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Molecular Design, Synthesis, and Evaluation of Novel Potent Apoptosis Inhibitors Inspired from Bongkrekic Acid

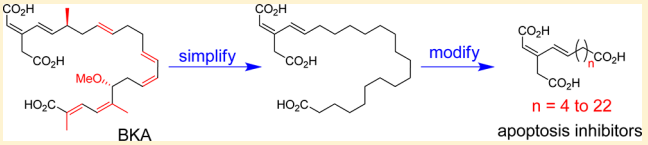
Katsuhiro Okuda,[†] Keisuke Hasui,[‡] Masato Abe,[†] Kenji Matsumoto,[†] and Mitsuru Shindo*[,†]

[†]Institute for Materials Chemistry and Engineering, Kyushu University, 6-1 Kasuga-koen, Kasuga, Fukuoka 816-8580, Japan

[‡]Interdisciplinary Graduate School of Engineering Sciences, Kyushu University, 6-1 Kasuga-koen, Kasuga, Fukuoka 816-8580, Japan

Supporting Information

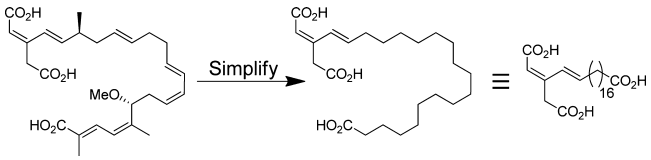
ABSTRACT: Bongkrekic acid (BKA) is an inhibitor of adenine nucleotide translocase (ANT). Since inhibition of ANT is connected to the inhibition of cytochrome *c* release from mitochondria, which then results in the suppression of apoptosis, it has been used as a tool for the mechanistic investigation of apoptosis. BKA consists of a long carbon chain with two asymmetric centers, a nonconjugated olefin, two conjugated dienes, three methyl groups, a methoxyl group, and three carboxylic acids. This complicated chemical structure has caused difficulties in synthesis, supply, and biochemical mechanistic investigations. In this study, we designed and synthesized more simple tricarboxylic acids that were inspired by the molecular structure of BKA. Their cytotoxicity and apoptosis-preventing activity in HeLa cells and the effect on the mitochondrial inner membrane potential ($\Delta\Psi$m) in HL-60 cells were then evaluated. All tested tricarboxylic acid derivatives including BKA showed little toxicity against HeLa cells. BKA and two of the synthesized derivatives significantly suppressed staurosporine (STS)-induced reductions in cell viability. Furthermore, STS-induced $\Delta\Psi$m collapse was significantly restored by pretreatment with BKA and a tricarboxylic acid derivative. Other derivatives, in which one of three carboxylic acids was esterified, exhibited potent toxicity, especially a derivative bearing a carbon chain of the same length as that of BKA. In conclusion, we have developed a new lead compound as an apoptosis inhibitor bearing three carboxylic acids connected with the proper length of a long carbon chain.



INTRODUCTION

Bongkrekic acid (BKA, Scheme 1) is a poisonous antibiotic produced by *Burkholderia Cocovenenans*. Its name comes from an Indonesian equivalent of tofu called tempeh bongkrek. BKA was isolated in 1934,[1] and its structure was elucidated between 1970 and 1973.[2−4] BKA was found to be an inhibitor of adenine nucleotide translocase (ANT), which mediates ADP/ATP exchange in mitochondria.[5,6] Further investigations have found that BKA inhibits the mitochondrial permeability transition pore (MPTP) via binding to ANT and fixes its conformation in the m-state, where its binding site faces the mitochondrial matrix. In an opposite manner, a different ANT inhibitor, atractyloside (ATR), and its derivative carboxyatractyloside (CATR), induced MPTP opening by changing the ANT conformation to its c-state where its binding site faced the cytosol side.[5,7−11]

Scheme 1. Chemical Structures of BKA and Its Simplified Analogue 10h



BKA inhibited chromatin condensation induced by valinomycin in BV-2, mouse microglia cells,[12] K^+ efflux in brain nonsynaptosomal mitochondria,[13] glucose-induced electrical activity in pancreatic beta-cells through the stimulation of ATP-sensitive potassium channel activity,[14] mitochondrial Ca^{2+} efflux, and Mg^{2+}, K^+, and adenine nucleotide release, which was promoted by a chelating agent EGTA and stimulated by menadione in rat liver mitochondria.[15] Furthermore, BKA reduced the uncoupling of the mitochondrial oxidative phosphorylation induced by K_{ATP} channel openers[16] and prevented losses in the mitochondrial inner membrane potential ($\Delta\Psi$m) induced by H_2O_2 in cerebellar granule neurons.[17] Recently, it was also reported that BKA inhibits mitochondrial membrane-bound glutathione transferase (mtMGST1) in rat livers through membrane components. This inhibition was observed only in mtMGST1 on the inner mitochondrial membrane, although mtMGST1 resides in both inner and outer mitochondrial membranes.[18]

These particular effects on mitochondria have been shown to suppress apoptosis.[19,20] BKA prevented a number of phenomena linked to apoptosis, such as depletion of reduced glutathione, generation of reactive oxygen species, translocation of NFκB, exposure of phosphatidylserine residues on the outer plasma membrane, cytoplasmic vacuolization, chromatin condensation, and oligonucleosomal DNA fragmentation.[21] It also protected multidrug-resistant tumor cells against apoptosis caused by 1,4-anthraquinone.[22] BKA protected MCF-7 cells

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against the proliferation inhibition effect of the chemotherapy drug Tamoxifen[23] and reduced ischemic-induced neuronal death.[24]

As a result, BKA has become a general tool for the elucidation of apoptosis mechanisms, appearing in hundreds of publications in spite of being an expensive and valuable reagent. To date, we have established an efficient synthetic method for BKA, which has gradually improved overall yield and reduced the steps in the longest linear sequence.[25−27] If more easily available potent apoptosis inhibitors could be designed and synthesized, bioactive compounds of this category would be useful for biochemical mechanistic investigations. In this study, we designed and synthesized simpler tricarboxylic acids as novel apoptosis inhibitors, inspired by the molecular structure of BKA. (Scheme 1), and their cytotoxicity and ability to prevent staurosporine (STS)-induced apoptosis in HeLa cells were evaluated. Furthermore, some of them were nominated for estimations of the effect on ΔΨm reduced by STS in HL-60 cells.

