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Synthesis and Evaluation of Novel Anticancer Compounds Derived from the Natural Product Brevilin A

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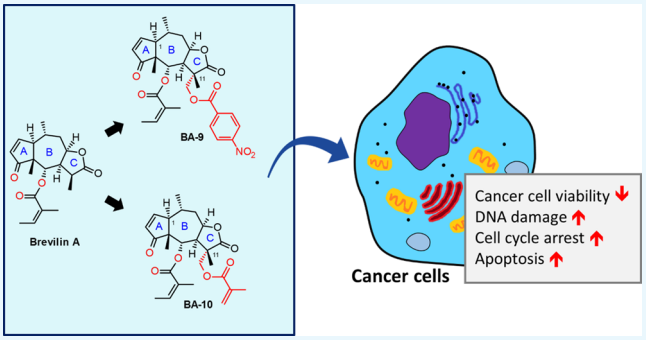
ABSTRACT: Cancer is the second leading cause of death globally, responsible for an estimated 9.6 million deaths in 2018, and this burden continues to increase. Therefore, there is a clear and urgent need for novel drugs with increased efficacy for the treatment of different cancers. Previous research has demonstrated that brevilin A (**BA**) exerts anticancer activity in various cancers, including human multiple myeloma, breast cancer, lung cancer, and colon carcinoma, suggesting the anticancer potential present in the chemical scaffold of **BA**. Here, we designed and synthesized a small library of 12 novel BA derivatives and evaluated the biological anticancer effects of the compounds in various cancer cell lines. The results of this structure−activity relationship study demonstrated that BA derivatives **BA-9** and **BA-10** possessed significantly improved anticancer activity toward lung, colon, and breast cancer cell lines. **BA-9** and **BA-10** could more effectively reduce cancer cell viability and induce DNA damage, cell-cycle arrest, and apoptosis when compared with **BA**. Our findings represent a significant step forward in the development of novel anticancer entities.



INTRODUCTION

According to the World Health Organization, cancer ranks second in global causes of death, with an estimated 9.6 million deaths attributed to this group of diseases in 2018. In males and females combined, lung cancer is the cancer with the highest rate of incidence (11.6% of total cases), followed by female breast cancer (11.6%), and colorectal cancer (10.2%). These three cancers also rank among the top five in terms of cancer mortality (lung, 18.4%; breast, 6.6%; colorectal, 9.2% of all cancer deaths). Due to circumstances including global population growth, an aging populace, and advances in social and economic development, cancer cases and deaths are on the rise, and cancer is set to soon overtake cardiovascular disease as the leading cause of death worldwide.[1]

Currently, the most commonly employed cancer treatments include surgery, radiotherapy, and chemotherapy, while targeted therapies and immunotherapies are receiving increasing attention. Accordingly, chemotherapeutic drugs, biologics, and immune-mediated therapies are major focuses in current efforts to treat cancer.[2] In the clinical situation, many cancer patients exhibit a poor response to conventional chemotherapy due to the development of resistance.[3] With the poor efficacy and considerable toxicity of many current chemotherapeutics, there is a clear and urgent need for novel, effective, and nontoxic (NT) drugs for the treatment of cancer.

Sesquiterpene lactones are natural bioactive compounds that are often used to treat cancer and inflammation in traditional Chinese medicine.[4,5] In recent years, researchers have conducted studies to investigate the anticancer mechanism of sesquiterpene lactones in various cancers.[4,6−8] Based on the structures of several sesquiterpene lactones with anticancer activity, a number of compounds with increased potency have been either isolated from natural products or partially synthesized. Several potential anticancer agents have emerged from this group of lead compounds and undergone further extensive investigation. Among these are brevilin A (**BA**), a sesquiterpene lactone that has been widely studied due to its potent bioactivities, including cancer cell cytotoxicity and antineoplastic efficacy in in vivo studies.[7,9−16] **BA** has been identified as one of the active compounds in *Centipeda minima*, a traditional Chinese herb that is frequently used for relieving stuffy nose, asthma, and cough.[17] Pharmacological studies have demonstrated that extracts from *C. minima* possess various bioactivities including antibacterial,[18] antiallergic,[19] antitu-

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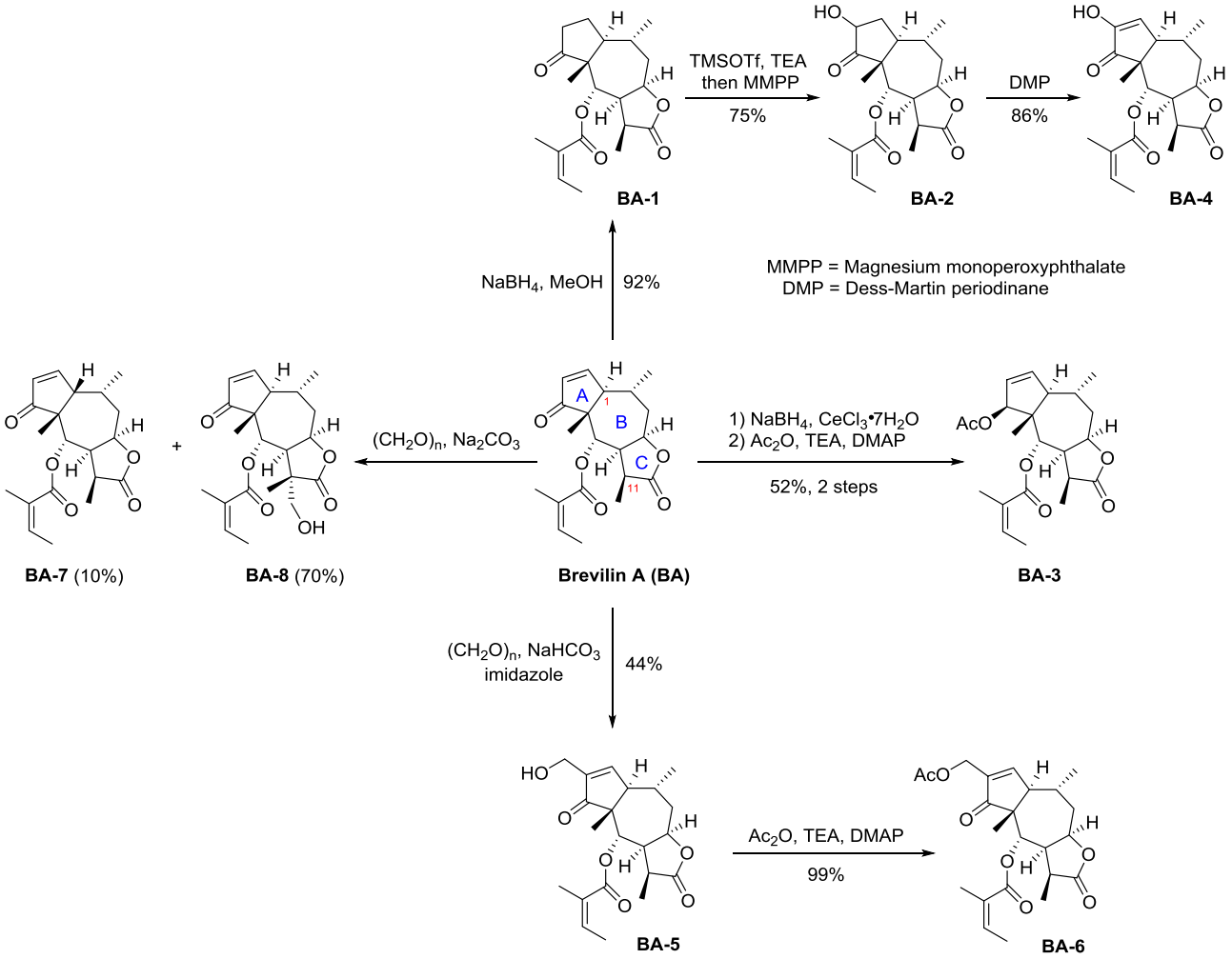
Scheme 1. Synthesis of BA-1−8



mor,[20] and anti-inflammatory effects.[21,22] Previous research revealed that **BA** could effectively reduce the growth of CT26 tumor cells via induction of apoptosis and promotion of autophagy through PI3K/AKT/mTOR signaling.[15] In addition, in HL60 cells, **BA** could induce an increase in reactive oxygen species (ROS), a decrease in mitochondrial membrane potential (MMP), activation of caspase 3/7, as well as induce cell apoptosis via inhibition of nuclear factor (NF)-κB activation.[16] Additional studies showed that **BA** could inhibit both STAT3 and JAK signaling[11] and could also cause the dissociation and degradation of Skp2 by binding to Skp1 in A549 lung cancer cells, resulting in G2/M phase arrest.[12] These findings support the development of **BA** as a novel anticancer drug and as a chemical scaffold or lead compound for the design and synthesis of new anticancer compounds.

