Chem Biol Drug Des 2015; **86:** 1304–1322

Research Article

Bongkrekic Acid Analogue, Lacking One of the Carboxylic Groups of its Parent Compound, Shows Moderate but pH-insensitive Inhibitory Effects on the Mitochondrial ADP/ATP Carrier

Atsushi Yamamoto[1], Keisuke Hasui[2], Hiroshi Matsuo[2], Katsuhiro Okuda[3], Masato Abe[3], Kenji Matsumoto[3], Kazuki Harada[4,5], Yuya Yoshimura[4,5], Takenori Yamamoto[4,5], Kazuto Ohkura[1], Mitsuru Shindo[3,*] and Yasuo Shinohara[4,5,*]

[1]Faculty of Pharmaceutical Sciences, Suzuka University of Medical Science, Minamitamagakicho-3500, Suzuka, Mie 513-8670, Japan
[2]Interdisciplinary Graduate School of Engineering Sciences, Kyushu University, 6–1 Kasuga-koen, Kasuga 816-8580, Japan
[3]Institute for Materials Chemistry and Engineering, Kyushu University, Kasugakoen-6, Kasuga, Fukuoka 816-8580, Japan
[4]Institute for Genome Research, Tokushima University, Kuramotocho-3, Tokushima 770-8503, Japan
[5]Faculty of Pharmaceutical Sciences, Tokushima University, Shomachi-1, Tokushima 770-8505, Japan
**Corresponding authors: Mitsuru Shindo, shindo@cm.kyushu-u.ac.jp; Yasuo Shinohara, yshinoha@genome.tokushima-u.ac.jp*

Bongkrekic acid, isolated from *Burkholderia cocovenenans*, is known to specifically inhibit the mitochondrial ADP/ATP carrier. However, the manner of its interaction with the carrier remains elusive. In this study, we tested the inhibitory effects of 17 bongkrekic acid analogues, derived from the intermediates obtained during its total synthesis, on the mitochondrial ATP/ATP carrier. Rough screening of these chemicals, performed by measuring their inhibitory effects on the mitochondrial ATP synthesis, revealed that 4 of them, KH-1, KH-7, KH-16, and KH-17, had moderate inhibitory effects. Further characterization of the actions of these 4 analogues on mitochondrial function showed that KH-16 had moderate; KH-1 and KH-17, weak; and KH-7, negligible side effects of both permeabilization of the mitochondrial inner membrane and inhibition of the electron transport, indicating that only KH-7 had a specific inhibitory effect on the mitochondrial ADP/ATP carrier. Although the parental bongkrekic acid showed a strong pH dependency of its action, the inhibitory effect of KH-7 was almost insensitive to the pH of the reaction medium, indicating the importance of the 3 carboxyl groups of bongkrekic acid for its pH-dependent action. A direct inhibitory effect of KH-7 on the mitochondrial ADP/ATP carrier was also clearly demonstrated.

Key words: ADP/ATP carrier, bongkrekic acid, mitochondria, mitochondrial solute carrier (SLC25a)

Abbreviations: BKA, bongkrekic acid; CATR, carboxyatractyloside.

Received 12 April 2015, revised 18 May 2015 and accepted for publication 27 May 2015

Most of the cellular ATP is synthesized by the process of oxidative phosphorylation in the mitochondria. During this process, energy of nutrient molecules is first converted into an electrochemical gradient of H^+ across the inner mitochondrial membrane. Then, using the electrochemical gradient of H^+ across this membrane as a driving force, ATP is synthesized by F_0F_1-ATP synthase. Therefore, to enable effective energy conversion, permeability of the inner mitochondrial membrane must be kept very low. However, various molecules such as those involved in the process of ATP synthesis or in the TCA cycle or β-oxidation must be conveyed across the inner mitochondrial membrane. The transport of various metabolites and ions across this inner membrane is known to be catalyzed by transporters specific for each individual metabolite. These transporters are thought to have arisen from a common ancestral gene, because they show structural similarities such as 6-times membrane spanning topology, and so they are referred to as the mitochondrial solute carrier family, SLC25a.

The ADP/ATP carrier has been the most extensively studied member of this solute carrier family [for recent review, see refs. (1–4)]. It was identified in 1964/65 (5–7), and its primary structure was determined in 1982 (8). Two decades later, in 2002, its crystal structure was revealed (9). Despite extensive studies, the catalytic mechanism of the ADP/ATP carrier still remains elusive. It is thought that the ADP/ATP carrier catalyzes the transports of ADP and ATP by changing its conformation between that facing the cytosolic side (c-state) and that facing the matrix side (m-state). Key molecules for understanding the catalytic

 doi: 10.1111/cbdd.12594

mechanism of this carrier are two specific inhibitors of it, that is, carboxyatractyloside (CATR) and bongkrekic acid (BKA), isolated as toxins from *Atractylis gummifera* and *Burkholderia cocovenenans*, respectively (10). These two inhibitors bind to the ADP/ATP carrier from the cytosolic side and matrix side, respectively, and fix the carrier in the c-state and m-state, respectively. Therefore, studies on the interaction between the ADP/ATP carrier and these inhibitors are expected to be effective for understanding the molecular mechanism of this nucleotide exchange. The detailed manner of interaction of the ADP/ATP carrier with CATR has been clarified by structural analysis of their co-crystal (9); however, that with BKA remains elusive.

For a better understanding of the interaction of the ADP/ATP carrier with BKA, studies on the actions of BKA analogues should be helpful. Thus, in this study, we examined the effects of a variety of BKA analogues, derived from the intermediates obtained during the process of BKA synthesis, on mitochondrial functions and the ADP/ATP carrier.