MATERIALS AND METHODS

Materials for Biological Assays. All new compounds were synthesized with the method based on BKA synthesis.[26] Representative methods for the compounds and their spectra data are described below, and the others are in Supporting Information. DMEM, RPMI-1640, STS, and carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP) were purchased from Wako Pure Chemical Industries (Osaka, Japan). WST-1 and 1-methoxy-5-methylphenazinium methylsulfate (1-methoxy-PMS) were from Dojindo Molecular Technologies, Inc. (Kumamoto, Japan). JC-1 was from Enzo Life Sciences, Inc. (Farmingdale, NY). Other chemicals used were of the highest quality commercially available.

Materials for Chemical Synthesis. The melting points were measured on a Yanako MP-S3. The ^{1}H- and ^{13}C NMR spectra were recorded using a JEOL JNM AL-400 spectrometer (400 and 100 MHz). The IR spectra were recorded on a Shimadzu FT/IR-8300 spectrometer using a KBr disk or a NaCl cell. Mass spectra were obtained on a JEOL JMS-700. Column chromatography was performed on silica gel (Kanto Chemical Co.). Thin-layer chromatography was performed on precoated plates (0.25 mm, silica gel Merck 60 F254). Reaction mixtures were stirred magnetically.

WST-1 Assay. HeLa human cervical cancer cells were cultured to subconfluency in DMEM supplemented 10% (v/v) heat-inactivated FBS, 100 units/mL penicillin G, and 100 μg/mL streptomycin. Cells were kept at 37 °C in 5% CO_2 and 95% humidified air. Cells suspended in culture medium were seeded on 96-well microtiter plastic culture plates at a density of 2.0×10^4 cells/100 μL/well. Media were exchanged to serum free DMEM on the next day, and incubation was continued for another 24 h. Test compounds dissolved in dimethylsulfoxide and diluted with the culture medium were added. To investigate the ability to prevent against apoptosis, staurosporine (STS) was added 2 h after the test compound had been added, and incubation was continued for another 22 h. Cell viability was assayed according to the WST-1 method of the modified MTT assay. This colorimetric assay measures the metabolic activity of viable cells based on cleavage of the tetrazolium salt WST-1 to water-soluble formazan by mitochondrial dehydrogenase in living cells. The assay was performed by incubation with 0.26 mM WST-1 and 0.01 mM 1-methoxy-PMS as an electron carrier in serum free DMEM for 0.5 h. After thorough shaking, the formazan produced by metabolically active cells in each sample was measured at a wavelength of 415 nm, with 620 nm as a reference, using a Microplate Reader Sunrise remote (Tecan Group Ltd., Männedorf, Switzerland). Absorbance readings were normalized against those of control wells with medium alone.

Measurement of Mitochondrial Membrane Potentials. The HL-60 promyelocytic leukemia cell line (RCB0041) was provided by RIKEN BRC through the National Bio-Resource Project of MEXT, Japan. Cells were cultured in RPMI-1640 supplemented 10% (v/v) heat-inactivated FBS, 100 units/mL penicillin G, and 100 μg/mL streptomycin. Cells were kept at 37 °C in 5% CO_2 and 95% humidified air. Cells were suspended in complete RPMI-1640 medium at a density of 4.0×10^5 cells/tube and preincubated for 1 h at 37 °C in the presence or absence of tricarboxylic acid derivatives. To investigate the ability to prevent against reductions in ΔΨm, 2 μM STS was added 1 h after preincubation of the test compound, and incubation was continued for another 3 h. Thirty minutes before finishing incubation, the fluorescent dye JC-1[28] was loaded for cells at a final concentration of 0.5 μM. In the case of CCCP exposure, it was added at the same time as JC-1 addition. Cells were centrifuged (900*g*, 3 min) and washed with PBS at room temperature to remove the drugs and unincorporated free dye. The fluorescence ratio, red aggregates/green monomers linked to the ΔΨm, of resuspended cells in PBS was immediately detected at 515 nm excitation and 595/535 nm emission using an RF-1500 spectrofluorophotometer (Shimadzu Co., Kyoto, Japan).

Statistical Analysis. Results were expressed as the mean ± SD. Statistical analysis was performed using a one-way ANOVA followed by the Tukey−Kramer test to assess the significance of differences in mean values between drug treatment groups and a suitable reference group. Significance was accepted when the *P* value was less than 0.05.

