leaves and roots of *P. exul* (Figure 1). Cytotoxicity of these compounds against human hepatocellular carcinoma (HepG2) and normal human embryo fibroblast (OUMS-36) cell lines was evaluated *in vitro* by the MTT assay.

2. Results and discussion

Compound **1** was obtained as an orange amorphous powder from the leaves of *P. exul*. Its IR spectrum showed absorption bands of ester carbonyl (1727 cm^{-1}), quinone ring (1647, 1633, 1604 cm^{-1}) and methyl ether (1230, 1032 cm^{-1}). The molecular formula of **1** was determined to be $C_{20}H_{30}O_5$, based on a pseudo-molecular $[M+Na]^+$ ion peak of *m/z* 373.1993 (calcd 373.1991) in the HRESI mass spectrum. The colour of the compound and the six degrees of unsaturation, calculable from its molecular formula, suggested that **1** was a benzoquinone with an ester function in its side chain. The ^{1}H NMR spectrum of **1** (Table S1, Figure S2) displayed *meta*-coupled signals of the quinone nucleus at δ_H 5.86 (1H, d, $J = 2.5$ Hz, H-5) and δ_H 6.46 (1H, d, $J = 2.5$ Hz, H-3), a methoxy singlet at δ_H 3.81 (3H, s, 6-OCH_3), as well as signals of methylene protons (H-1′) adjacent to quinone ring at δ_H 2.42 (1H, dd, $J = 14.0$, 9.3 Hz) and δ_H 2.87 (1H, ddd, $J = 14.0$, 3.3, 1.3 Hz), which showed ^{1}H-^{1}H COSY correlations (Figures S1 and S6) with an oxymethine proton resonated at δ_H 5.00 (1H, m, H-2′), α-methylene protons

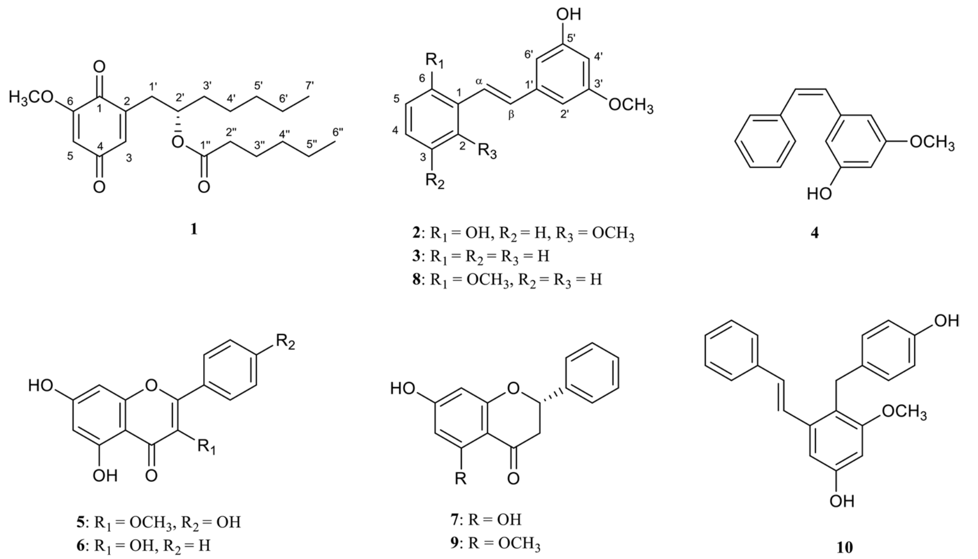

Figure 1. Chemical structures of compounds **1-10**.