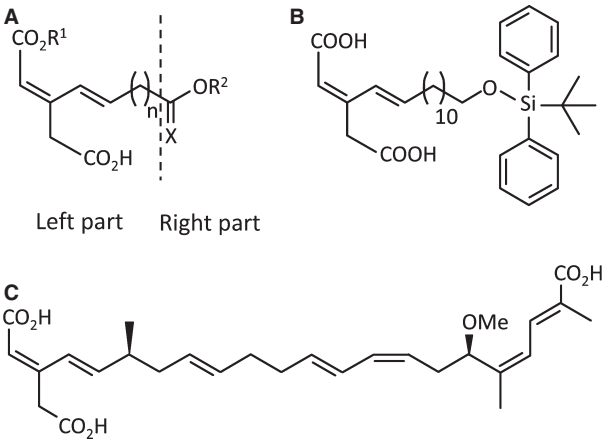


Figure 1: General structure of the bongkrekic acid (BKA) analogues used in the present study (A) and chemical structure of KH-7 (B) and parental BKA (C) For the structural properties of the BKA analogues, see the text. For the substituents R^1, R^2, X, or chain length n of individual analogues, see Table 1.

Materials and Methods

Chemicals and reagents

Authentic BKA was provided by Prof. Hans J. Duine (Delft University, The Netherlands). [2,8-^{3}H] adenosine 5′-diphosphate (code NET241), hereafter referred to just as [^{3}H]ADP, was purchased from PerkinElmer, Inc., Waltham, MA, USA

The BKA analogues, compounds KH-1~14, summarized in Figure 1A and Table 1, were prepared as indicated below (see also ref. 11 and Schemes 1 and 2). The method for synthesis of KH-15, 16, and 17 was already reported previously (12).

Materials for chemical synthesis

The ^{1}H- and ^{13}C-NMR spectra were recorded using a JNM LA-400 spectrometer, JEOL Ltd., Tokyo, Japan (400 and 100 MHz). The IR spectra were recorded on a Shimadzu FT/IR-8300 spectrometer (Shimadzu Co., Kyoto, Japan) 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., Tokyo, Japan). Thin-layer chromatography was performed on precoated plates (0.25 mm, silica gel 60 F254, Merck & Co, Inc., Kenilworth, NJ, USA.). Reaction mixtures were stirred magnetically.

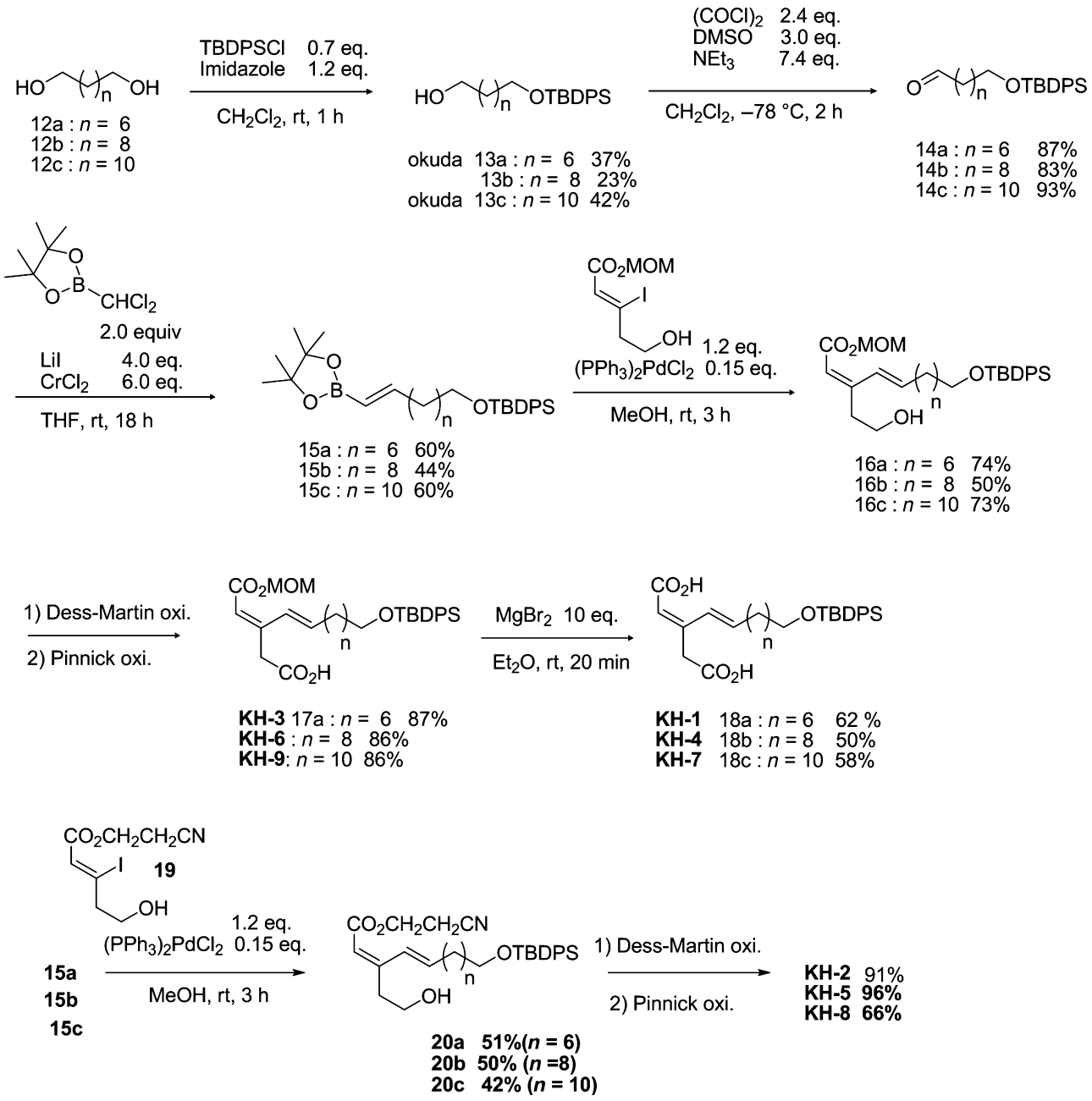
8-((tert-butyldiphenylsilyl)oxy)octan-1-ol (13a)