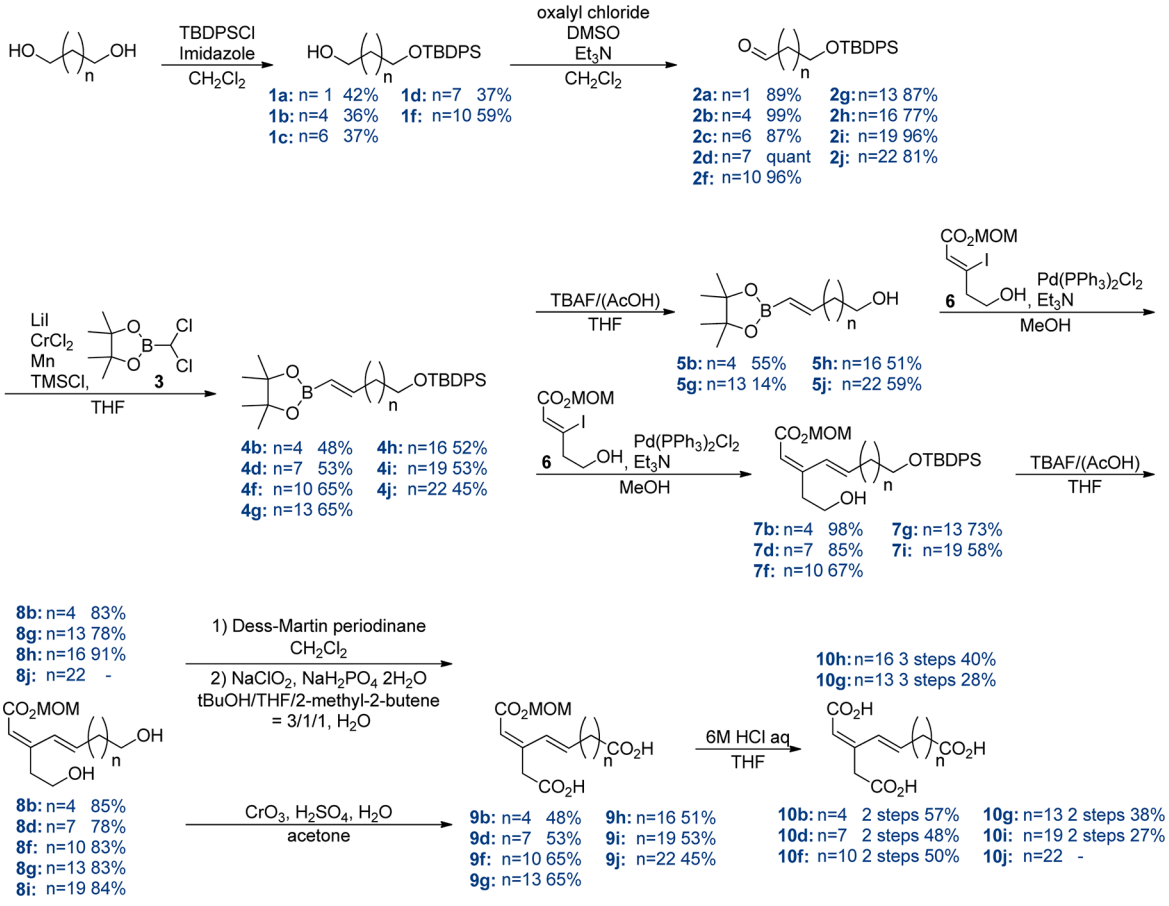
Chemical Synthesis. *3-((tert-Butyldiphenylsilyl)oxy)propan-1-ol (**1a**).* Imidazole (2.15 g, 31.5 mmol) was added to a solution of 1,3-propanediol (2.0 g, 26.3 mmol) in CH_2Cl_2 (130 mL). The reaction mixture was stirred at room temperature for 35 min, and TBDPSCl (4.82 mL, 18.4 mmol) was added. The reaction mixture was stirred at room temperature for 10 min, and then the saturated aqueous solution of $NaHCO_3$ was added. The aqueous layer was extracted with CH_2Cl_2, and the organic layer was washed with brine, dried over $MgSO_4$, filtered, and concentrated *in vacuo*. The crude product was purified by silica gel column chromatography (hexane/EtOAc = 3/1) to give a colorless oil (3.44 g, 42%). ^{1}H NMR (400 MHz in $CDCl_3$) δ: 1.06 (s, 9H), 1.76−1.81 (m, 2H), 3.81−3.85 (m, 4H), 7.35−7.42 (m, 6H), 7.66−7.68 (m, 4H).

*3-((tert-Butyldiphenylsilyl)oxy)propanal (**2a**).* Me_2SO (1.48 mL, 20.9 mmol) in CH_2Cl_2 (4 mL) was added to a solution of oxalyl chloride (1.48 mL, 16.7 mmol) in CH_2Cl_2 (60 mL) at −78 °C and was stirred for 20 min, and alcohol **1a** (2.18 g, 6.93 mmol) in CH_2Cl_2 (6 mL) was added at −78 °C under an argon atmosphere. The reaction mixture was stirred at −78 °C for 20 min, and triethylamine (6.86 mL, 48.8 mmol) was added at −78 °C. The reaction mixture was stirred at room temperature for 30 min, and the saturated aqueous solution of $NaHCO_3$ was then added. The aqueous layer was extracted with CH_2Cl_2, and the organic layer was washed with brine, dried over $MgSO_4$, filtered, and concentrated *in vacuo*. The crude product was purified by silica gel column chromatography (hexane/EtOAc = 4/1) to give a pale yellow oil (1.84 g, 89%). ^{1}H NMR (400 MHz in $CDCl_3$) δ: 1.04 (s, 9H), 2.59 (td, *J* = 2.0, 6.0 Hz, 2H), 4.02 (t, *J* = 6.0 Hz, 2H), 7.36−7.44 (m, 6H), 7.65−7.67 (m, 4H), 9.80 (t, *J* = 2.0 Hz, 1H).

*(12-Hydroxydodecyl)triphenylphosphonium bromide (**12f**).* PPh_3 (5.12 g, 19.5 mmol) was added to a solution of 12-bromododecan-1-ol **11f** (5.18 g, 19.5 mmol) in acetonitrile (73 mL). The reaction mixture was stirred under reflux for 90 h and evaporated. The crude product was purified by silica gel column chromatography (MeOH/$CHCl_3$ = 1/9) and recrystallization (EtOH/EtOAc) to give a white powder (9.63 g, 94%). ^{1}H NMR (400 MHz in $CDCl_3$) δ: 1.20−1.28 (m, 14H), 1.51−1.63 (m, 6H), 3.62 (t, *J* = 6.8 Hz, 2H), 3.76 (brs, 2H), 7.71−7.87 (m, 15H).