The present study focuses on the anticancer potential of **BA** and its derivatives. Our study has demonstrated advances in the development of brevilin-type sesquiterpene lactones (**BA** and its derivatives **BA-9** and **BA-10**) as potential anticancer agents, including their structural characterization, synthesis and synthetic modification, and antitumor potential, with the mechanism of action and structure−activity relationships (SARs) also being investigated. Results from our study can stimulate further interest in developing sesquiterpene lactones and their derivatives as novel anticancer agents.

RESULTS AND DISCUSSION

BA analogues were prepared by direct derivatization of **BA**. As shown in Scheme 1, reduction of **BA** using sodium borohydride resulted in selective 1,4-reduction of the enone in the A ring and gave **BA-1** at a 92% yield. Subsequent α-hydroxylation of the ketone in the A ring using trimethylsilyl triflate and triethylamine followed by oxidation with magnesium monoperoxyphthalate (MMPP) provided **BA-2**, which was further oxidized to afford **BA-4** in good yields. Luche reduction of **BA** followed by acetylation gave **BA-3**. The Morita−Baylis−Hillman reaction of **BA** using imidazole and paraformaldehyde afforded **BA-5**, and acetylation of the newly installed C11-hydroxylmethyl provided **BA-6**. Aldol reaction between **BA** and paraformaldehyde in the presence of sodium carbonate provided **BA-8** at a 70% yield along with 10% of a side-product, which was identified as **BA-7** (C1-epimer of **BA**) after careful analysis of the NMR data. With **BA-8** prepared, the C11-hydroxylmethyl was acetylated with *p*-nitrobenzoyl chloride, methacrylic anhydride, acetic anhydride, and succinic anhydride to afford **BA-9−12**, respectively (Scheme 2).

Research has demonstrated that **BA** exerts anticancer effects in various cancer types, including human multiple myeloma, breast, lung, and colon.[7,9−16] To examine and compare the anticancer activities of **BA** and its derivatives, the cytotoxicities of the compounds were assessed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay in four

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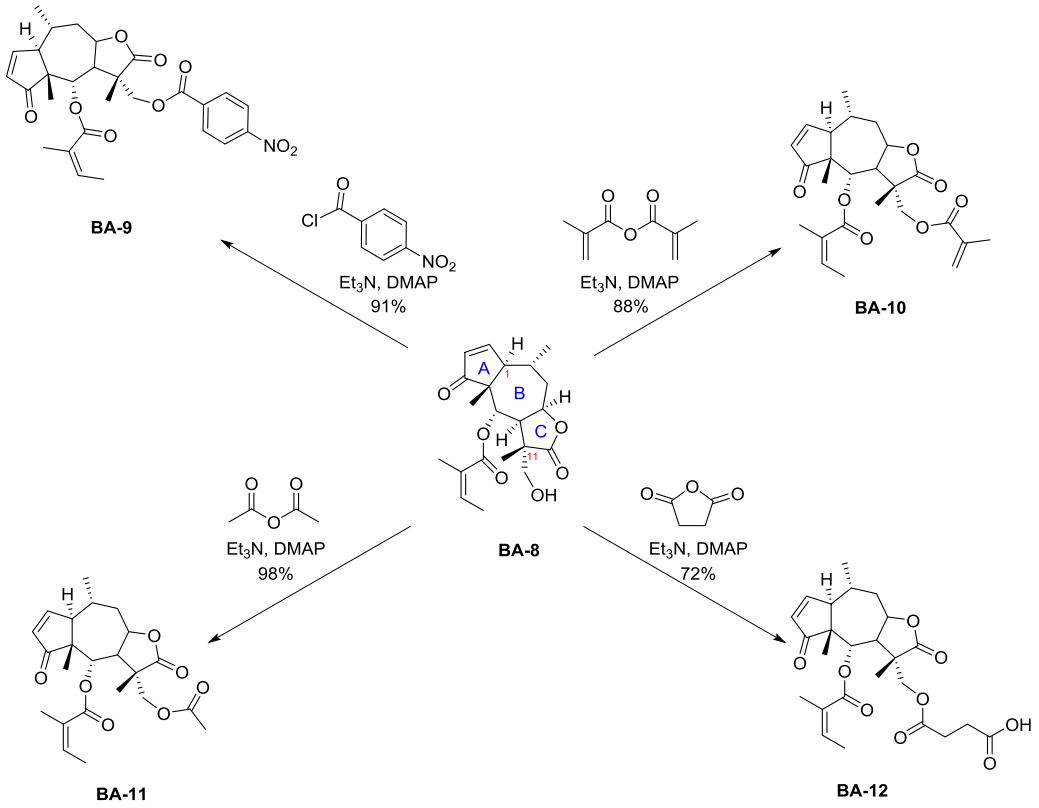
Scheme 2. Synthesis of BA-9−12



cancer cell lines, A549 (lung cancer), SW480 (colorectal cancer), MDA-MB-231 (triple negative breast cancer), and MCF-7 (estrogen receptor-positive breast cancer). Table 1 and Figure S1 show the half-maximal inhibitory concentration (IC_{50}) values and dose−response curves of **BA** and its derivatives.

In the structure−activity relationship (SAR) study, the effects of modification of the A and C rings were examined by two series of BA derivatives. These BA analogues were prepared by direct derivatization of the isolated natural **BA**. BA analogue series 1 contains derivatives with A ring modifications (Figure 1). When the alkene or carbonyl of the enone in the A ring was reduced (**BA-1−3**), the in vitro cytotoxicity was lost completely. Introducing a hydroxyl or hydroxylmethyl to the α-position of the enone in the A ring (**BA-4** and **BA-5**) also led to complete loss of activity. Acetylated derivative **BA-6** exhibited poor-to-moderate cytotoxicity in different cancer cell lines. Moreover, the *cis*-AB ring junction derivative **BA-7** exhibited lower activity than **BA** in most of the tested cell lines. In general, the BA derivatives in series 1 did not exhibit improved cytotoxicity in the four cancer cell lines when compared to **BA**. These results indicated that the enone in the A ring is essential for cytotoxicity, and functional group modification of the A ring could generally lead to significant loss of anticancer activity.

Table 1. Effect of BA Compounds on Viability of a Panel of Cancer Cell Lines[a]

compound name	A549 (μM)	SW480 (μM)	MDA-MB-231 (μM)	MCF-7 (μM)
BA	10.086	13.631	7.033	12.500
BA-1	NT	NT	>50	>50
BA-2	NT	NT	NT	NT
BA-3	NT	NT	NT	NT
BA-4	NT	NT	NT	NT
BA-5	NT	NT	NT	NT
BA-6	>50	15.418	12.037	27.101
BA-7	22.899	>50	27.750	>50
BA-8	13.722	26.395	20.700	>50
BA-9	6.239	5.147	4.647	5.847
BA-10	6.392	8.566	6.385	6.352
BA-11	15.050	49.955	21.010	>50
BA-12	NT	NT	NT	NT

[a]A549, SW480, MDA-MB-231, and MCF-7 cells were treated with various doses of BA and its derivatives for 48 h. Cell viability was measured by the MTT assay. Results are expressed as IC_{50} values in micromolar. NT, nontoxic at the highest tested dose.

On the other hand, several derivatives in BA analogue series 2 (**BA-8−12**, prepared by introducing different substituents to the α-position of the γ-lactone D ring) exhibited a significantly enhanced cytotoxicity in the tested cancer cell lines. Most notably, in all tested cell lines, compounds **BA-9** and **BA-10** showed lower IC_{50} values than their parental compound, **BA**. In the MCF-7, SW480, and A549 cell lines, the IC_{50} values of **BA-9** and **BA-10** were almost half of that of **BA**: the IC_{50} values of **BA** in MDA-MB-231, MCF-7, SW480, and A549 were 7.033, 12.5, 13.631, and 10.086 μM, respectively, while the IC_{50} values of **BA-9** and **BA-10** were, respectively, 4.647 and 6.385 μM in MDA-MB-231, 5.847 and 6.352 μM in MCF-7, 5.147 and 8.566 μM in SW480, and 6.239 and 6.392 μM in A549. These results indicated that **BA-9** and **BA-10** exhibited potential anticancer efficacy, which, importantly, may be improved compared to **BA**.