To a solution of 1.8-octanediol (10 g, 68.4 mmol) in CH_2Cl_2 (230 mL) were added imidazole (5.60 g, 82.1 mmol) and TBDPSCl (13.1 g, 47.8 mmol). The reaction mixture was stirred at RT for 12 h and added sat, $NaHCO_3$ aq, extracted with CH_2Cl_2, and washed with brine, dried over $MgSO_4$. The crude product was purified by silica gel column chromatography (Hexane/EtOAc = 4/1) to give colorless oil (9.81 g, 37%):^{1}H-NMR (400 MHz in $CDCl_3$) δ: 1.05 (s, 9H), 1.20–1.29 (m, 7H), 1.52–1.59 (m, 5H), 3.61–3.67 (m, 4H), 7.26–7.44 (m, 6H), 7.68 (m, 4H); ^{13}C-NMR

Table 1: Structural features of the bongkrekic acid analogues used in the present study

Code	R^1	R^2	X	*n*	Alias or reference
KH-1	H	Si(^{t}Bu)Ph_2	H,H	6	18a
KH-2	CH_2CH_2CN	Si(^{t}Bu)Ph_2	H,H	6	21a
KH-3	CH_2OCH_3	Si(^{t}Bu)Ph_2	H,H	6	17a
KH-4	H	Si(^{t}Bu)Ph_2	H,H	8	18b
KH-5	CH_2CH_2CN	Si(^{t}Bu)Ph_2	H,H	8	21b
KH-6	CH_2OCH_3	Si(^{t}Bu)Ph_2	H,H	8	17b
KH-7	H	Si(^{t}Bu)Ph_2	H,H	10	18c
KH-8	CH_2CH_2CN	Si(^{t}Bu)Ph_2	H,H	10	21c
KH-9	CH_2OCH_3	Si(^{t}Bu)Ph_2	H,H	10	17c
KH-10	CH_2OCH_3	Si(^{t}Bu)Me_2	H,H	10	27a
KH-11	CH_2OCH_3	$SiPh_3$	H,H	10	27b
KH-12	CH_2OCH_3	CPh_3	H,H	10	27c
KH-13	CH_2OCH_3	H	H,H	10	28
KH-14	CH_2OCH_3	CH_2OCH_3	O	10	35
KH-15	H	H	O	10	Ref. (12)
KH-16	CH_2OCH_3	H	O	16	Ref. (12)
KH-17	H	H	O	16	Ref. (12)

This table summarizes the structural properties of the 17 BKA analogues (see Figure 1A for their general structure). The symbols with 'KH-xx' represent the names of the individual chemicals; and structures or atoms shown in the columns 'R^1', 'R^2', and 'X' are the substituents of the individual chemicals at these positions. The numbers shown in column '*n*' represent the number of repeated alkyl chain. The actual structure of one compound, KH-7, of which R^1, R^2, X, and n are H, Si(^{t}Bu)Ph_2, H2, and 10, respectively, is shown in Figure 1B. The codes or reference No. shown in the column 'Alias or reference' indicate the alias of individual chemicals used in the process of their synthesis (see Schemes 1 and 2) or the reference No. for a study in which the procedures for the synthesis of these analogues are described.