*(Z)-15-((tert-Butyldiphenylsilyl)oxy)pentadec-12-en-1-ol (**13g**).* *n*BuLi (2.6 M in hexane, 5.50 mL, 14.3 mmol) was added to a suspension of phosphonium salts **12f** (3.47 g, 7.15 mmol) in THF (40 mL) at 0 °C under an argon atmosphere. The reaction mixture was stirred under 0 °C for 10 min, and aldehyde **2a** (1.49 g, 4.77 mmol) in THF (8 mL) was added at 0 °C. The mixture was stirred at 0 °C for 10 min, and NH_4Cl was added and extracted with EtOAc, and the organic layer was washed with brine and dried over $MgSO_4$. The crude product was purified by silica gel column chromatography (hexane/EtOAc = 4/1) to give a colorless oil (1.83 g, 80%). ^{1}H NMR (400

Scheme 2. Synthesis of Tricarboxylic Acid Derivatives 10 Bearing Different Carbon Chain Lengths (n = 4, 7, 10, 13, 16, 19, and 22)



MHz in $CDCl_3$) δ: 1.05 (s, 9H), 1.27 (brs, 16H), 1.53−1.58 (m, 4H), 1.96 (q, J = 6.8 Hz, 2H), 2.31 (q, J = 17.2 Hz, 2H), 3.61−3.68 (m, 4H), 5.33−5.46 (m, 2H), 7.35−7.43 (m, 6H), 7.66−7.68 (m, 4H). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 19.2, 25.7, 26.9, 27.3, 29.2, 29.3, 29.4, 29.5, 29.6, 29.7, 30.8, 32.8, 63.1, 63.7, 125.5, 127.6, 127.7, 131.9, 134.0, 135.6. IR (Neat): 3354, 2928, 2855 cm^{-1}; mass (FAB) m/z 481 [M + H]$^+$. HRMS calcd for $C_{31}H_{49}O_2Si$: 481.3502; found, 481.3495.

*15-((tert-Butyldiphenylsilyl)oxy)pentadecan-1-ol (**1g**).* Alcohol **13g** (0.98 g, 2.04 mmol) in MeOH (5 mL) was added to a suspension of Pd/C (10%, 109 mg, 0.10 mmol) in MeOH (15 mL) under H_2. The reaction mixture was stirred at room temperature for 2 h, filtered, and concentrated to afford a colorless oil (892 mg, 91%). ^{1}HNMR (400 MHz in $CDCl_3$) δ: 1.04 (s, 9H), 1.25 (brs, 22H), 1.56 (m, 4H), 3.64−3.67 (m, 4H), 7.35−7.41 (m, 6H), 7.66−7.68 (m, 4H). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 19.2, 25.7, 25.8, 26.9, 29.4, 29.4, 29.6, 29.7, 32.6, 32.8, 63.1, 64.0, 127.5, 127.7, 129.4, 129.8, 134.2, 135.5.

*15-((tert-Butyldiphenylsilyl)oxy)pentadecanal (**2g**).* Me_2SO (0.41 mL, 5.71 mmol) in CH_2Cl_2 (1 mL) was added to a solution of oxalyl chloride (0.40 mL, 4.57 mmol) in CH_2Cl_2 (16 mL) at −78 °C and stirred for 20 min, and then alcohol **1g** (919 mg, 1.90 mmol) in CH_2Cl_2 (3 mL) was added at −78 °C under argon atmosphere. The reaction mixture was stirred at −78 °C for 20 min, and triethylamine (1.87 mL, 13.3 mmol) was added at −78 °C. The reaction mixture was stirred at room temperature for 30 min, and then the saturated aqueous solution of $NaHCO_3$ was added. The aqueous layer was extracted with CH_2Cl_2, and the organic layer was washed with brine, dried over $MgSO_4$, filtered, and concentrated *in vacuo*. The crude product was purified by silica gel column chromatography (hexane/EtOAc = 4/1) to give a colorless oil (797 mg, 87%). ^{1}H NMR (400 MHz in $CDCl_3$) δ: 1.05 (s, 9H), 1.20−1.33 (m, 20H), 1.52−1.64 (m, 4H), 2.41 (td, J = 2.0, 6.8 Hz, 2H), 3.65 (t, J = 6.8 Hz, 2H), 7.35−7.43 (m, 6H), 7.66−7.68 (m, 4H), 9.76 (t, J = 2.0 Hz, 1H)). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 19.2, 22.1, 25.8, 26.9, 29.2, 29.3, 29.4, 29.4, 29.6, 29.6, 29.6, 29.6, 32.6, 43.9, 64.2, 127.5, 129.4, 134.2, 135.6, 202.9. IR (Neat): 2928, 2855, 1728 cm^{-1}; mass (FAB) m/z 423 [M − C_4H_9]$^+$. HRMS calcd for $C_{27}H_{39}O_2Si$: 423.2719; found, 423.2718.

*(E)-tert-Butyldiphenyl((16-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)hexadec-15-en-1-yl)oxy)silane (**4g**).* Aldehyde **2g** (770 mg, 1.60 mmol) in THF (2 mL), pinacol borate **3**[29] (470 mg, 2.24 mmol) in THF (2 mL), and TMSCl (1.22 mL, 9.61 mmol) were added to a suspension of $CrCl_2$ (200 mg, 1.60 mmol), LiI (1.29 g, 9.61 mmol), and Mn (260 mg, 6.41 mmol) in THF (12 mL) under a nitrogen atmosphere. The mixture was stirred at room temperature for 16.5 h, and then the saturated aqueous solution of $NaHCO_3$ was added. The aqueous layer was extracted with EtOAc, and the organic layer was washed with brine, dried over $MgSO_4$, filtered, and concentrated *in vacuo*. The crude product was purified by silica gel column chromatography (hexane/EtOAc = 20/1) to give a colorless oil (624 mg, 65%). ^{1}H NMR (400 MHz in $CDCl_3$) δ: 1.04 (s, 9H), 1.24 (s, 20H), 1.36−1.41 (m, 2H), 1.52−1.59 (m, 2H), 2.14 (q, J = 6.8 Hz, 2H), 3.65 (t, J = 6.8 Hz, 2H), 5.42 (d, J = 18.4 Hz, 1H), 6.63 (dt, J = 6.8, 18.4 Hz, 1H), 7.35−7.43 (m, 6H), 7.66−7.68 (m, 4H). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 19.2, 24.8, 25.8, 26.9, 28.2, 29.2, 29.4, 29.5, 29.6, 29.6, 29.7, 32.6, 35.8, 64.0, 83.0, 127.5, 129.4, 134.2, 135.6, 154.8. IR (Neat): 2926, 2855, 1638 cm^{-1}; mass (FAB) m/z 589 [M − CH_3]$^+$. HRMS calcd for $C_{37}H_{58}BO_3Si$: 589.4248; found, 589.4265.