To further confirm the anticancer efficacies of **BA-9** and **BA-10**, we employed the clonogenic assay to determine the effect of the derivatives on the survival and proliferation of cancer cells. As demonstrated in our results (Figure 2), **BA-9** and **BA-10**

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ACS Omega 2020, 5, 14586−14596

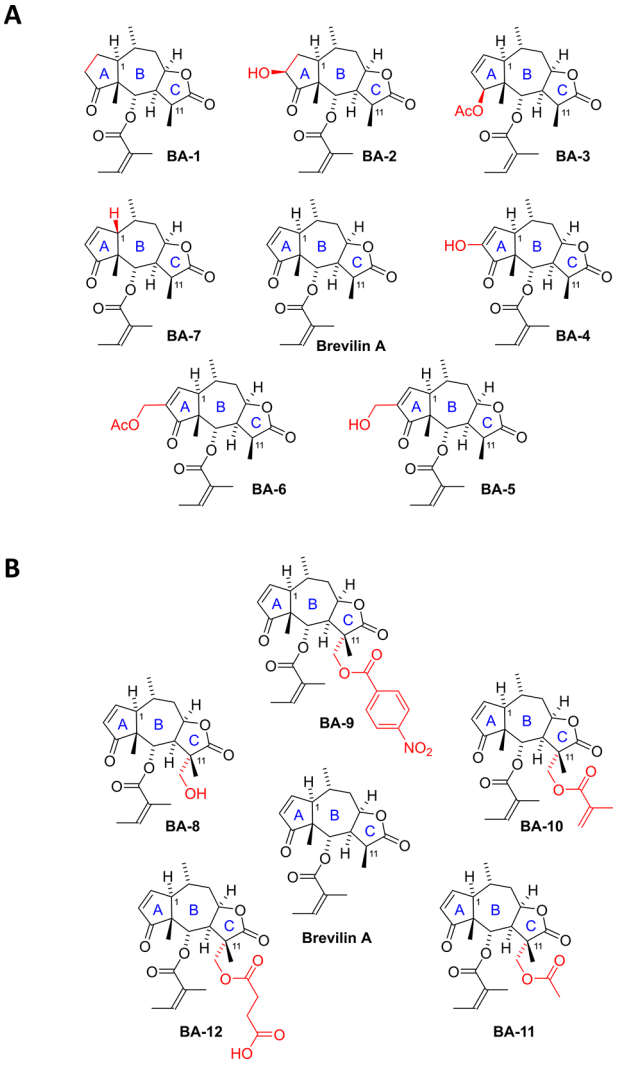

Figure 1. BA analogue series. (A) Series 1: derivatives with A ring modifications; (B) series 2: derivatives with C ring modifications.

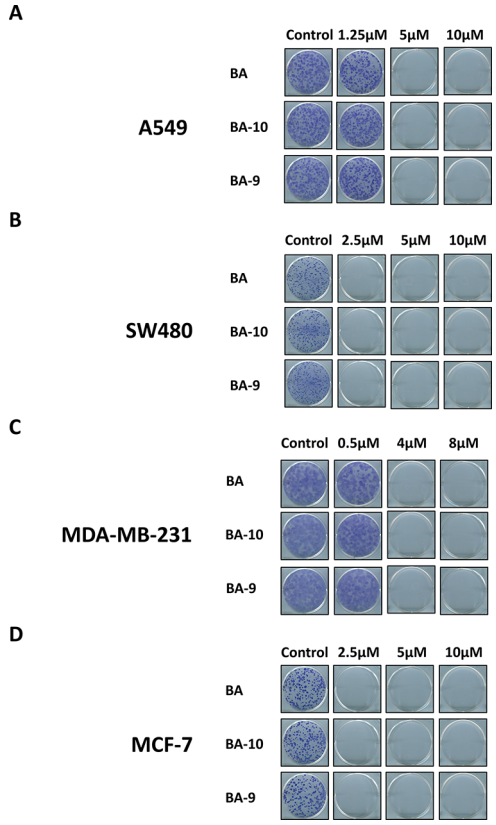

Figure 2. Effect of BA compounds on colony formation in a panel of cancer cell lines. (A) A549, (B) SW480, (C) MDA-MB-231, and (D) MCF-7 cells were treated with the indicated doses of **BA**, **BA-10**, and **BA-9** for 14 days. At the end of the experiment, cells were fixed with 4% paraformaldehyde for 4 h and stained with 2% Giemsa blue solution overnight. Stained cells were briefly rinsed in Milli-Q water and then imaged.

could efficiently suppress cancer cell growth in all tested cancer cell lines, with results comparable to **BA**. Derivatives **BA-9** and **BA-10** could completely inhibit cancer cell colony formation from doses as low as 5 μM in A549, 2.5 μM in SW480, 4 μM in MDA-MB-231, and 2.5 μM in MCF-7. These results further confirmed the anticancer effects of **BA-9** and **BA-10**.

As previous studies have reported that **BA** could induce cell-cycle arrest and apoptosis in cancer,[12,15,16,23] we next investigated whether **BA-9** or **BA-10** could exhibit improved abilities for cell-cycle arrest or induction of apoptosis. A549, SW480, MDA-MB-231, and MCF-7 cells were treated with various concentrations of **BA**, **BA-9**, or **BA-10** and then assessed via propidium iodide (PI)/RNase staining for cell-cycle analysis and annexin V−phycoerythrin (PE)/7-aminoactinomycin D (7-AAD) staining for apoptosis analysis. As shown in Figure 3, **BA** could induce G2/M arrest in SW480, MDA-MB-231, and MCF-7 in a dose-dependent manner. Interestingly, **BA-9** and **BA-10** not only induced G2/M arrest but did so with a greater effect when compared to their parental compound (**BA**). **BA-10** showed the most potent induction of cell-cycle arrest in all tested cancer cell lines.

We also examined in depth the effect of the compounds on induction of apoptosis. Our results showed that **BA**, **BA-9**, and **BA-10** could all significantly and dose-dependently increase the proportion of apoptotic (annexin V-positive) cells in the treated cancer cell lines (Figure 4). Notably, **BA-9** and **BA-10** demonstrated much greater induction of apoptosis than their parental compound, **BA**. Of the three compounds, **BA-9** was the most potent in terms of induction of apoptotic cell death, with the proportion of apoptotic cells reaching 58.0, 37.9, 43.7, and 31.1% in A549, SW480, MDA-MB-231, and MCF-7 cell lines, respectively.

Studies show that unrepaired DNA damage can be toxic and promote cell elimination pathways such as apoptotic death, thus functioning as a tumor-suppressive pathway.[24,25] Therefore, we conducted Western blot analysis to investigate protein expressions of the major apoptotic markers cleaved poly(ADP-ribose) polymerase (PARP) and cleaved caspase 3 and an important DNA damage marker, pH2A.X. H2AX is very sensitive to DNA damage and is involved in its detection and the early cellular response by undergoing phosphorylation and recruiting DNA repair proteins to the site of damage. As shown in Figure 5, **BA**, **BA-9**, and **BA-10** could upregulate the expression of cleaved caspase 3 and cleaved PARP while downregulating their total form in lung, colon, and breast cancer cell lines. Importantly, **BA-9** and **BA-10** greatly and dose-dependently enhanced the expression of pH2A.X in the tested cancer cell lines when compared to **BA**, indicating that **BA-9** and **BA-10** were more effective in induction of DNA damage and cancer cell death.