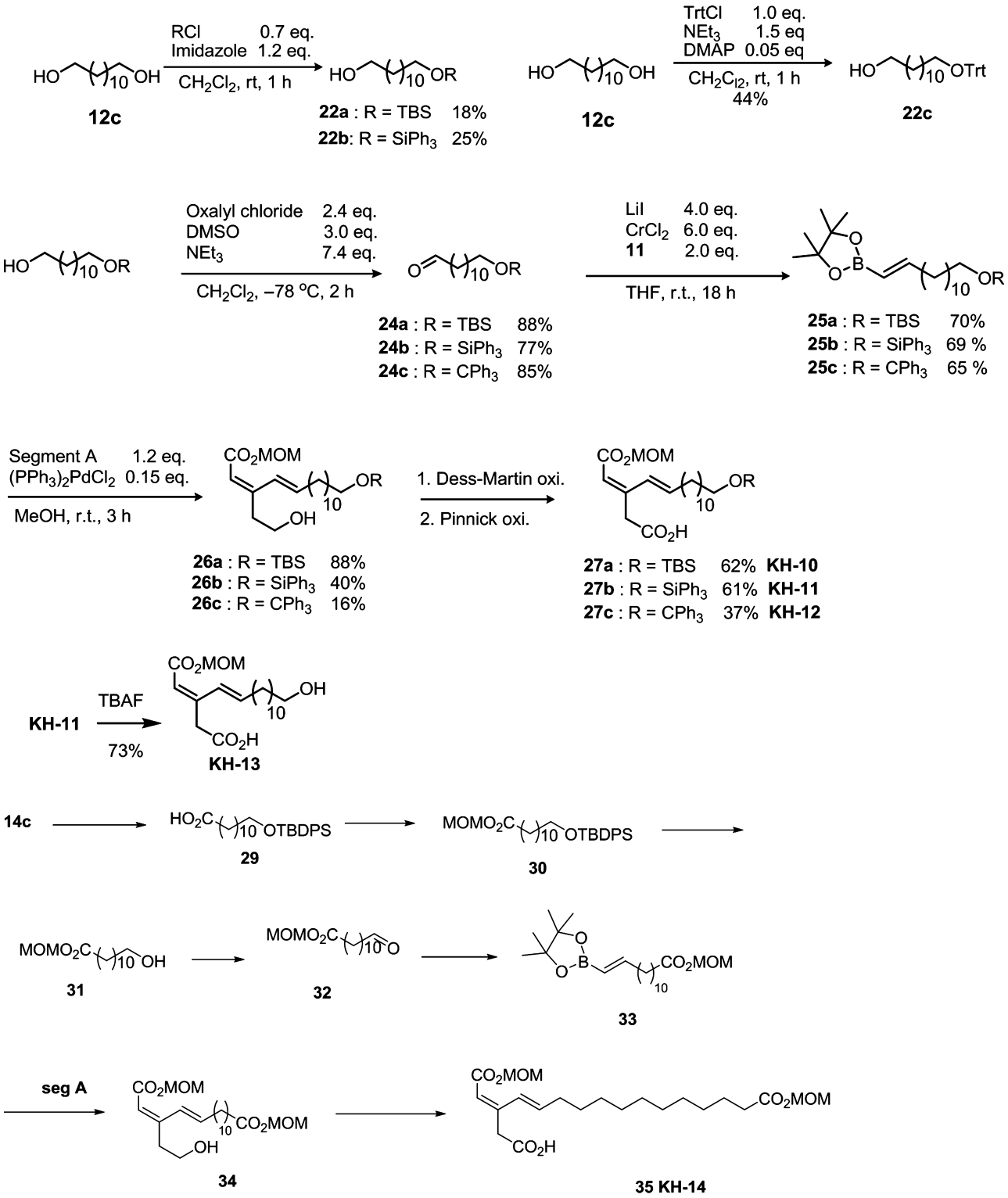


Scheme 1: Outline of the procedure used for the synthesis of KH-1~9.

(100 MHz, $CDCl_3$) δ: 19.1, 25.6, 25.7, 25.8, 26.8, 29.3, 32.5, 32.7, 62.8, 63.9, 127.5, 129.4, 134.0, 135.5; IR (Neat): 3342, 2930, 2850, 1589 cm^{-1}.

8-((tert-butyldiphenylsilyl)oxy)octanal (14a)

To a solution of oxalyl chloride (2.70 mL, 31.2 mmol) in CH_2Cl_2 (70 mL) was added DMSO (2.77 mL, 39.0 mmol) at −78 °C. and stirred for 20 min, and alcohol (5.0 g, 13.0 mmol) in CH_2Cl_2 (10 mL) was added at −78 °C. The reaction mixture stirred at −78 °C for 20 min, and NEt_3 (13.5 mL, 96.2 mmol) was added at −78 °C. The mixture was stirred at RT for 1 h and quenched with sat, $NaHCO_3$ aq., extracted with CH_2Cl_2, and washed with brine, dried over $MgSO_4$. The crude product was purified by silica gel column chromatography (Hexane/EtOAc = 5:1) to give a yellow oil. (4.32 g, 87%): ^{1}H-NMR (400 MHz in $CDCl_3$) δ: 1.03 (s, 9H), 1.23–1.30 (m, 8H),1.52–1.61 (m, 2H), 2.41 (m, 2H), 3.65 (t, J = 9.4 Hz, 2H), 7.35–7.42 (m, 6H), 7.65–7.68 (m, 4H), 9.76 (t, J = 2.8 Hz, 1H).



Scheme 2: Outline of the procedure used for the synthesis of KH-10~14.

(E)-tert-butyldiphenyl((9-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)non-8-en-1-yl)oxy)silane (15a)

To a suspension of $CrCl_2$ (5.77 g, 47.0 mmol) and LiI (4.20 g, 31.4 mmol) in THF (20 mL) were added pinacol borane (2.48 g, 11.8 mmol) in THF (10 mL) and aldehyde (3.00 g, 7.84 mmol) in THF (10 mL). The mixture was stirred at RT for 3 h, and added sat.$NaHCO_3$ aq, extracted with EtOAc, and washed with brine, dried over $MgSO_4$.

The crude product was purified by silica gel column chromatography (Hexane/EtOAc = 10/1) to give pale yellow oil (2.37 g, 60%): ^{1}H-NMR (400 MHz in $CDCl_3$) δ: 1.04 (s, 9H), 1.27–1.39(m, 20H), 1.53–1.56 (m, 2H), 2.13 (q, J = 7.2 Hz, 4H), 3.64 (t, J = 6.4 Hz, 6H), 5.42 (d, J = 18.4 Hz, 1H), 6.63 (td, J = 6.4, 18 Hz, 1H), 7.36–7.42 (m, 6H), 7.66–7.68 (m, 4H); ^{13}C-NMR (100 MHz, $CDCl_3$) δ:19.1, 24.7, 24.8, 25.7, 26.8, 28.2, 29.4, 29.3, 32.5, 35.8, 63.9, 82.8, 127.5, 129.4, 134.0, 135.5, 154.7; IR (Neat): 2933, 2852, 1641 cm^{-1}.

(2Z,4E)-methoxymethyl-12-((tert-butyldiphenylsilyl)oxy)-3-(2-hydroxyethyl)dodeca-2,4-dienoate (16a)

To a suspension of $Pd(PPh_3)_2Cl_2$ (225 mg, 0.32 mmol) in MeOH (4.0 mL) were added Segment A (540 mg, 1.89 mmol) and boronic ester (800 mg, 1.58 mmol) at RT. The mixture stirred at RT for 10 min, and NEt_3 (1.55 mL, 11.0 mmol) was added. The reaction mixture was stirred at RT for 6 h, and evaporated. The crude product was purified by silica gel column chromatography (Hexane/EtOAc = 2/1) to give a yellow oil (626 mg, 74%): ^{1}H-NMR (400 MHz in $CDCl_3$) δ: 1.05 (s, 9H), 1.25–1.28(m, 4H), 1.35–1.42 (m, 2H), 1.53–1.58 (m, 4H), 2.21 (q, J = 7.6 Hz, 2H), 2.63 (t, J = 6.4 Hz, 2H), 3.48 (s, 3H), 3.65 (t, J = 6.6 Hz, 2H), 3.79 (q, J = 6.8 Hz, 2H), 5.28 (s, 2H), 5.69 (s, 1H),6.22 (td, J = 7.6 Hz, 16.4 Hz, 1H), 7.36–7.44 (m, 6H), 7.53 (d, J = 16.4 Hz, 1H), 7.66–7.68 (m, 4H); ^{13}C-NMR (100 MHz, $CDCl_3$) δ:19.1, 25.6, 26.8, 28.9, 29.0, 29.2, 32.4, 33.4, 37.5, 57.5, 61.8, 63.9, 89.8, 115.7, 126.5, 127.5, 129.4, 134.0, 135.5, 140.0, 153.1, 165.4; IR (Neat): 3421, 2930, 2858, 1716, 1635, 1597 cm^{-1}.