*(2Z,4E)-Methoxymethyl 19-((tert-Butyldiphenylsilyl)oxy)-3-(2-hydroxyethyl)nonadeca-2,4-dienoate (**7g**).* Segment A **6**[26] (341 mg, 1.19 mmol) in MeOH (3 mL) and boronic ester **4g** (594 mg, 0.99 mmol) in THF (10 mL) were added to a suspension of $Pd(PPh_3)_2Cl_2$ (139 mg, 0.20 mmol) in MeOH (7 mL) at room temperature. The mixture was stirred at room temperature for 10 min, and triethylamine (0.98 mL, 6.94 mmol) was added. The reaction mixture was stirred at room temperature for 40 h, filtered, and concentrated *in vacuo*. The

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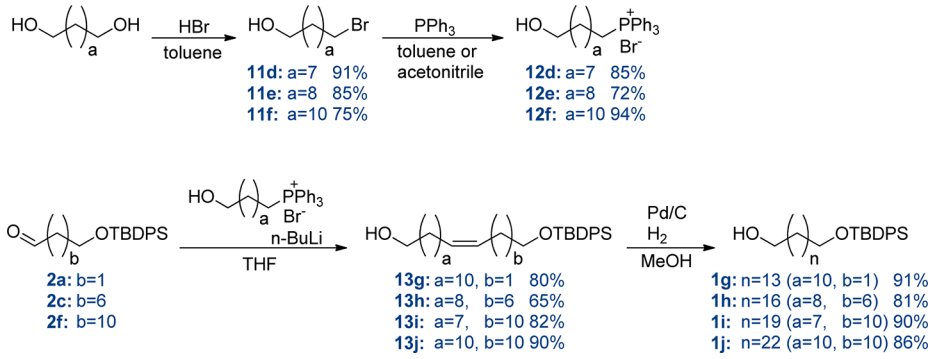
Scheme 3. Synthesis of *tert*-Butyldiphenylsilylated Long Chain Alkane Diol 2



crude product was purified by silica gel column chromatography (hexane/EtOAc = 3/1) to give a yellow oil (463 mg, 73%). ^{1}H NMR (400 MHz in $CDCl_3$) δ: 1.04 (s, 9H), 1.24−1.27 (m, 20H), 1.40−1.43 (m, 2H), 1.53−1.56 (m, 2H), 2.21 (q, J = 6.8 Hz, 2H), 2.63 (t, J = 6.8 Hz, 2H), 3.48 (s, 3H), 3.65 (t, J = 6.4 Hz, 2H), 3.78 (q, J = 6.4 Hz, 2H), 5.27 (s, 2H), 5.68 (s, 1H), 6.22 (dt, J = 6.8, 16.4 Hz, 1H), 7.34−7.41 (m, 6H), 7.52 (d, J = 16.8 Hz, 1H), 7.66−7.68 (m, 4H); ^{13}C NMR (100 MHz, $CDCl_3$) δ: 19.2, 25.8, 26.9, 29.0, 29.3, 29.4, 29.6, 29.6, 29.7, 32.6, 33.5, 37.5, 57.6, 61.9, 64.0, 89.9, 115.8, 126.5, 127.5, 129.4, 134.2, 135.6, 140.2, 153.1, 165.5. IR (Neat): 3418, 2928, 2855, 1715, 1699, 1633 cm^{-1}; mass (FAB) m/z 575 $[M - C_2H_3O_2]^+$. HRMS calcd for $C_{37}H_{55}O_3Si$: 575.3920; found, 575.3920.

*(2Z,4E)-Methoxymethyl 19-hydroxy-3-(2-hydroxyethyl)-nonadeca-2,4-dienoate (**8g**).* TBAF (1 M in THF, 1.38 mL, 1.38 mmol) was added to a solution of **7g** (440 mg, 0.69 mmol) in THF (6 mL) at room temperature. The reaction mixture was stirred at room temperature for 16 h and quenched with the saturated aqueous solution of NH_4Cl. The aqueous layer was extracted with EtOAc, and the organic layer was washed with brine, dried over $MgSO_4$, filtered, and concentrated *in vacuo*. The crude product was purified by silica gel column chromatography (hexane/EtOAc = 1/1) to give a colorless powder (227 mg, 83%); mp 48−52 °C. ^{1}H NMR (400 MHz in $CDCl_3$) δ: 1.25−1.31 (m, 20H), 1.42−1.45 (m, 2H), 1.53−1.58 (m, 2H), 2.22 (q, J = 6.8 Hz, 2H), 2.63 (t, J = 6.4 Hz, 2H), 3.48 (s, 3H), 3.64 (t, J = 6.4 Hz, 2H), 3.78 (t, J = 6.8 Hz, 2H), 5.28 (s, 2H), 5.68 (s, 1H), 6.23 (dt, J = 6.8, 16.4 Hz, 1H), 7.52 (d, J = 16.4 Hz, 1H). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 25.7. 28.9, 29.3, 29.4, 29.4, 29.5, 29.5, 29.6, 29.6, 32.8, 33.5, 37.5, 57.5, 61.9, 63.0, 89.9, 115.7, 126.5, 140.1, 153.1, 165.4. IR (KBr): 3423, 2928, 2849, 1695, 1629, 1591 cm^{-1}. EA calcd for $C_{23}H_{42}O_5$: C 69.31%, H 10.62%; found, C 68.96%, H 10.52%.