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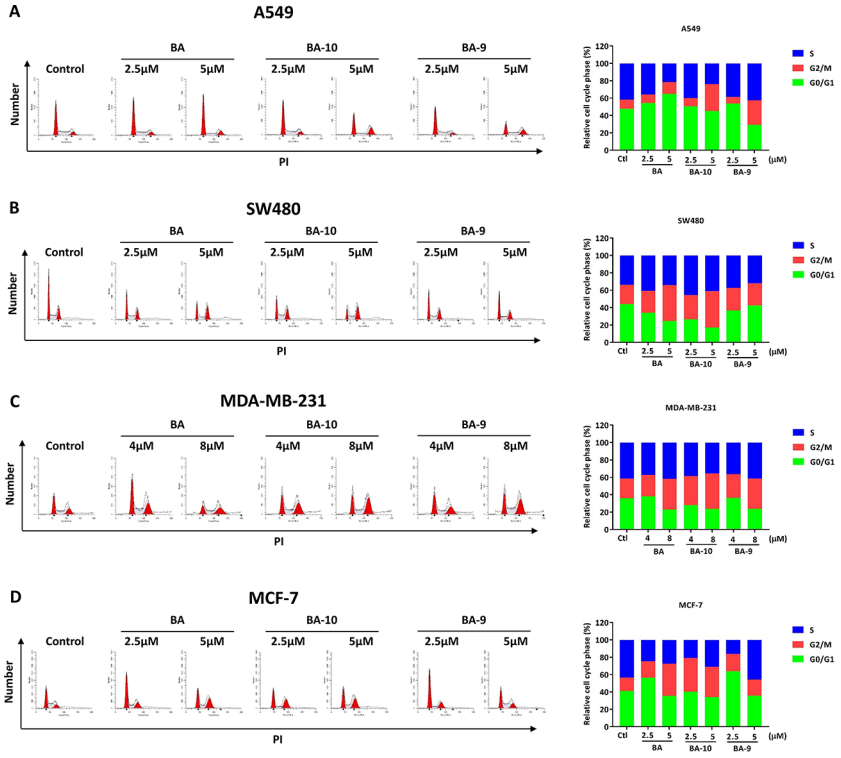

Figure 3. Effect of BA compounds on cell-cycle arrest in a panel of cancer cell lines. (A) A549, (B) SW480, (C) MDA-MB-231, and (D) MCF-7 cells were treated with the indicated doses of **BA**, **BA-10**, and **BA-9** for 24 h.

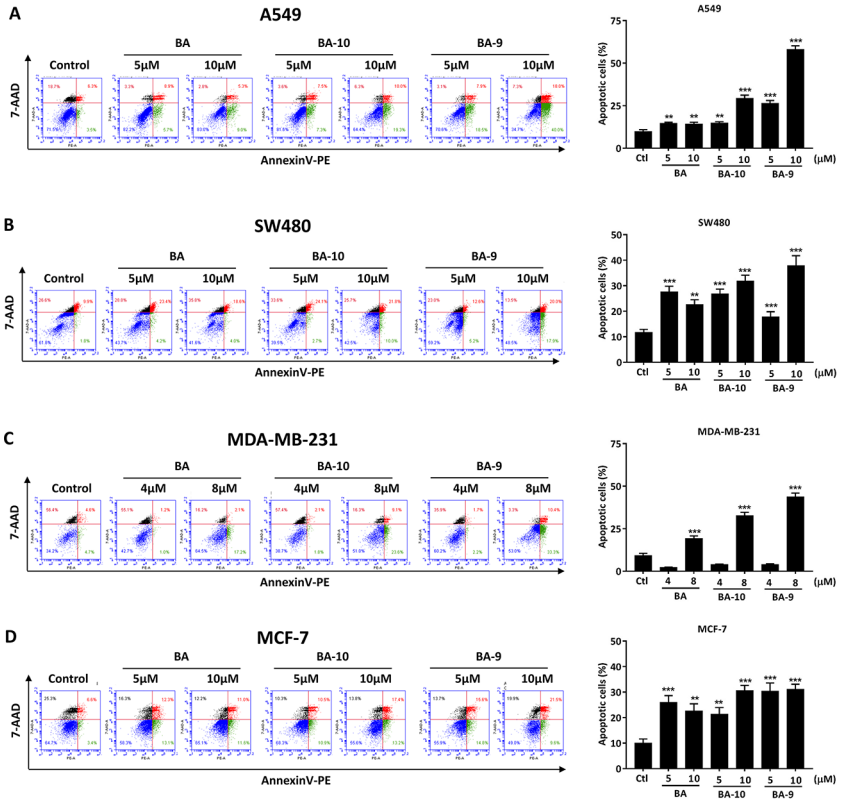

Figure 4. Effect of BA compounds on apoptosis induction in a panel of cancer cell lines. (A) A549, (B) SW480, (C) MDA-MB-231, and (D) MCF-7 cells were treated with the indicated concentrations of **BA**, **BA-10**, or **BA-9** for 48 h and subjected to the Annexin V−PE/7-AAD staining assay. $^{**}P < 0.01$, $^{***}P < 0.001$ compared to the control.

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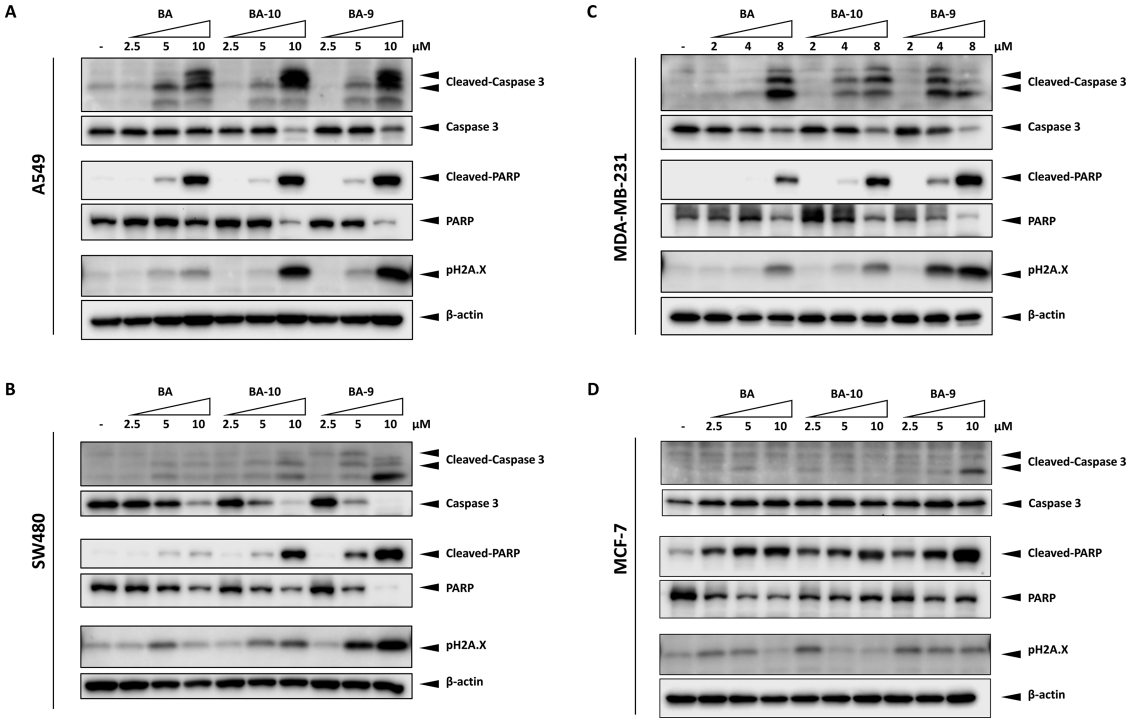

Figure 5. Effect of BA compounds on apoptosis induction and DNA damage in a panel of cancer cell lines. (A) A549, (B) SW480, (C) MDA-MB-231, and (D) MCF-7 cells were treated with the indicated concentrations of **BA**, **BA-10**, or **BA-9** for 48 h. The protein levels of apoptotic markers, cleaved caspase 3, caspase 3, cleaved PARP, and PARP and DNA damage marker pH2A.X were detected by Western blotting. β-Actin was used as an internal loading control. Representative immunoblots are shown.

CONCLUSIONS

In this study, we reported the design, synthesis, and biological evaluation of **BA** and 12 derivatives. In in vitro analyses, we examined the anticancer activity of the compounds in a panel of cancer cell lines. Notably, we identified two BA derivatives, **BA-9** and **BA-10**, that exerted greater anticancer activity than their parental compound, **BA**. We further demonstrated that these two derivatives could significantly and dose-dependently reduce cell proliferation and induce apoptosis and DNA damage in different cancer types and cell lines.

Altogether, we present strong evidence of the potent anticancer activities of **BA-9** and **BA-10**. Based on our findings, **BA-9** and **BA-10** are promising candidates for further development as therapeutics with high efficacy against multiple cancer types.