(3Z,4E)-12-((tert-butyldiphenylsilyl)oxy)-3-(2-(methoxymethoxy)-2-oxoethylidene)dodec-4-enoic acid (KH-3, 17a)

To a solution of alcohol (200 mg, 0.371 mmol) in CH_2Cl_2 (14.8 mL) was added DMP (314 mg, 0.742 mmol). The reaction mixture was stirred at RT for 0.5 h, and added sat. $Na_2S_2O_3$ aq., extracted with CH_2Cl_2, washed with brine, dried over $MgSO_4$ and gave a crude product.

To a solution of the crude product in tBuOH/THF/2-methyl-2-butene = 3/1/1 (7.6 mL) were added $NaClO_2$ (43 mg, 0.47 mmol) and NaH_2PO_4•$2H_2O$ (103 mg, 0.66 mmol) in H_2O (7.6 mL). The reaction mixture was stirred at RT for 0.5 h, and extracted with EtOAc, and washed with brine, dried over $MgSO_4$. The crude product was purified by silica gel column chromatography (Hexane/EtOAc = 2/1) to give colorless oil (180 mg, 87%): ^{1}H-NMR (400 MHz in $CDCl_3$) δ: 1.04 (s, 9H), 1.24–1.28(m, 6H), 1.42 (brs, 2H), 1.53–1.54 (m, 2H), 2.22 (q, J = 7.2 Hz, 2H), 3.38 (s, 2H), 3.48 (s, 3H), 3.65 (t, J = 6.6 Hz, 2H), 3.65 (t, J = 6.8 Hz, 2H), 5.29 (s, 1H), 5.74 (s, 2H),6.22 (td, J = 7.2 Hz, 16.4 Hz, 1H), 7.36–7.44 (m, 6H), 7.56 (d, J = 16.4 Hz, 1H), 7.67 (m, 4H); ^{13}C-NMR (100 MHz, $CDCl_3$) δ:19.1, 25.4, 26.8, 28.6, 28.8, 28.9, 32.2, 33.4, 40.0, 57.6, 64.0, 90.1, 118.1, 126.3, 127.6, 129.5, 133.9, 135.6, 140.8, 147.7, 165.0; IR (Neat): 3244, 2930, 2856, 1716, 1695, 1633, 1602 cm^{-1}.

(Z)-3-((E)-9-((tert-butyldiphenylsilyl)oxy)non-1-en-1-yl)pent-2-enedioic acid (KH-1, 18a)

To a solution of ester (10 mg, 0.018 mmol) in Et_2O (1.7 mL) was added $MgBr_2$ (33 g, 0.18 mmol). The reaction mixture was stirred at RT for 20 min, and added 3 M HCl and H_2O, extracted with EtOAc, and washed with brine, dried over $MgSO_4$. The crude product was purified by silica gel column chromatography ($CHCl_3$/MeOH=/19/1) to give colorless solid (5.7 mg, 62%):^{1}H-NMR (400 MHz in $CDCl_3$) δ: 1.04 (s, 9H), 1.23–1.28(m, 6H), 1.41 (brs, 2H), 1.52–1.54 (m, 2H), 2.21 (q, J = 7.6 Hz, 2H), 3.38 (s,2H), 3.65 (t, J = 13.6 Hz, 2H), 5.73 (s,1H), 6.22 (td, J = 7.2, 16.0 Hz, 1H), 7.36–7.42 (m, 6H), 7.51 (d, J = 15.6 Hz, 1H), 7.67 (m, 4H); ^{13}C-NMR (100 MHz, $CDCl_3$) δ:20.0, 26.8, 27.3, 29.9, 30.1, 30.2, 33.5, 34.3, 41.2, 65.0, 119.8, 127.9, 128.7, 130.8, 135.1, 136.7, 140.3, 149.1, 169.3, 174.4; IR (Neat): 3508, 2929, 2856, 1697, 1631, 1604 cm^{-1}.

10-(tert-butyldiphenylsilyloxy)decan-1-ol (13b)

To a solution of 1.10-decanediol (10 g, 58.0 mmol) in CH_2Cl_2 (290 mL) were added imidazole (4.73 g, 69.6 mmol) and TBDPSCl (9.56 g, 34.8 mmol). The reaction mixture was stirred at RT for 40 min, and added sat, $NaHCO_3$ aq, extracted with CH_2Cl_2, and washed with brine, dried over $MgSO_4$. The crude product was purified by silica gel column chromatography (Hexane/EtOAc=4/1) to give colorless oil (5.39 g, 23%): ^{1}H-NMR (400 MHz in $CDCl_3$) δ: 1.04 (s, 9H), 1.26 (brs, 16H), 3.62–3.3.67 (m, 4H), 7.37–7.7.42 (m, 6H), 7.65–7.68 (m, 4H); ^{13}C-NMR (100 MHz, $CDCl_3$) δ: 19.1, 25.7, 26.8, 29.3, 29.4, 32.5, 62.9, 63.9, 127.5, 129.4, 134.1, 135.5; IR (Neat): 3340, 2929, cm^{-1}.

10-((tert-butyldiphenylsilyl)oxy)decanal (14b)

To a solution of oxalyl chloride (2.60 mL, 30.2 mmol) in CH_2Cl_2 (74 mL) was added DMSO (2.68 mL, 37.8 mmol) at −78 °C. and stirred for 20 min, and alcohol (5.22 g, 12.6 mmol) in CH_2Cl_2 (10 mL) was added at −78 °C. The reaction mixture stirred at −78 °C for 20 min, and NEt_3 (7.47 mL, 53.2 mmol) was added at −78 °C. The mixture was stirred at RT for 1 h, and quenched with sat, $NaHCO_3$ aq., extracted with CH_2Cl_2, and washed with brine, dried over $MgSO_4$. The crude product was purified by silica gel column chromatography (Hexane/EtOAc =4:1) to give a yellow oil. (4.27 g, 83%):^{1}H-NMR (400 MHz in $CDCl_3$) δ: 1.04 (s, 9H), 1.26 (brs, 12H), 1.52–1.62 (m, 3H), 2.41 (m, 2H), 3.64 (t, J = 6.3 Hz, 2H), 7.35–7.7.42 (m, 6H), 7.65–7.68 (m, 4H), 9.76 (t, J = 2.8 Hz, 1H).