*(3Z,4E)-3-(2-(Methoxymethoxy)-2-oxoethylidene)nonadec-4-enedioic acid (**9g**).* Alcohol **8g** (208 mg, 0.52 mmol) in acetone (2 mL) was added dropwise to a solution of Jones reagent (2.5 M in acetone, 1.67 mL, 4.18 mmol) in acetone (8.5 mL) at 0 °C. The reaction mixture was stirred at 0 °C for 30 min and quenched with *i*PrOH, and H_2O was added. The aqueous layer was extracted with Et_2O, and the organic layer was washed with brine, dried over $MgSO_4$, filtered, and concentrated *in vacuo*. The crude product was purified by recrystallization (EtOAc/hexane) to give a colorless powder (88.7 mg, 40%); mp 99−101 °C. ^{1}H NMR (400 MHz in $CDCl_3$) δ: 1.26−1.30 (m, 18H), 1.41−1.43 (m, 2H), 1.60−1.64 (m, 2H), 2.23 (q, J = 7.2 Hz, 2H), 2.36 (t, J = 6.8 Hz, 2H), 3.38 (s, 2H), 3.48 (s, 3H), 5.29 (s, 2H), 5.74 (s, 1H), 6.21 (dt, J = 6.8, 16.8 Hz, 1H), 7.55 (d, J = 16.8 Hz, 1H). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 24.4, 28.5, 28.8, 28.8, 28.9, 29.0, 29.1, 29.2, 29.3, 29.4, 33.4, 33.9, 40.4, 57.6, 90.1, 118.1, 126.3, 141.1, 147.8, 165.1, 176.6, 180.5. IR (KBr): 2922, 2849, 1701, 1634, 1603 cm^{-1}. EA calcd for $C_{23}H_{38}O_7$: C 64.76%, H 8.98%; found, C 64.79%, H 8.94%.

*(2Z,4E)-3-(Carboxymethyl)nonadeca-2,4-dienedioic acid (**10g**).* HCl (6 M, 2.8 mL, 16.7 mmol) was added to a solution of ester **9g** (60 mg, 0.14 mmol) in THF (2.8 mL) at room temperature. The reaction mixture was stirred at room temperature for 1 h, and H_2O was then added, extracted with Et_2O, dried over $MgSO_4$, filtered, and concentrated *in vacuo*. The crude product was purified by recrystallization (CH_3CN) to give a colorless powder (51 mg, 95%); mp 129−133 °C. ^{1}H NMR (400 MHz in CD_3OD) δ: 1.29 (brs, 18H), 1.41−1.45 (m, 2H), 1.57−1.61 (m, 2H), 2.19 (q, J = 6.8 Hz, 2H), 2.27 (t, J = 7.2 Hz, 2H), 3.34 (s, 2H), 5.70 (s, 1H), 6.20 (dt, J = 6.8, 16.8 Hz, 1H), 7.49 (d, J = 15.6 Hz, 1H). ^{13}C NMR (100 MHz, CD_3OD) δ: 26.1, 30.0, 30.2, 30.4, 30.6, 30.6, 30.7, 30.7, 30.7, 34.3, 35.0, 41.1, 119.8, 127.9, 140.3, 149.1, 169.4, 174.3, 177.7 . IR (KBr): 2920, 2851, 1703, 1632, 1603 cm^{-1}. EA calcd for $C_{21}H_{34}O_6$: C 65.94%, H 8.96%; found, C 65.69%, H 8.99%.

RESULTS

Synthesis of Tricarboxylic Acids. We have reported a convergent strategy for the total synthesis of BKA.[26] A series of tricarboxylic acids bearing a fatty acid chain were synthesized by the modified convergent strategy (Scheme 2). A conjugated double-bond was formed via Suzuki−Miyaura coupling between Segment A (**6**) and pinacol borate bearing various lengths of protected or unprotected fatty alcohols **4** and **5**. Suzuki−Miyaura coupling prior to deprotection (4→7→8) afforded the target diols **8** in higher yield than vice versa (4→5→8). Oxidation of the diols **8** was tried with two distinct methods via 2 step-oxidation by Dess−Martin oxidation, followed by Pinnick oxidation, and via straightforward oxidation with Jones reagent to provide the carboxylic acids **9** at a similar level of yield. Several noncommercially available 1,*n*-diols **1** having a long carbon chain (n = more than 10) were synthesized by the Wittig reaction, followed by catalytic hydrogenation (Scheme 3).

Biological Assays for Tricarboxylic Acid Derivatives 10. To evaluate the cytotoxicity of BKA and tricarboxylic acid derivatives **10**, the WST-1 assay was conducted after 22 h exposure to each compound in HeLa cells. As shown in Figure 1, all tested compounds including BKA showed little toxicity against HeLa cells. The most toxic compound among these was **10g** (n = 13), but 85% of cells were still alive even at a concentration of 200 μM.

The apoptosis preventing activities of BKA and derivatives **10** were estimated by the WST-1 assay under the conditions of 2 h of pretreatment of test compounds followed by 22 h of exposure to 100 nM of STS, which is well-known as an apoptosis inducer (Figure 2).[30,31] Under these conditions, STS reduced cell viability at most 74%, and cell viability significantly recovered by pretreatment with 200 μM BKA. As for tricarboxylic acid derivatives, **10f** (n = 10) and **10g** (n = 13) significantly suppressed STS-induced reductions in cell viability. The other compounds (**10b**, **10d**, **10i**, and **10j**) did not show substantial recovery. Apoptosis inducible ability of STS was confirmed by modified TdT-mediated dUTP nick-end labeling

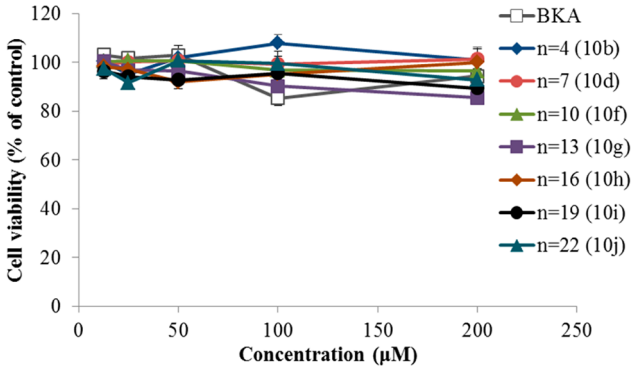


Figure 1. Cytotoxicity of BKA and tricarboxylic acid derivatives **10** in HeLa cells. Cultured HeLa cells were exposed to these compounds for 22 h. Cell viability was determined by the WST-1 assay. Each value represents the mean ± SD of three (control $n = 6$) experiments.