EXPERIMENTAL SECTION

Isolation of Brevilin A. The isolation of **BA** has been reported in our previous studies.[26] In brief, the ethanolic extract of *C. minima* (CME) was resolved in water and then partitioned with ethyl acetate. The ethyl acetate extract was subjected to column chromatography on silica gel with chloroform–methanol (1:0, 50:1, 20:1, 10:1, 0:1, v/v) for gradient elution to obtain five fractions A–E. Fraction G was separated by continuous column chromatography using silica gel, pre-high-performance liquid chromatography (pre-HPLC), and Sephadex LH-20, sequentially.

General Information for Synthesis of New Compounds. All air- and water-sensitive reactions were carried out under a nitrogen atmosphere with dry solvents under anhydrous conditions, unless otherwise noted. Reactions were monitored by thin-layer chromatography (TLC) carried out on 0.25 mm silica gel plates (60F-254) that were analyzed by UV irradiation (254 nm) and by staining with either $KMnO_4$ (200 mL of H_2O, 1.5 g of $KMnO_4$, 10 g of K_2CO_3, and 1.25 mL of 10% aqueous NaOH) or anisaldehyde (450 mL of 95% EtOH, 25 mL of conc. H_2SO_4, 15 mL of acetic acid, and 25 mL of anisaldehyde). Silica gel (60, particle size 0.040–0.063 mm) was used for flash column chromatography. All chemicals were purchased commercially and used without further purification. Tetrahydrofuran (THF) was distilled from sodium and benzophenone, and CH_2Cl_2 was distilled from calcium hydride. Yields refer to the isolated yields after silica gel flash column chromatography, unless otherwise stated. NMR spectra were recorded on a 300 MHz (^{1}H, 300 MHz; ^{13}C, 75 MHz), 400 MHz (^{1}H, 400 MHz; ^{13}C, 100 MHz), or 500 MHz (^{1}H, 500 MHz; ^{13}C, 125 MHz) spectrometer. The following abbreviations were used to explain the multiplicities: s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; and br, broad. High-resolution mass spectra (HRMS) were obtained from a matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometer. Melting points were uncorrected and determined on a micromelting point meter. Purity was assessed via HPLC analysis, and all derivatives were shown to have a purity ≥95% (see the Supporting Information).

*Synthesis of **BA-1**.* To a stirred solution of brevilin A (2.2 g, 6.4 mmol) in methanol (300 mL) was added $NaBH_4$ (270 mg, 6.9 mmol) in three portions at 0 °C. After stirring at 0 °C for 2 h, the reaction was quenched by addition of saturated $NaHCO_3$ aqueous solution (30 mL). The aqueous phase was concentrated under reduced pressure and extracted with ethyl acetate (200 mL × 3). The combined organic extracts were washed with brine, dried over $MgSO_4$, filtered, and concentrated. Silica gel flash column chromatography (hexane/ethyl acetate = 8:1) of the residue gave a white solid powder (2.02 g, 5.89 mmol, 92%)

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as the product. **BA-1**: ^{1}H NMR (500 MHz, $CDCl_3$) δ 6.07 (dd, J = 7.5, 1.5 Hz, 1H), 5.46 (s, 1H), 4.75 (t, J = 6.0 Hz, 1H), 3.05−3.09 (m, 1H), 2.87 (dd, J = 10.0, 6.5 Hz, 1H), 2.46 (dd, J = 19.0, 10.5 Hz, 1H), 2.35 (dq, J = 15.0, 1.5 Hz, 1H), 2.24−2.26 (m, 1H), 2.05−2.15 (m, 4H), 1.99 (dd, J = 7.5, 1.5 Hz, 3H), 1.81 (s, 3H), 1.70 (dd, J = 14.5, 11.5 Hz, 1H), 1.56−1.57 (m, 1H), 1.54 (d, J = 14.5 Hz, 3H), 1.07 (d, J = 7.0 Hz, 3H), 0.88 (s, 3H); ^{13}C{^{1}H} NMR (125 MHz, $CDCl_3$) δ 217.56, 179.20, 166.19, 139.23, 127.25, 79.55, 72.47, 53.74, 48.35, 47.92, 40.20, 38.89, 35.26, 27.14, 22.46, 20.69, 20.39, 15.81, 12.09, 10.54; HRMS (electrospray ionization (ESI)/[M + H]$^+$) calcd for $C_{20}H_{29}O_5$: 349.2015, found 349.2018.

Synthesis of ***BA-2***. To a stirred solution of **BA-1** (400 mg, 1.15 mmol) and triethylamine (2.95 mL, 8.60 mmol) in dichloromethane (30 mL) was added TBSOTf (0.93 mL, 4 mmol) slowly at 0 °C. After stirring at room temperature for 3 h, the reaction was quenched by addition of water (15 mL) and the aqueous phase was extracted with dichloromethane (15 mL × 3). The combined organic extracts were washed with brine, dried over $MgSO_4$, filtered, and concentrated. Silica gel flash column chromatography (hexane/ethyl acetate = 30:1) of the residue gave a colorless oil as the crude product. To a stirred solution of the above crude product in dichloromethane (10 mL) was added *meta*-chloroperoxybenzoic acid (*m*-CPBA) (297 mg, 1.29 mmol, 75%) slowly at 0 °C. The reaction was stirred at 0 °C until TLC showed completion of the reaction. The reaction was then quenched by addition of a saturated $NaHCO_3$ aqueous solution, and the aqueous phase was extracted with ethyl acetate (20 mL × 3). The combined organic extracts were washed with brine, dried over $MgSO_4$, filtered, and concentrated. Silica gel flash column chromatography (hexane/ethyl acetate = 30:1) of the residue gave a colorless oil (340 mg, 1 mmol, 83%) as the product. **BA-2**: ^{1}H NMR (500 MHz, $CDCl_3$) δ 6.07 (dddd, J = 8.8, 7.3, 5.7, 1.5 Hz, 1H), 5.44 (s, 1H), 4.72 (t, J = 6.4 Hz, 1H), 4.20 (d, J = 7.8 Hz, 1H), 3.03 (dt, J = 10.1, 7.4 Hz, 1H), 2.85 (dd, J = 10.2, 6.5 Hz, 1H), 2.51 (ddd, J = 12.8, 11.0, 7.0 Hz, 1H), 2.46 (s, 1H), 2.32 (ddd, J = 15.2, 6.6, 1.9 Hz, 1H), 2.13−2.02 (m, 1H), 2.02−1.99 (m, 1H), 1.98 (ddd, J = 4.6, 3.5, 1.5 Hz, 3H), 1.87 (ddd, J = 14.1, 12.8, 7.9 Hz, 2H), 1.79 (p, J = 1.6 Hz, 3H), 1.69 (ddd, J = 15.3, 11.5, 1.3 Hz, 1H), 1.47 (d, J = 7.4 Hz, 3H), 1.03 (d, J = 6.6 Hz, 3H), 0.90 (s, 3H). ^{13}C{^{1}H} NMR (125 MHz, $CDCl_3$) δ 216.1, 179.2, 166.2, 139.8, 127.2, 79.4, 71.4, 69.6, 53.9, 48.0, 45.3, 40.1, 38.6, 31.3, 27.1, 20.6, 20.2, 15.8, 12.3, 10.5. HRMS (ESI/[M + H]$^+$) calcd for $C_{20}H_{29}O_6$: 365.1959, found 365.1956.