(E)-tert-butyldiphenyl(13-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)tridec-12-enyloxy)silane (15b)

To a suspension of $CrCl_2$ (4.60 g, 37.4 mmol) and LiI (4.56 g, 34.1 mmol) in THF (50 mL) were added pinacol borane (3.58 g, 17.0 mmol) in THF (10 mL) and aldehyde (3.50 g, 8.52 mmol) in THF (15 mL). The mixture was stirred at RT for 14 h, and added sat.$NaHCO_3$ aq, extracted with EtOAc, and washed with brine, dried over $MgSO_4$. The crude product was purified by silica gel column chromatography (Hexane/EtOAc=10/1) to give yellow oil (2.03 g, 44%): ^{1}H-NMR (400 MHz in $CDCl_3$) δ: 1.04 (s, 9H), 1.24–1.57(m, 26H), 2.13 (m, 2H), 3.64 (t, J = 6.4 Hz, 2H), 5.42 (d, J = 18.1 Hz, 1H), 6.63 (td, J = 6.5, 17.8 Hz, 1H), 7.35–7.44 (m, 6H), 7.65–7.68 (m, 4H); ^{13}C-NMR (100 MHz, $CDCl_3$) δ: 19.1, 24.7, 25.6, 26.8, 28.1, 29.0, 29.1, 32.5, 35.7, 63.8, 82.8, 127.5, 129.4, 134.0, 135.5, 154.6; IR (Neat): 2929, 2858, 1637, cm^{-1}.

(2Z,4E)-methoxymethyl-14-((tert-butyldiphenylsilyl)oxy)-3-(2-hydroxyethyl)tetradeca-2,4-dienoate (16b)

To a suspension of $Pd(PPh_3)_2Cl_2$ (204 mg, 0.29 mmol) in MeOH (3.3 mL) were added Segment A (497 mg, 1.74 mmol) and boronic ester (800 g, 1.45 mmol) at RT. The mixture stirred at RT for 10 min, and NEt_3 (1.44 mL, 10.2 mmol) was added. The reaction mixture was stirred at RT for 3 h, and evaporated. The crude product was purified by silica gel column chromatography (Hexane/EtOAc=2/1) to give a yellow oil (414 mg, 50%): ^{1}H-NMR (400 MHz in $CDCl_3$) δ: 1.04 (s, 9H), 1.16–1.60 (m, 14H), 2.20 (q, J = 6.8 Hz, 2H), 2.63 (t, J = 6.4 Hz, 2H), 3.42 (s, 3H), 3.65 (t, J = 6.5 Hz, 2H), 3.79 (q, J = 6.8 Hz, 2H), 5.28 (s, 1H), 5.69 (s, 2H),6.22 (td, J = 6.8, 16.0 Hz, 1H), 7.30–7.44 (m, 6H), 7.48 (d, J = 16.2 Hz, 1H), 7.67 (m, 4H); ^{13}C-NMR (100 MHz, $CDCl_3$) δ:19.1, 25.7, 26.8, 28.9, 29.1, 29.2, 29.3, 29.4, 32.5, 33.4, 37.5, 57.5, 61.8, 63.9, 89.8, 115.7, 126.5, 127.5, 129.4, 134.0, 135.5, 140.0, 153.1, 165.4; IR (Neat): 3481, 2929, 2856, 1718, 1134 cm^{-1}.

(3Z,4E)-16-(tert-butyldiphenylsilyloxy)-3-(2-(methoxymethoxy)-2-oxoethylidene)hexadec-4-enoic acid (KH-6, 17b)

To a solution of alcohol (30 mg, 0.053 mmol) in CH_2Cl_2 1.1 mL) was added DMP (45 mg, 0.11 mmol). The reaction mixture was stirred at RT for 1 h, and added sat. $Na_2S_2O_3$ aq., extracted with CH_2Cl_2, washed with brine, dried over $MgSO_4$ and gave a crude product.

To a solution of the crude product in *t*BuOH/THF/2-methyl-2-butene = 3/1/1 (1.1 mL) were added $NaClO_2$ (24 mg, 0.27 mmol) and NaH_2PO_4•$2H_2O$ (58 mg, 0.37 mmol) in H_2O (1.1 mL). The reaction mixture was stirred at RT for 1 h, and extracted with EtOAc, and washed with brine, dried over $MgSO_4$. The crude product was purified by silica gel column chromatography (Hexane/EtOAc=2/1) to give colorless oil (17 mg, 55%):^{1}H-NMR (400 MHz in $CDCl_3$) δ: 1.04 (s, 9H), 1.26–1.28 (m, 10H), 1.43 (brs, 2H), 1.51–1.56 (m, 2H), 2.22 (q, J = 6.8 Hz, 2H), 3.38 (s, 2H), 3.48 (s, 3H), 3.65 (t, J = 6.6 Hz, 2H), 5.28 (s, 2H), 5.74 (s, 1H), 6.20 (td, J = 6.8 Hz, 16.8 Hz, 1H), 7.35–7.44 (m, 6H), 7.56 (d, J = 16 Hz, 1H), 7.68 (m, 4H); ^{13}C-NMR (100 MHz, CD_3OD) δ: 20.0, 26.8, 27.4, 29.9, 30.2, 30.3, 30.4, 30.6, 33.6, 34.4, 57.7, 65.0, 90.9, 118.6, 127.7, 128.7, 130.8, 135.1, 136.6, 141.3, 150.6, 166.6, 174.1; IR (Neat): 3512, 2929, 1716, 1633, 1602 cm^{-1}; Mass (FAB) m/z 581 (M^++1); HRMS calcd for $C_{48}H_{49}O_6Si$: 581.3298 found 581.3285.