of an alkyl ester at δ_H 2.21 (2H, td, $J = 14.0$, 6.5 Hz, H-2′′), methylene (δ_H 1.24-1.59, 14H, m) and terminal methyl groups [δ_H 0.87 (3H, t, $J = 6.5$ Hz, H-7′) and δ_H 0.88 (3H, t, $J = 6.5$ Hz, H-6′′)] of two alkyl chains. The ^{13}C NMR (Table S1, Figure S3) and HSQC (Figure S4) spectra of **1** displayed twenty carbon resonances including signals of 1,4-benzoquinone carbonyls (δ_C 181.6, C-1 and δ_C 187.2, C-4), one ester carbonyl (δ_C 173.6, C-1′′), one oxymethine (δ_C 71.9, C-2′), one methoxy (δ_C 56.3, 6-OCH_3), nine methylene and two methyl carbons. HMBC correlations (Figures S1 and S5) from H-1′ signals to C-1 (δ_C 181.6) and C-3 (δ_C 134.4) confirmed linkage of alkyl side chain to C-2 of the quinone moiety, while the 6-methoxy position was established from the HMBC cross peak observed between its proton signal and that of C-6 (δ_C 158.9). These spectral data revealed that compound **1** was a 1,4-benzoquinone substituted at C-2 with an esterified hydroxyalkyl side chain and at C-6 with a methoxy group similar to some benzoquinones found in *Ardisia cornudentata* (Tian et al. 1987), except for differences in the length of their side chains. The presence of a hexanoyl ester in the side chain of **1** was established from a base ion peak of *m/z* 115.0765 ($C_6H_{11}O_2^+$, calcd 115.0759) in the negative-mode HRESI mass spectrum. Therefore, based on its molecular formula, the oxyalkyl side chain of **1** should compose of seven carbons and be esterified with hexanoic acid at position 2′. The experimental ECD spectrum of **1** was similar with that of calculated (2′*S*)-1, indicating *S* configuration of C-2′ (Figure S11). Consequently, the structure of compound **1** was determined as 2-[2*S*-hexanoyloxyheptyl]-6-methoxy-1,4-benzoquinone, a new compound trivially named paphionone.

Compound **2** was obtained as a brown amorphous powder from the roots of *P. exul*. The molecular formula was determined as $C_{16}H_{16}O_4$, consistent with nine degrees of unsaturation, from its $[M+H]^+$ ion peak at *m/z* 273.1132 (calcd 273.1126) in the HRESI mass spectrum. Its IR spectrum showed a hydroxy absorption band at 3338 cm^{-1}. The ^1H NMR spectrum of **2** (Table S2 and Figure S12) showed signals of a *trans*-double bond at δ_H 7.12 (1H, d, $J=16.8$ Hz, H-β) and δ_H 7.25 (1H, d, $J=16.8$ Hz, H-α), a set of *ortho*-coupled aromatic protons [δ_H 6.50 (1H, d, $J=8.2$ Hz, H-3), δ_H 6.54 (1H, t, $J=8.2$ Hz, H-5) and δ_H 7.01 (1H, d, $J=8.2$ Hz, H-4)], another set of *meta*-coupled aromatic protons [δ_H 6.34 (1H, t, $J=2.2$ Hz, H-4′), δ_H 6.62 (1H, t, $J=2.2$ Hz, H-6′) and δ_H 6.64 (1H, t, $J=2.2$ Hz, H-2′)] and two methoxy groups at δ_H 3.81 (3H, s, 3′-OCH_3) and δ_H 3.85 (3H, s, 2-OCH_3), while its ^{13}C NMR (Table S2 and Figure S13) and HSQC (Figure S14) spectra displayed signals assigned to two methyls, eight methines and six quaternary carbons. These spectroscopic data and the molecular formula suggested that **2** was a *trans*-stilbene bearing two hydroxy and two methoxy groups. The patterns of aromatic proton signals indicated that one ring of this stilbene was 1,2,3-trisubstituted, whereas another ring was 1,3,5-trisubstituted. On the 1,2,3-trisubstituted phenyl ring, one methoxy group could be located at C-2 (δ_C 158.5) based on HMBC correlations (Figures S1 and S15) between H-α, H-4 and 2-OCH_3 signals with this carbon, which further suggested that a hydroxy group should be placed at C-6 (δ_C 154.2). Similarly, a methoxy and a hydroxy substituent could be assigned to C-3′ and C-5′, respectively, of the 1,3,5-trisubstituted aromatic ring of **2**, based on HMBC cross peaks observed from both H-2′ and 3′-OCH_3 signals to C-3′ (δ_C 161.1). Therefore, the structure of compound **2** was elucidated as a new *trans*-stilbenoid, (*E*)-6,5′-dihydroxy-2,3′-dimethoxystilbene.