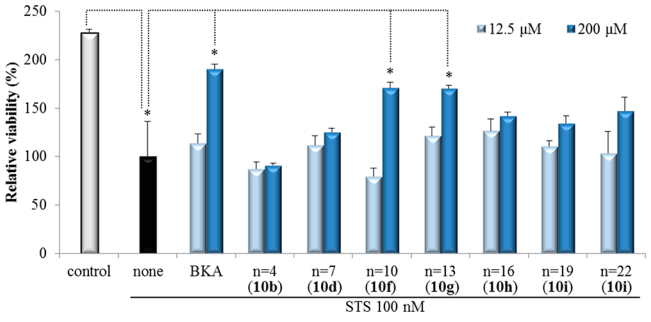


Figure 2. Effect of BKA and tricarboxylic acid derivatives 10 on cell viability in HeLa cells. Cultured HeLa cells were preincubated with these compounds for 2 h. Apoptosis was induced in cells by exposure to 100 nM STS for 22 h together with the test compound. Cell viability was determined by the WST-1 assay. Each value represents the mean ± SD of three experiments. The significance of differences was assessed using the Tukey–Kramer test. *$P < 0.05$ was significantly different from the reference group.

(TUNEL) assay, and then DNA fragmentation was observed in a dose-dependent manner (Figure S1, Supporting Information).

Since some of the tricarboxylic acid derivatives possessed BKA-like activity, further assessment was conducted to confirm their availabilities. Since it has been reported that BKA is capable of preventing disruptions in the inner mitochondrial transmembrane potential ($\Delta\Psi$m),[20] the ability to prevent disruptions in the $\Delta\Psi$m induced by STS in HL-60 cells was evaluated using the derivatives **10** (Figure 3). The fluorescence ratio of the $\Delta\Psi$m sensitive dye JC-1 represents the relative $\Delta\Psi$m in cells.[32] The STS-induced $\Delta\Psi$m collapse was significantly restored by pretreatment with BKA as reported.[17] In the case of the tricarboxylic acid derivatives, $\Delta\Psi$m tended to increase as carbon chains got shorter, but only **10d** ($n = 7$) exhibited significant $\Delta\Psi$m recovery. In contrast, **10j** ($n = 22$) strongly promoted reductions in $\Delta\Psi$m. CCCP (carbonyl cyanide *m*-chlorophenylhydrazone) was used as a positive control and completely induced uncoupling on mitochondria. When the cell viabilities of these cells were measured right after the JC-1 assay using trypan blue, more than 90% of cells were still alive even in only STS- or CCCP-treated groups (data not shown); however, apoptotic changes in morphology were observed in STS treated cells (Figure S2, Supporting Information).

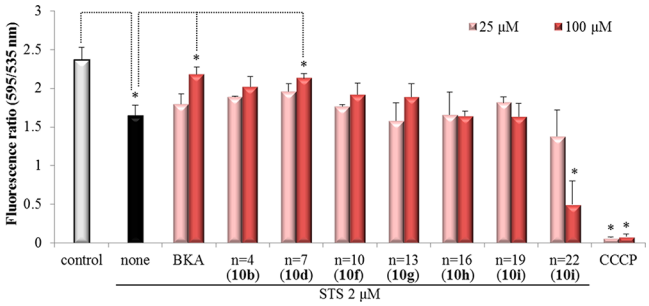


Figure 3. Effect of BKA and tricarboxylic acid derivatives **10** on reductions in $\Delta\Psi$m caused by STS in HL-60 cells. Cultured HL-60 cells were preincubated with these compounds for 1 h. $\Delta\Psi$m collapse was induced in cells by exposure to 2 μM STS for 3 h together with the test compound. Values were evaluated using the $\Delta\Psi$m sensitive fluorescence dye JC-1. Each value represents the mean ± SD of three experiments. The significance of differences was assessed using the Tukey–Kramer test. *$P < 0.05$ was significantly different from the reference group.

Biological Assays for MOM Esters 9. Cytotoxicity and the ability to prevent apoptosis of MOM esters were also evaluated (Figure 4). These compounds are not only synthetic

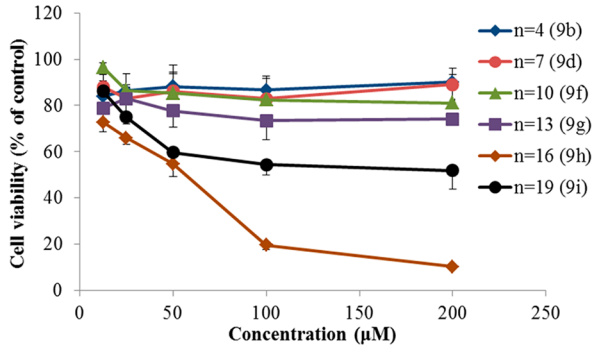


Figure 4. Cytotoxicity of mono-MOM ester derivatives **9** in HeLa cells. Cultured HeLa cells were exposed to these compounds for 22 h. Cell viability was determined by the WST-1 assay. Each value represents the mean ± SD of three (control $n = 6$) experiments.

intermediates but also more hydrophobic derivatives than the corresponding tricarboxylic acid. Compound **9j** ($n = 22$) was not tested because we were not able to purify it enough due to its detergent-like properties. As shown in Figure 4, **9h** ($n = 16$) exhibited potent toxicity in HeLa cells, which stood out among the other derivatives. Compound **9i** ($n = 19$) also somewhat exhibited toxicity, however, which was weaker than that of **9h**, although the derivatives became more toxic as their carbon chain length got longer.

The only compound that showed a slight ability to prevent STS-induced apoptosis was **9d** (n = 7) (Figure 5). Compounds **9h** and **9i** magnified reductions in cell viability, and the other derivatives had no effect on cells.