(Z)-3-((E)-11-((tert-butyldiphenylsilyl)oxy)undec-1-en-1-yl)pent-2-enedioic acid (KH-4, 18b)

To a solution of ester (84 mg, 0.14 mmol) in Et_2O (11 mL) was added $MgBr_2$ (258 g, 0.14 mmol). The reaction mixture was stirred at RT for 20 min, and added 3M HCl and H_2O, extracted with EtOAc, and washed with brine, dried over $MgSO_4$. The crude product was purified by silica gel column chromatography ($CHCl_3$/MeOH=/19/1) to give colorless solid (37.7 mg, 50%): ^{1}H-NMR (400 MHz in $CDCl_3$) δ: 1.04 (s, 9H), 1.21–1.41 (m, 10H), 1.41 (brs, 2H), 1.53–1.56 (m, 2H), 2.21 (q, J = 6.8 Hz, 2H), 3.37 (s,2H), 3.65 (t, J = 6.6 Hz, 2H), 5.72 (s,1H), 6.22 (dt, J = 7.2, 16.0 Hz, 1H), 7.36–7.42 (m, 6H), 7.50 (d, J = 16.4 Hz, 1H), 7.67 (m, 4H); ^{13}C-NMR (100 MHz, CD_3OD) δ: 19.2, 25.7, 26.9, 28.8, 29.2, 29.3, 29.4, 32.5, 33.5, 40.3, 64.0, 117.9, 126.3, 127.5, 129.4, 134.1, 135.6, 141.5, 148.9, 171.2, 176.2; IR (Neat): 3497, 2926, 2852, 1699, 1631, 1606 cm^{-1}.

12-(tert-butyldiphenylsilyloxy)dodecan-1-ol (13c)

To a solution of 1.12-dodecanediol (5.00 g, 24.7 mmol) in CH_2Cl_2 were added imidazole (2.01 g, 29.6 mmol) and TBDPSCl (4.75 g, 17.3 mmol). The reaction mixture was stirred at RT for 2 h, and added sat, $NaHCO_3$ aq, extracted with CH_2Cl_2, and washed with brine, dried over $MgSO_4$. The crude product was purified by silica gel column chromatography (Hexane/EtOAc=3/1) to give colorless oil (3.83 g, 35%): ^{1}H-NMR (400 MHz in $CDCl_3$) δ: 1.05 (s, 9H), 1.25–1.31(m, 18H), 1.53–1.57 (m, 6H), 3.65 (t, J = 6.6 Hz, 4H), 7.36–7.42 (m, 6H), 7.67 (m, 4H):^{13}C-NMR (100 MHz, $CDCl_3$) δ:19.2, 25.7, 26.8, 29.3, 29.4, 29.5, 29.6, 32.5, 32.7, 63.0, 64.0, 127.5, 129.4, 134.1, 135.5; IR (Neat): 3336, 2928, 2854, 1589 cm^{-1}.

12-(tert-butyldiphenylsilyloxy)dodecanal (14c)

To a solution of oxalyl chloride (0.95 mL, 11.0 mmol) in CH_2Cl_2 (30 mL) was added DMSO (0.97 mL, 13.7 mmol) at −78 °C. and stirred for 20 min, and alcohol **13c**

(2.02 g, 4.58 mmol) in CH_2Cl_2 (10 mL) was added at −78 °C. The reaction mixture stirred at −78 °C for 20 min, and NEt_3 (4.83 mL, 34.4 mmol) was added at −78 °C. The mixture was stirred at RT for 1 h, and quenched with sat, $NaHCO_3$ aq., extracted with CH_2Cl_2, and washed with brine, dried over $MgSO_4$. The crude product was purified by silica gel column chromatography (Hexane/EtOAc =4: 1) to give a yellow oil. (1.86 g, 93%): ^{1}H-NMR (400 MHz in $CDCl_3$) δ: 1.07 (s, 9H), 1.25–1.29 (m, 15H), 1.52–1.64 (m, 7H), 2.42 (t, J = 8.0 Hz, 2H), 3.65 (t, J = 6.6 Hz, 2H), 7.36–7.44 (m, 6H), 7.67 (m, 4H), 9.76 (t, J = 4.0 Hz, 1H).

(E)-tert-butyldiphenyl(13-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)tridec-12-enyloxy)silane (15c)

To a suspension of $CrCl_2$ (4.05 g, 33.0 mmol) and LiI (2.94 g, 22.0 mmol) in THF (50 mL) were added pinacol borane **11** (2.20 g, 11.0 mmol) in THF (5.0 mL) and aldehyde **14c** (2.41 g, 5.50 mmol) in THF (5.0 mL). The mixture was stirred at RT for 3 h, and added sat.$NaHCO_3$ aq, extracted with EtOAc, and washed with brine, dried over $MgSO_4$. The crude product was purified by silica gel column chromatography (Hexane/EtOAc=10/1) to give yellow oil (1.87 g, 60%): ^{1}H-NMR (400 MHz in $CDCl_3$) δ: 1.04 (s, 9H), 1.26–1.42(m, 28H), 1.52–1.59 (m, 2H), 2.14 (q, J = 6.8 Hz, 4H), 3.65 (t, J = 6.6 Hz, 6H), 5.42 (d, J = 18.4 Hz, 1H), 6.63 (td, J = 6.8, 18.0 Hz, 1H), 7.36–7.43 (m, 6H), 7.66–7.68 (m, 4H) 01-193;^{13}C-NMR (100 MHz, $CDCl_3$) δ:19.2, 24.7, 25.7, 26.8, 28.1, 29.2, 29.3, 29.4, 29.5, 29.6, 32.5, 35.8, 63.9, 82.9, 127.5, 129.4, 134.1, 135.5, 154.8; IR (Neat): 2978, 2928, 1637 cm^{-1}.