Eight known stilbenoids and flavonoids were also isolated and identified as follows: pinosylvin monomethyl ether (**3**) (Ngo and Brown 1998), 5-hydroxy-3-methoxy-*cis*-stilbene (**4**) (Ngo and Brown 1998) and isokaempferide (**5**) (Gohari et al. 2003) from the leaves and galangin (**6**) (Bertelli et al. 2012), pinocembrin (**7**) (Neacsu et al. 2007), (*E*)-5-hydroxy-3,2′-dimethoxystilbene (**8**) (Lertnitikul et al. 2016), alpinetin (**9**) (Itokawa et al. 1981) and (*E*)-5′-hydroxy-2′-(4-hydroxybenzyl)-3′-methoxystilbene (**10**) (Garo et al. 2007) from the roots of *P. exul*. They were identified by comparison of their 1 D and 2 D NMR data with reported values. The isolated compounds were assayed for their *in vitro* cytotoxic effect against human hepatocellular carcinoma (HepG2) cell line and a normal human embryo fibroblast cell line (OUMS-36) by the MTT method (Twentyman and Luscombe 1987), with cisplatin as the positive control. All of them, except the benzoquinone **1**, showed varying degrees of cytotoxicity against HepG2 cell line, but were not cytotoxic to human fibroblast cell line (Table S3). The *trans*-stilbene **10**, which has an additional *para*-hydroxybenzyl moiety, was strongly cytotoxic to the cancer cells with an IC_{50} value of 4.80 ± 1.10 μM and selectivity index of 20.83. Although its chemical structure is similar to **3**, the presence of a *para*-hydroxybenzyl substituent appears to increase its cytotoxic effect against HepG2 cells more than 10 folds. Recently, potent cytotoxicity of similarly substituted *trans*-stilbenes from *Paphiopedilum dianthum* towards a number of cancer cell lines has also been observed (Lertnitikul et al. 2023), and another *para*-hydroxybenzyl substituted stilbene previously obtained from *P. exul* was shown to induce apoptosis and autophagy of non-small cell lung cancer cells (Tungsukruthai et al. 2021). In addition, the new *trans*-stilbene **2** and two flavonoids with unsubstituted B-ring (**6** and **7**) were moderately cytotoxic to HepG2 cells. However, replacing the 5-hydroxy substituent of flavanone **7** with a methoxy group, as in **9**, considerably decreased its cytotoxicity towards the tested cancer cells.

3. Experimental

3.1. General experimental procedures

UV spectra were measured on a Shimadzu UV-160A spectrophotometer (Shimadzu Corp., Kyoto, Japan) in MeOH. IR spectra were obtained on a Perkin-Elmer Spectrum One FTIR spectrometer (Perkin-Elmer, Inc., Waltham, MA, USA). HRESIMS spectra were recorded on a Bruker Daltonics microTOF mass spectrometer (Bruker Corp., Billerica, MA, USA). NMR spectra were recorded in $CDCl_3$ on a Varian Unity INOVA-500 (Varian, Inc., Palo Alto, CA, USA) or a Bruker Avance NEO 400 MHz NMR spectrometer, and the chemical shifts were referenced relative to the residual $CDCl_3$ signal. Column chromatography (CC) was conducted using silica gel (40–63 µm and 63–200 µm; Merck, KGaA, Darmstadt, Germany) and Sephadex LH-20 (Pharmacia Biotech AB, Uppsala, Sweden). TLC was performed on precoated silica gel 60 F_{254} aluminium plates (Merck). TLC spots were detected under UV light (254 or 365 nm), then sprayed with 10% sulphuric acid in 95% ethanol and heated.

3.2. Plant material

The fresh whole plants of *P. exul* (Ridl.) Rolfe were purchased from Chatuchak market, Bangkok, in January 2015, and identified by comparison with authentic specimen (QBG No. 13143) at the herbarium of the Botanical Garden Organisation, Ministry of Natural Resources and Environment, Thailand. A voucher specimen (RS15011) has been deposited at the herbarium of the Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok, Thailand. The leaves and roots were separated from the plants, washed with water and then dried in hot-air oven at 50 °C.