Synthesis of ***BA-3***. To a stirred solution of brevilin A (2.20 g, 6.4 mmol) and $CeCl_3{\cdot}7H_2O$ (1.19 g, 3.2 mmol) in methanol (300 mL) was added $NaBH_4$ (270 mg, 6.9 mmol) in three portions at 0 °C. After stirring at 0 °C for 2 h, the reaction was quenched by addition of saturated $NaHCO_3$ aqueous solution (30 mL). The aqueous phase was concentrated under reduced pressure to remove methane and extracted with ethyl acetate (200 mL × 3). The combined organic extracts were washed with brine, dried over $MgSO_4$, filtered, and concentrated. To a stirred solution of the residue in dichloromethane (200 mL) and triethylamine (6.26 mL, 45 mmol) was added acetic anhydride (1.42 mL, 15 mmol) followed by 4-dimethylaminopyridine (DMAP) (83.5 mg, 0.69 mmol) at room temperature. The reaction was stirred at room temperature until TLC showed completion of the reaction. The reaction was then quenched by addition of a saturated NH_4Cl aqueous solution (50 mL), and the aqueous phase was extracted with ethyl acetate (200 mL × 3). The combined organic extracts were washed with brine, dried over $MgSO_4$, filtered, and concentrated. Silica gel flash column chromatography (hexane/ethyl acetate = 10:1) of the residue gave a white solid powder (1.30 g, 3.33 mmol, 52% for two steps) as the product. **BA-3**: ^{1}H NMR (500 MHz, $CDCl_3$) δ 6.19 (dd, J = 7.5, 1.5 Hz, 1H), 5.94 (d, J = 6.0 Hz, 1H), 5.66 (d, J = 3.5 Hz, 1H), 5.60−5.63 (m, 1H), 5.28 (s, 1H), 4.78 (dd, J = 7.0, 5.5 Hz, 1H), 3.05−3.09 (m, 1H), 2.97−3.00 (m, 1H), 2.77 (dd, J = 11.5, 1.5 Hz, 1H), 2.31−2.40 (m, 1H), 2.11−2.20 (m, 1H), 2.05−2.10 (m, 6H), 1.96 (s, 3H), 1.67−1.72 (m, 1H), 1.47 (d, J = 7.5 Hz, 3H), 1.14 (d, J = 7.0 Hz, 3H), 1.04 (s, 3H); ^{13}C{^{1}H} NMR (125 MHz, $CDCl_3$) δ 179.13, 170.78, 166.56, 139.51, 133.41, 128.80, 127.49, 81.85, 79.25, 72.36, 54.58, 54.34, 47.98, 40.03, 39.34, 26.37, 20.88, 20.83, 20.74, 15.90, 12.28, 10.67; HRMS (ESI/[M + H]$^+$) calcd for $C_{22}H_{31}O_6$: 391.2121, found 391.2130.

Synthesis of ***BA-4***. To a stirred solution of **BA-2** (340 mg, 1 mmol) in dichloromethane (20 mL) was added Dess−Martin periodinane (590 mg, 1.3 mmol) at 0 °C. After stirring at room temperature for 30 min, the mixture was filtered through a plug of silica gel and concentrated. Silica gel flash column chromatography (hexane/ethyl acetate = 4:1) of the residue gave a yellow oil (312 mg, 0.86 mmol, 86%) as the product. **BA-4**: ^{1}H NMR (500 MHz, pyridine-d_5) δ 6.69 (d, J = 2.5 Hz, 1H), 5.96−5.85 (m, 2H), 4.86 (td, J = 6.2, 2.0 Hz, 1H), 3.35 (dq, J = 10.2, 7.5 Hz, 1H), 3.18 (dd, J = 10.9, 2.5 Hz, 1H), 3.12 (dd, J = 10.3, 6.5 Hz, 1H), 2.40 (ddd, J = 15.3, 6.0, 2.5 Hz, 1H), 2.17−2.00 (m, 1H), 1.96 (dq, J = 7.3, 1.5 Hz, 3H), 1.77 (p, J = 1.6 Hz, 3H), 1.74−1.52 (m, 5H), 1.27 (s, 3H), 1.05 (d, J = 6.6 Hz, 3H); ^{13}C{^{1}H} NMR (125 MHz, pyridine-d_5) δ 206.9, 180.2, 167.5, 153.7, 139.6, 129.1, 128.9, 80.8, 73.6, 54.9, 50.4, 49.6, 42.2, 41.5, 27.7, 21.5, 20.7, 19.3, 16.7, 12.3; HRMS (ESI/[M + H]$^+$) calcd for $C_{20}H_{27}O_6$: 363.1802, found 363.1805.

Synthesis of ***BA-5***. To a stirred solution of brevilin A (2.0 g, 5.8 mmol) in THF (100 mL) and saturated $NaHCO_3$ aqueous solution (100 mL) were added imidazole (790 mg, 11.6 mmol) and formaldehyde (525 mg, 174 mmol) at room temperature. After stirring for 6 h, the reaction was quenched by addition of saturated NaCl (50 mL). The aqueous phase was extracted with ethyl acetate (250 mL × 3). The combined organic extracts were washed with brine, dried over $MgSO_4$, filtered, and concentrated. Silica gel flash column chromatography (hexane/ethyl acetate = 4:1) of the residue gave a white solid powder (959 mg, 2.55 mmol, 44%) as the product. **BA-5**: ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.51 (d, J = 1.0 Hz, 1H), 6.05−6.08 (m, 1H), 5.51 (s, 1H), 4.79 (t, J = 5.0 Hz, 1H), 4.34 (dd, J = 21.0, 14.5 Hz, 2H), 3.10−3.15 (m, 1H), 3.04 (dd, J = 11.0, 2.0 Hz, 1H), 2.94−2.97 (m, 1H), 2.48−2.52 (m, 2H), 2.20−2.23 (m, 1H), 1.91 (d, J = 7.5 Hz, 3H), 1.75 (s, 3H), 1.66−1.74 (m, 1H), 1.56 (d, J = 7.5 Hz, 3H), 1.26 (s, 3H); ^{13}C{^{1}H} NMR (125 MHz, $CDCl_3$) δ 208.91, 179.14, 166.37, 155.63, 140.37, 139.02, 127.25, 79.53, 71.86, 57.46, 55.75, 52.76, 48.90, 40.96, 40.46, 25.86, 20.46, 19.75, 17.57, 15.68, 10.97; HRMS (ESI/[M + Na]$^+$) calcd for $C_{21}H_{28}O_6Na$: 399.1784, found 399.1783.

Synthesis of ***BA-6***. To a stirred solution of **BA-5** (600 mg, 1.60 mmol) and triethylamine (2 mL, 15 mmol) in dichloromethane (20 mL) was added acetic anhydride (0.30 mL, 3.20 mmol) followed by DMAP (19.4 mg, 0.16 mmol) at room temperature. The reaction was stirred at room temperature until TLC showed completion of the reaction. The reaction was then quenched by addition of a saturated NH_4Cl aqueous solution (10 mL), and the aqueous phase was extracted with ethyl acetate (50 mL × 3). The combined organic extracts were washed with brine, dried over $MgSO_4$, filtered, and concentrated. Silica gel

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flash column chromatography (hexane/ethyl acetate = 10:1) of the residue gave a white solid powder (656 mg, 1.58 mmol, 99%) as the product. **BA-6**: ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.51 (s, 1H), 6.04 (qd, J = 8.4, 1.5 Hz, 1H), 5.49 (s, 1H), 4.70−4.78 (m, 2H), 2.90−3.18 (m, 3H), 2.49 (ddd, J = 15.3, 5.7, 2.1 Hz, 1H), 2.18−2.23 (m, 1H), 2.08 (s, 3H), 1.89 (dd, J = 7.2, 1.5 Hz, 3H), 1.72 (s, 3H), 1.55 (d, J = 7.5 Hz, 3H), 1.23 (d, J = 6.9 Hz, 3H), 1.04 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 207.01, 178.88, 170.42, 166.15, 157.81, 138.99, 136.20, 127.04, 79.26, 71.75, 57.68, 55.40, 52.68, 48.63, 40.78, 40.30, 25.61, 20.64, 20.31, 19.62, 17.28, 15.54, 10.82; HRMS (ESI/$[M + H]^+$) calcd for $C_{23}H_{31}O_7$: 419.2070, found 419.2071.