DISCUSSION

BKA is well-known as a selective inhibitor for ANT that mediates ADP/ATP exchange in the inner mitochondrial membrane.[5,6] Since the inhibition of ANT could be linked to oxidative phosphorylation impairments, BKA treatment may cause cell death. However, it did not exhibit potent cytotoxicity in HeLa and HL-60 cells. This paradoxical phenomenon may

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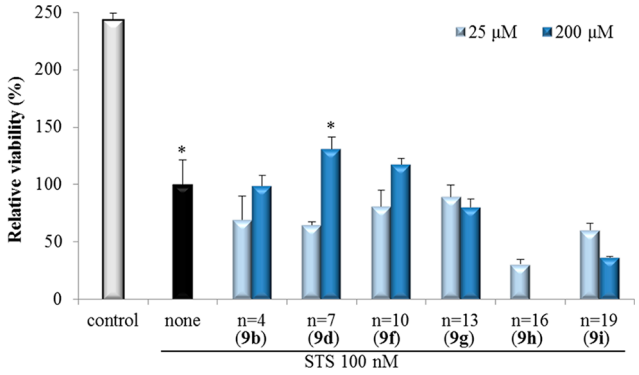

Figure 5. Effect of mono-MOM ester derivatives **9** on cell viability in HeLa cells. Cultured HeLa cells were preincubated with these compounds for 2 h. Apoptosis was induced in cells by exposure to 100 nM STS for 22 h together with the test compound. Cell viability was determined by the WST-1 assay. Each value represents the mean ± SD of three experiments. The significance of differences was assessed using the Tukey–Kramer test. *$P < 0.05$ was significantly different from the reference group.

happen because these kinds of cancer cells use aerobic glycolysis with reduced mitochondrial oxidative phosphorylation for glucose metabolism.[33] Under such ATP supplied conditions, BKA prevents MPTP opening and cytochrome *c* release from mitochondria, thus suppressing apoptosis induction.[24] There are several apoptotic pathways without mitochondrial uncoupling, for instance, a pathway through caspase 8 activation. We considered that this is one of the reasons why BKA could not completely rescue cells from STS-induced apoptosis.

Synthesized tricarboxylic acids and their MOM esters were applied to the WST-1 assay to estimate their cytotoxicity, and only MOM esters **9h** and **9i**, bearing a long carbon chain, were found to be positioned as toxic compounds. Conformational searches using the MMFF force field (CONFLEX ver. 5; CONFLEX Corporation, Tokyo, Japan) indicated that BKA would adopt cyclic conformations, rather than linear ones, partially due to hydrogen bonding with two carboxylic acid moieties. In the case of the tricarboxylic acids synthesized herein, cyclic conformations may be possible when their carbon chain is long enough (n = around 13 or more). Although it is not clear whether cyclic conformations are essential for binding to target proteins, three carboxylic acid moieties and appropriate spatial positioning among these moieties would be crucial for the inhibition of apoptosis.

The most effective apoptosis inhibitor among these derivatives was **10f**, and **10g** was as potent as **10f**. The MOM ester **9d** also showed a slight inhibitory activity. Contrary to our expectations, the tricarboxylic acid **10h**, which was designed and synthesized as a BKA equivalent bearing the same carbon chain length, did not exhibit significant activity. However, the fact that a compound with a shorter carbon chain possesses higher activity is desirable for further investigation.

Usually cationic and lipophilic compounds selectively localize on mitochondria because of the high negative transmembrane electrical potential maintained by functional mitochondria.[34] An anionic compound like carboxylic acid would not reach the inner mitochondrial membrane where ANT localizes and works except by passive diffusion. Thus, an efficacious carrier for the delivery of anionic compounds into mitochondria[35] may be able to magnify the activity of these derivatives. A simple structure is helpful for such a delivery system and for the design of further derivatives.

Once the MOM ester moiety of derivatives **9** is hydrolyzed by intracellular esterase or under acidic conditions like the intermembrane space of mitochondria, it becomes an active compound, tricarboxylic acid. As such, it had been considered as the easier membrane permeable precursor of tricarboxylic acid derivatives. However, some of the cytotoxicities and preventing activities of **9f** and **9g** were weaker than those of the corresponding tricarboxylic acids **10f** and **10g**. It should be considered that the carbon chain may be shortened by β-oxidation in the mitochondrial matrix and that the ester moiety would not be hydrolyzed by the intracellular esterase.

Only **10d** could significantly suppress reductions in $\Delta\Psi$m, and **10b**, **10f**, and **10g** tended to recover the potential to some degree. The optimal carbon chain length for apoptosis prevention was n = 10 and for $\Delta\Psi$m collapse inhibition was n = 7. This variety may come from differences in cells having distinct sensitivities, metabolic abilities, and amounts of protein expression. Furthermore, it could be considered that the apoptosis preventing mechanism of these derivatives including BKA were not only ANT inhibition but also the inhibition or activation of other target molecules.

In conclusion, our results indicate that the BKA carbon chain, which has an olefin, two conjugated olefins, three methyl groups, and a methoxyl group, can be simplified to a saturated carbon hydrate chain but that the chain length is critical to its activity. According to our previous unpublished data, the carboxylic acid group at the unbranched terminal is essential for making the compound nontoxic. It is highly possible that ANT and/or the target of these derivatives recognize the three carboxylic acid moieties and then bind to some of them. We have successfully achieved the design and synthesis in more easily available potent apoptosis inhibitors, and these compounds would be useful for biochemical mechanistic investigations. Since there are several points that need to be uncovered about ANT as a component of MPTP, a novel reagent effect to ANT will play an important role as an apoptosis research tool. Further structure–activity relationship studies are ongoing, and investigations may elucidate a pharmacophore of BKA.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Experimental procedures, DNA fragmentation caused by STS addition in HeLa cells, and apoptotic changes in morphology in HL-60 cells exposed with STS. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Tel: +81-92-583-7802. Fax: +81-92-583-7875. E-mail: shindo@cm.kyushu-u.ac.jp.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

BKA, bongkrekic acid; ANT, adenine nucleotide translocase; MPTP, mitochondrial permeability transition pore; $\Delta\Psi m$, mitochondrial inner membrane potential; STS, staurosporine; CCCP, carbonyl cyanide *m*-chlorophenyl hydrazone; 1-methoxy-PMS, 1-methoxy-5-methylphenazinium methylsulfate; TBDPSCl, *tert*-butyldiphenylchlorosilane; TMSCl, trimethylchlorosilane; TBAF, tetrabutyl ammonium fluoride; PPh_3, triphenylphosphine; *n*BuLi, *n*-butyl lithium; HRMS, high resolution mass spectrometry; EA, elemental analysis

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