3.3. Extraction and isolation

The dried, powdered *P. exul* leaves (28 g) were macerated in MeOH (1 $L \times 3$, 3 days each). The crude MeOH extract was concentrated under reduced pressure, then mixed with distilled water and partitioned with EtOAc. The EtOAc extract (19 g) was separated by silica gel CC, eluted with gradient mixtures of CH_2Cl_2-EtOAc (1:0→4:1), into fractions A-H. Fraction B (0.52 g) was further separated by Sephadex LH-20 CC, washed down with CH_2Cl_2-MeOH (1:1), into subfractions B1–B3. Silica gel CC of subfraction B2 (0.27 g), eluted with CH_2Cl_2, gave subfractions B21-B24. Subfraction B22 (85 mg) was chromatographed on a silica gel column, using gradient mixtures of *n*-hexane-acetone (4:1→3:2) as eluents, to yield subfractions B221-B223. Repeated silica gel CC of subfraction B222 (23 mg), eluted with *n*-hexane-acetone (4:1), afforded compound **1** (5.5 mg). Subfraction B3 (0.12 g) was separated on a silica gel column, eluted with CH_2Cl_2, into subfractions B31-B33. Purification of subfraction B32 (76 mg) on another silica gel column, washed down with *n*-hexane-EtOAc (4:1), yielded compounds **3** (23.4 mg) and **4** (9.3 mg), respectively. Fraction F (0.21 g) was chromatographed over a silica gel column, eluted with gradient mixtures of CH_2Cl_2-acetone (20:1→5:1), to give subfractions F1-F3. Sephadex LH-20 CC of subfraction F2 (0.15 g), eluted with CH_2Cl_2-MeOH (1:1), afforded compound **5** (5.7 mg).

The dried roots of *P. exul* (380 g) were macerated in MeOH (3 *L* × 3, 3 days each). Removal of the solvent under reduced pressure gave crude MeOH extract (100 g). A portion (50.0 g) of this extract was separated by silica gel CC, eluted with *n*-hexane-acetone (3:1), into fractions A-H. Fraction C (2.9 g) was subjected to silica gel CC, eluted with CH_2Cl_2-acetone (40:1), to give subfractions C1-C6. Subfractions C6 and C5 yielded compounds **6** (0.1 g) and **7** (90.7 mg), respectively. Subfraction C2 (0.9 g) was separated on a Sephadex LH-20 column, washed down with CH_2Cl_2-MeOH (1:1), into subfractions C21-C22. Repeated silica gel CC of subfraction C22 (0.65 g), eluted with CH_2Cl_2, gave subfractions C221-C224. Subfraction C221 was further separated on a silica gel column, eluted with *n*-hexane-CH_2Cl_2 (1:1), into subfractions C2211-C2215. Purification of subfractions C2213-C2215 on silica gel columns, eluted with either *n*-hexane-CH_2Cl_2 (1:1) or CH_2Cl_2, afforded compound **8** (in total, 79.8 mg). Separation of fraction E (9.9 g) by silica gel CC, eluted with *n*-hexane-acetone (2:1), gave subfractions E1-E6. Subfraction E3 (3.75 g) was chromatographed twice on silica gel columns, washed down with CH_2Cl_2-acetone (30:1), to give subfractions E31-E33. Silica gel CC of subfractions E32 and E33, eluted with CH_2Cl_2-acetone (30:1), afforded compounds **10** (15.3 mg) and **2** (43.6 mg), respectively. Sephadex LH-20 CC of subfraction E5 (0.8 g), using MeOH as the eluent, gave subfractions E51-E54. Repeated silica gel CC of subfraction E53, eluted with CH_2Cl_2-acetone (20:1) and then CH_2Cl_2-EtOAc (9:1), yielded compound **9** (1.1 mg).

3.3.1. Paphionone (1)

Orange amorphous powder; $[\alpha]_D^{20}$ +166 (*c* 0.01, MeOH); ECD (*c* 0.01, MeOH) λ_{max} ($\Delta\varepsilon$) 221 (+1.0), 298 (–0.5), 342 (+0.2) nm; UV (MeOH) λ_{max} (log ε) 265 (4.32) nm; IR (KBr) ν_{max} 2929, 1727, 1647, 1633, 1604, 1230, 1032, 455 cm^{-1}; ^1H and ^{13}C NMR data, see Table S1; HRESIMS *m/z* 373.1993 $[M+Na]^+$ (calcd for $C_{20}H_{30}O_5Na^+$, 373.1991).