*Synthesis of **BA-7** and **BA-8**.* To a stirred solution of brevilin A (105 mg, 0.3 mmol) in THF (5 mL) and saturated Na_2CO_3 aqueous solution (5 mL) was added formaldehyde (10 mg, 0.33 mmol) at room temperature. The reaction was heated to 55 °C and stirring for 36 h. After consumption of the starting material, the reaction was quenched by addition of saturated NaCl (10 mL). The aqueous phase was extracted with ethyl acetate (10 mL × 3). The combined organic extracts were washed with brine, dried over $MgSO_4$, filtered, and concentrated. Silica gel flash column chromatography (hexane/ethyl acetate = 2:1) of the residue gave white solid powders **BA-7** (11 mg, 0.03 mmol, 10%) and **BA-8** (79 mg, 0.21 mmol, 70%). **BA-7**: ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.63 (dd, J = 6.0, 2.0 Hz, 1H), 6.11 (dd, J = 6.0, 2.8 Hz, 1H), 6.03 (m, 1H), 5.41 (s, 1H), 4.90 (ddd, J = 12.6, 6.4, 3.0 Hz, 1H), 2.90 (dt, J = 12.8, 2.3 Hz, 1H), 2.67 (dt, J = 13.6, 6.8 Hz, 1H), 2.56 (dd, J = 13.3, 6.4 Hz, 1H), 2.37 (ddd, J = 15.1, 12.6, 7.7 Hz, 1H), 2.02 (dt, J = 13.5, 6.9 Hz, 1H), 1.96−1.83 (m, 3H), 1.85−1.80 (m, 1H), 1.74−1.66 (m, 3H), 1.50 (d, J = 6.9 Hz, 3H), 1.34 (d, J = 6.8 Hz, 3H), 1.26 (d, J = 7.6 Hz, 4H); ^{13}C{^{1}H} NMR (125 MHz, $CDCl_3$) δ 209.1, 177.2, 165.8, 162.3, 139.0, 130.5, 127.3, 77.3, 77.0, 76.7, 76.2, 72.0, 54.7, 52.6, 51.8, 37.2, 35.9, 29.7, 26.7, 20.7, 20.3, 18.1, 15.6, 13.7; HRMS (ESI/$[M + H]^+$) calcd for $C_{20}H_{27}O_5$: 347.1853, found 347.1859. **BA-8**: ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.63 (dd, J = 5.5, 1.5 Hz, 1H), 6.09 (dd, J = 6.0, 3.0 Hz, 1H), 6.02−6.08 (m, 1H), 5.33 (s, 1H), 4.95−4.99 (m, 1H), 3.79 (s, 2H), 3.19 (s, 1H), 3.12 (d, J = 7.5 Hz, 1H), 2.91 (d, J = 12.0 Hz, 1H), 2.36−2.43 (m, 1H), 2.00−2.08 (m, 1H), 1.82−1.89 (m, 1H), 1.69 (s, 3H), 1.35 (s, 3H), 1.28 (d, J = 7.0 Hz, 3H), 1.24 (s, 3H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 209.69, 179.00, 167.04, 162.25, 140.29, 130.40, 126.98, 77.34, 73.43, 67.84, 54.57, 52.42, 49.68, 38.29, 26.40, 20.20, 20.06, 19.06, 15.78; HRMS (ESI/$[M + H]^+$) calcd for HRMS (ESI/$[M + H]^+$) calcd for $C_{21}H_{29}O_6$: 377.1959, found 377.1965.

*Synthesis of **BA-9**.* To a stirred solution of **BA-8** (600 mg, 1.60 mmol) and triethylamine (2 mL, 15 mmol) in dichloromethane (20 mL) was added *p*-nitrobenzoyl chloride (594 mg, 3.20 mmol) followed by DMAP (19.4 mg, 0.16 mmol) at room temperature. The reaction was stirred at room temperature for 2 days. After the reaction was completed, the solution was quenched by addition of a saturated NH_4Cl aqueous solution (10 mL) and the aqueous phase was extracted with ethyl acetate (50 mL × 3). The combined organic extracts were washed with brine, dried over $MgSO_4$, filtered, and concentrated. Silica gel flash column chromatography (hexane/ethyl acetate = 4:1) of the residue gave a faint-yellow amorphous solid (765 mg, 1.46 mmol, 91%) as the product. **BA-9**: ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.27 (d, J = 9.0 Hz, 2H), 8.21 (d, J = 9.0 Hz, 2H), 7.67 (dd, J = 6.0, 2.0 Hz, 1H), 6.11 (dd, J = 6.0, 3.0 Hz, 1H), 5.94−5.98 (m, 1H), 5.51 (s, 1H), 5.06−5.10 (m, 1H), 4.54 (s, 2H), 3.19 (s, 1H), 3.01−3.04 (m, 2H), 2.51 (dq, J = 15.0, 4.5 Hz, 1H), 2.16−2.19 (m, 1H), 1.81−1.84 (m, 1H), 1.71 (d, J = 6.0 Hz, 3H), 1.65 (s, 3H), 1.62 (s, 3H), 1.30 (d, J = 6.5 Hz, 3H), 1.21 (s, 3H); ^{13}C{^{1}H} NMR (125 MHz, $CDCl_3$) δ 209.12, 177.30, 165.93, 164.07, 161.70, 150.74, 139.41, 134.93, 130.89, 130.10, 126.94, 123.48, 77.60, 72.72, 68.10, 54.81, 53.46, 50.37, 39.64, 29.63, 26.14, 20.18, 19.79, 18.81, 18.40, 15.45; HRMS (ESI/$[M + Na]^+$) calcd for $C_{28}H_{31}NO_9Na$: 548.1897, found 548.1901.

*Synthesis of **BA-10**.* To a stirred solution of **BA-8** (600 mg, 1.60 mmol) and triethylamine (2 mL, 15 mmol) in dichloromethane (20 mL) was added methacrylic anhydride (0.50 mL, 3.20 mmol) followed by DMAP (19.4 mg, 0.16 mmol) at room temperature. The reaction was stirred at room temperature for 2 h. After the reaction was completed, the solution was quenched by addition of a saturated NH_4Cl aqueous solution (10 mL) and the aqueous phase was extracted with ethyl acetate (50 mL × 3). The combined organic extracts were washed with brine, dried over $MgSO_4$, filtered, and concentrated. Silica gel flash column chromatography (hexane/ethyl acetate = 8:1) of the residue gave a white amorphous solid (606 mg, 1.41 mmol, 88%) as the product. **BA-10**: (58 mg, 0.13 mmol, 88%), ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.64 (dd, J = 6.1, 1.9 Hz, 1H), 6.15−6.05 (m, 2H), 6.06−5.94 (m, 1H), 5.55 (p, J = 1.6 Hz, 1H), 5.49 (s, 1H), 5.02 (ddd, J = 9.7, 7.8, 2.3 Hz, 1H), 4.30 (q, J = 11.1 Hz, 2H), 2.99 (dt, J = 11.6, 2.6 Hz, 1H), 2.91 (d, J = 7.5 Hz, 1H), 2.52−2.39 (m, 1H), 2.14 (d, J = 6.9 Hz, 1H), 1.90 (d, J = 1.3 Hz, 3H), 1.84 (dq, J = 7.3, 1.6 Hz, 3H), 1.76 (m, 1H), 1.67 (p, J = 1.6 Hz, 3H), 1.55 (s, 3H), 1.30−1.24 (m, 4H), 1.16 (s, 3H). ^{13}C{^{1}H} NMR (100 MHz, $CDCl_3$) δ 209.4, 177.8, 166.6, 165.7, 161.9, 139.4, 135.6, 130.0, 127.0, 126.4, 77.7, 77.3, 77.2, 77.0, 76.7, 72.5, 67.5, 54.9, 53.5, 50.5, 39.7, 29.7, 26.1, 20.3, 19.8, 18.9, 18.3, 18.1, 15.6. HRMS (ESI/$[M + H]^+$) calcd for $C_{25}H_{23}O_7$: 445.2221, found 445.2225.

*Synthesis of **BA-11**.* To a stirred solution of **BA-8** (600 mg, 1.60 mmol) and triethylamine (2 mL, 15 mmol) in dichloromethane (20 mL) was added acetic anhydride (0.30 mL, 3.20 mmol) followed by DMAP (19.4 mg, 0.16 mmol) at room temperature. The reaction was stirred at room temperature until TLC showed completion of the reaction. The reaction was then quenched by addition of a saturated NH_4Cl aqueous solution (10 mL), and the aqueous phase was extracted with ethyl acetate (50 mL × 3). The combined organic extracts were washed with brine, dried over $MgSO_4$, filtered, and concentrated. Silica gel flash column chromatography (hexane/ethyl acetate = 10:1) of the residue gave a white solid powder (655 mg, 1.57 mmol, 98%) as the product. **BA-11**: ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.69 (dd, J = 6.0, 1.5 Hz, 1H), 6.14 (dd, J = 11.0, 2.5 Hz, 1H), 6.07 (dd, J = 7.5, 1.5 Hz, 1H), 5.49 (s, 1H), 5.04−5.08 (m, 1H), 4.31 (dd, J = 34.0, 11.0 Hz, 2H), 3.03 (d, J = 11.5 Hz, 1H), 2.98 (d, J = 7.5 Hz, 1H), 2.48−2.54 (m, 1H), 2.15−2.19 (m, 1H), 2.08 (s, 3H), 1.93 (dd, J = 7.5, 1.5 Hz, 3H), 1.82−1.86 (m, 1H), 1.81 (s, 3H), 1.74 (s, 3H), 1.56 (s, 3H), 1.33 (d, J = 3.0 Hz, 3H), 1.21 (s, 3H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 209.37, 177.80, 170.28, 165.86, 161.87, 139.10, 130.14, 127.17, 77.53, 72.64, 67.19, 54.80, 53.40, 50.18, 39.56, 30.20, 29.67, 26.16, 20.66, 20.30, 19.84, 18.95, 18.44, 15.60; HRMS (ESI/$[M + H]^+$) calcd for $C_{23}H_{31}O_7$: 419.2070, found 419.2073.