3.3.2. (E)-6,5′-dihydroxy-2,3′-dimethoxystilbene (2)

Brown amorphous powder; UV (MeOH) λ_{max} (log ε) 308 (4.81) nm; IR (KBr) ν_{max} 3338, 2935, 1468, 1341, 1149, 1079, 778 cm^{-1}; ^1H and ^{13}C NMR data, see Table S2; HRESIMS *m/z* 273.1132 $[M+H]^+$ (calcd for $C_{16}H_{17}O_4^+$, 273.1126).

3.4. Computational detail

In this research, computational methodologies were employed to optimise the conformers of paphionone (**1**). Specifically, Density Functional Theory (DFT) calculations at the B3LYP/6-31g(d,p) level were utilised for this purpose. Subsequently, the Electron Circular Dichroism (ECD) spectra were computed *via* time-dependent DFT (TD-DFT) at the B3LYP/6-31 + G(d,p) level, incorporating solvation effects modelled with the Continuum Model (PCM) employing methanol. All computational analyses were executed using the Gaussian16 software (Frisch et al. 2016). Furthermore, the ECD spectra were simulated through the application of overlapping Gaussian functions, parameterised with a fitting parameter (σ = 0.25 eV) using the SpecDis1.64 program (Bruhn et al. 2013), employing the length gauge representation for enhanced reliability.

3.5. Cell culture

Human hepatocellular carcinoma cell line (HepG2; ATCC® HB-8065™) was purchased from American Type Culture Collection (Rockville, MD, USA) and normal human embryo fibroblast cell line (OUMS-36) was obtained from Japanese Collection of Research Bioresources Cell Bank (Tokyo, Japan). HepG2 cells were grown in Eagle's Minimum Essential Medium (EMEM) supplemented with 10% Foetal Bovine Serum (FBS), 1% non-essential amino acids, 1% penicillin-streptomycin and 1% pyruvate (Burgess and Marcel 2001). OUMS-36 cells were cultured in Dulbecco's Modified Eagle's Medium/Ham's Nutrient Mixture F-12 (DMEM-F-12) with 10% FBS and 1% penicillin-streptomycin (Powthong et al. 2022). The cell lines were incubated at 37 °C in a humidified atmosphere containing 5% CO_2. They were sub-cultured every 3 days and maintained at 70% confluence.

3.6. Cytotoxicity assay

Cells were seeded in 96-well microplates at a density of 5×10^3 cells/well and cultured for 24 h, then treated with the isolated compounds (at 0–100 μM) or cisplatin (positive control) for 72 h. Then, the cells were washed and incubated in serum-free medium containing MTT reagent (0.05 mg/ml) at 37 °C for 4 h to allow the formation of formazan crystals. These crystals were dissolved with DMSO and the optical density (OD) was measured at 570 nm, using a Fisher Scientific Multiskan FC microplate photometer. Cell viability was calculated according to formula: % cell survival = [OD test/OD control] × 100. Half maximal inhibitory concentration (IC_{50}) values were estimated from linear regression analysis of concentration-response curve. Each experiment was done in triplicate and repeated twice. The results were expressed as mean ± standard deviation (SD).

4. Conclusion

A new alkyl benzoquinone, paphionone (**1**), a new stilbene, (*E*)-6,5′-dihydroxy-2,3′-dimethoxystilbene (**2**), and eight known flavonoids and stilbenes (**3**-**10**) were isolated from the leaves and roots of *P. exul* (Orchidaceae). Compound **10** was strongly cytotoxic, whereas compounds **2**, **6**, **7** were moderately cytotoxic to hepatic cancer (HepG2) cell line. None of the isolated compounds were toxic to normal human fibroblast (OUMS-36) cell line. Similar to previous reports, the *para*-hydroxybenzyl substituted stilbene **10** exhibited potent cytotoxic activity against the tested cancer cells, and the significance of this structural feature on the anticancer effect of *trans*-stilbenoids should be further studied.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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