*Synthesis of **BA-12**.* To a stirred solution of **BA-8** (600 mg, 1.60 mmol) and triethylamine (2 mL, 15 mmol) in dichloromethane (20 mL) was added succinic anhydride (320 mg, 3.20 mmol) followed by DMAP (19.4 mg, 0.16 mmol) at room temperature. The reaction was stirred at room temperature for 2 days. After the reaction was completed, the solution was quenched by addition of a 2 N HCl aqueous solution (15 mL)

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and the aqueous phase was extracted with ethyl acetate (50 mL × 3). The combined organic extracts were washed with brine, dried over $MgSO_4$, filtered, and concentrated. Silica gel flash column chromatography (hexane/ethyl acetate = 1:1) of the residue gave a white solid powder (532 mg, 1.15 mmol, 72%) as the product. **BA-12**: ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.66 (dd, J = 6.0, 1.9 Hz, 1H), 6.10 (dd, J = 6.0, 2.9 Hz, 1H), 6.04 (dq, J = 7.3, 1.5 Hz, 1H), 5.45 (s, 1H), 5.01 (ddd, J = 9.4, 7.5, 2.4 Hz, 1H), 4.30 (q, J = 10.9 Hz, 2H), 2.99 (dt, J = 11.7, 2.4 Hz, 1H), 2.92 (d, J = 7.6 Hz, 1H), 2.64 (d, J = 1.6 Hz, 4H), 2.48−2.39 (m, 1H), 2.14−2.08 (m, 1H), 1.97−1.82 (m, 3H), 1.79 (ddd, J = 15.8, 7.1, 2.6 Hz, 1H), 1.70 (q, J = 1.6 Hz, 3H), 1.51 (s, 3H), 1.31−1.23 (m, 7H), 1.17 (s, 3H). ^{13}C{^{1}H} NMR (125 MHz, $CDCl_3$) δ 209.6, 177.8, 171.4, 166.0, 162.1, 139.5, 130.1, 127.1, 77.6, 77.3, 77.0, 76.8, 72.6, 67.6, 54.8, 53.4, 50.2, 39.4, 29.7, 29.3, 28.9, 28.7, 26.1, 20.3, 19.8, 18.9, 18.4, 15.6.HRMS (ESI/[M + H]$^+$) calcd for $C_{25}H_{33}O_9$: 477.2119, found 477.2122.

Cell Lines and Culture Reagents. A549 (human alveolar basal epithelial adenocarcinoma), SW480 (colon adenocarcinoma), and MDA-MB-231 and MCF-7 (human breast adenocarcinomas) were purchased from the American Type Culture Collection (Manassas, VA). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) or Roswell Park Memorial Institution (RPMI)-1640 medium (Life Technologies) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and penicillin/streptomycin (50 U/mL), at 37 °C and 5% CO_2. Cell lines were tested and confirmed to be free of mycoplasma contamination.

Cell Viability Assessment. The cell viability of different cancer cell lines under drug treatment was determined via the MTT assay. Cells were seeded at a density of 6×10^3 (A549), 4×10^3 (SW480), or 5×10^3 (MDA-MB-231 or MCF-7) cells per well in 96-well plates. After 24 h, cells were treated with various concentrations (0−50 μM) of BA compounds for another 48 h. Cells were then treated with 0.5 mg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma, St. Louis, MO) at 37 °C for 4 h. Media was removed after incubation, and dimethyl sulfoxide (DMSO) (Duksan, Korea) was added to each well to dissolve the formazan crystals. Absorbance at 570 nm of each well was measured using a CLARIOstar monochromator microplate reader (BMG LABTECH, Germany). The IC_{50} values of different BA compounds in the cell lines were calculated using Prism 7 software (GraphPad Software, CA). Experiments were conducted in triplicate.

Colony Formation Assay. A549, SW480, MDA-MB-231, and MCF-7 cells were seeded in triplicate at a density of 1×10^3 cells per well in six-well plates. After 24 h of incubation, cells were treated with various concentrations of **BA**, **BA-9**, or **BA-10** for 14 days. The medium containing the desired concentration of BA compounds was refreshed once on day 7. At the end of the experiment, cells were fixed with 4% paraformaldehyde for 4 h and stained with 2% Giemsa blue solution overnight. Stained cells were briefly rinsed with Milli-Q water and then imaged. Experiments were conducted in triplicate.

Cell-Cycle Analysis. A549, SW480, MDA-MB-231, and MCF-7 cells were seeded in 60 mm culture plates at a density of $4-6 \times 10^5$ cells per plate. After 24 h, cells were treated with various concentrations of **BA**, **BA-9**, or **BA-10** for another 24 h. Cells were then harvested, fixed, and permeabilized in 70% cold ethanol overnight at −20 °C. Samples were washed twice with cold PBS and incubated with a PI/RNase staining reagent (BD Pharmingen, San Jose, CA) at room temperature for 15 min prior to cell-cycle analysis. Flow cytometric analysis was conducted using a BD FACSVia flow cytometer (BD, San Jose, CA), and histograms plotted were analyzed with ModFit LT 5.0 flow cytometry modeling software (Verity Software House, ME). Experiments were conducted in triplicate.

Annexin V−7-AAD/PE Double Staining Assay. A549, SW480, MDA-MB-231, and MCF-7 cells were seeded in 60 mm culture plates at a density of $4-6 \times 10^5$ cells per plate. After 48 h treatment with various concentrations of **BA**, **BA-9**, or **BA-10**, cells were harvested and suspended in Annexin-binding buffer. Apoptosis was assessed via flow cytometry with a BD FACSVia flow cytometer using the PE annexin V apoptosis detection kit (BD, San Jose, CA), according to the manufacturer's instructions. Experiments were conducted in triplicate.

Western Blot Analysis. A549, SW480, MDA-MB-231, and MCF-7 cells were seeded in 60 mm culture plates at a density of $4-6 \times 10^5$ cells per plate and treated with various concentrations of **BA**, **BA-9**, or **BA-10** for 48 h. Cells were harvested and cell pellets were lysed in a radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris−HCl, pH 7.4, 150 mM NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA), 1% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS)). The DC protein assay (Bio-Rad, Hercules, CA) was used to determine protein concentrations. Equal amounts of cell lysates were electrophoresed through SDS-polyacrylamide gel electrophoresis (PAGE) gels and transferred onto poly(vinylidene difluoride) (PVDF) membranes (Bio-Rad). The blots were then blocked in 5% nonfat skim milk and probed with the following diluted primary antibodies overnight: cleaved PARP, PARP, cleaved caspase 3, caspase 3, pH2A.X (Cell Signaling Technology), and β-actin (Santa Cruz Biotechnology). Blots were then incubated with the corresponding goat antirabbit or goat antimouse (Life Technologies) horseradish peroxidase (HRP)-conjugated secondary antibodies. Protein bands were visualized using Clarity ECL or Clarity Max Western blotting substrates (Bio-Rad). Images were obtained using a ChemiDoc Imaging System (Bio-Rad), and protein expression was analyzed using Image Lab software (Bio-Rad). Experiments were conducted in triplicate.

Statistical Analysis. Statistical analyses were performed using one-way analysis of variance (ANOVA). Data are presented as mean ± standard deviation (SD) of three independent experiments. *P < 0.05, **P < 0.01, and ***P < 0.001 were considered as significant differences.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.0c01276.

Effects of **BA-1−12** on viability of different cancer cells, purity quality control of **BA-1−12**, ^{1}H and ^{13}C NMR spectra of **BA-1−12** (PDF)

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

ROS, reactive oxygen species; MMP, mitochondrial membrane potential; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PI, propidium iodide; PE, phycoerythrin; 7-AAD, 7-aminoactinomycin D

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