(2Z,4E)-methoxymethyl-16-(tert-butyldiphenylsilyloxy)-3-(2-hydroxyethyl)hexadeca-2,4-dienoate (16c)

To a suspension of $Pd(PPh_3)_2Cl_2$ (250 mg, 0.36 mmol) in MeOH (20 mL) were added Segment A (560 mg, 1.96 mmol) and boronic ester **15c** (1.00 g, 1.78 mmol) at RT. The mixture stirred at RT for 10 min, and NEt_3 (1.76 mL, 12.5 mmol) was added. The reaction mixture was stirred at RT for 3 h, and evaporated. The crude product was purified by silica gel column chromatography (Hexane/EtOAc=2/1) to give a yellow oil (781 mg, 73%): ^{1}H-NMR (400 MHz in $CDCl_3$) δ: 1.05 (s, 9H), 1.20–1.28 (m, 9H), 1.39–1.42 (m,3H), 1.53–1.58 (m, 6H), 2.22 (q, J = 7.6 Hz, 2H), 2.63 (t, J = 6.6 Hz, 2H), 3.48 (s, 3H), 3.65 (t, J = 7.0 Hz, 2H), 3.78 (q, J = 6.6 Hz, 2H), 5.28 (s, 2H), 5.68 (s, 1H),6.22 (dt, J = 6.8 Hz, 16.4 Hz, 1H), 7.36–7.44 (m, 6H), 7.53 (d, J = 16.0 Hz, 1H), 7.67 (m, 4H); ^{13}C-NMR (100 MHz, $CDCl_3$) δ:19.1, 25.7, 26.8, 28.9, 29.2, 29.3, 29.4, 29.5, 32.5, 33.5, 37.5, 57.5, 61.8, 64.0, 89.8, 115.7, 126.5, 127.5, 129.4, 134.1, 135.5, 140.1, 153.1, 165.4; IR (Neat): 3481, 2929, 2856, 1718 cm^{-1}.

(3Z,4E)-16-(tert-butyldiphenylsilyloxy)-3-(2-(methoxymethoxy)-2-oxoethylidene)hexadec-4-enoic acid (KH-9, 17c)

To a solution of alcohol **16c** (200 mg, 0.336 mmol) in CH_2Cl_2 (6.7 mL) were added DMP (570 mg, 1.34 mmol). The reaction mixture was stirred at RT for 1 h, and added sat. $Na_2S_2O_3$ aq., extracted with CH_2Cl_2, washed with brine, dried over $MgSO_4$ and gave a crude product.

To a solution of the crude product in *t*BuOH/THF/2-methyl-2-butene =3/1/1 (6.7 mL) were added $NaClO_2$ (152 mg, 1.69 mmol) and NaH_2PO_4•$2H_2O$ (368 mg, 2.36 mmol) in H_2O (6.7 mL). The reaction mixture was stirred at RT for 1 h, and extracted with EtOAc, and washed with brine, dried over $MgSO_4$. The crude product was purified by silica gel column chromatography (Hexane/EtOAc=2/1) to give colorless oil (173 mg, 86%):^{1}H-NMR (400 MHz in $CDCl_3$) δ: 1.04 (s, 9H), 1.24–1.31(m, 12H), 1.43 (brs, 2H), 1.53–1.58 (m,4H), 2.20 (q, J = 7.2 Hz, 2H), 3.38 (s, 2H), 3.48 (s, 3H), 3.65 (t, J = 6.6 Hz, 2H), 5.28 (s, 2H), 5.74 (s, 1H), 6.20 (dt, J = 6.8 Hz, 16 Hz, 1H), 7.36–7.42 (m, 6H), 7.56 (d, J = 16.4 Hz, 1H), 7.67 (m, 4H); ^{13}C-NMR (100 MHz, CD_3OD) δ: 20.0, 26.9, 27.4, 29.9, 30.2, 30.3, 30.5, 30.6, 30.7, 33.6, 34.4, 41.1, 57.7, 65.0, 90.9, 118.6, 127.8, 128.7, 130.8, 135.1, 136.7, 141.3, 150.6, 166.7, 174.0; IR (Neat): 3292, 2933, 2854, 1710, 1635, 1602 cm^{-1}; Mass (FAB) m/z 631 (M^++Na); HRMS calcd for $C_{36}H_{52}NaO_6Si$: 631.3431 found 631.3427.

(Z)-3-((E)-13-((tert-butyldiphenylsilyl)oxy)tridec-1-en-1-yl)pent-2-enedioic acid (KH-7, 18c)

To a solution of ester **17c** (56 mg, 0.092 mmol) in Et_2O (7.0 mL) was added $MgBr_2$ (102 mg, 0.55 mmol). The reaction mixture was stirred at RT for 20 min, and added 3M HCl and H_2O, extracted with EtOAc, and washed with brine, dried over $MgSO_4$. The crude product was purified by silica gel column chromatography ($CHCl_3$/MeOH=/19/1) to give colorless solid (30 mg, 58%): ^{1}H-NMR (400 MHz in $CDCl_3$) δ: 1.04 (s, 9H), 1.24 (brs, 14H), 1.41–1.43 (m, 2H), 1.51–1.61 (m, 2H), 2.22 (q, J = 7.6 Hz, 2H), 3.38 (s,2H), 3.65 (t, J = 6.6 Hz, 2H), 5.73 (s,1H), 6.22 (dt, J = 7.2, 16.0 Hz, 1H), 7.35–7.43 (m, 6H), 7.51 (d, J = 16.4 Hz, 1H), 7.66–7.68 (m, 1H); ^{13}C-NMR (100 MHz, CD_3OD): 19.2, 25.8, 26.9, 28.9, 29.2, 29.3, 29.4, 29.5, 29.6, 29.7, 33.5, 40.2, 64.1, 117.8, 126.3, 127.6, 129.4, 134.1, 135.6, 141.5, 148.9, 170.9, 175.9; IR (Neat): 3349, 2926, 2852, 1697, 1631, 1606 cm^{-1}.

(2Z,4E)-2-cyanoethyl 12-((tert-butyldiphenylsilyl) oxy)-3-(2-hydroxyethyl)dodeca-2,4-dienoate (20a)

To a suspension of $Pd(PPh_3)_2Cl_2$ (166 mg, 0.24 mmol) in MeOH (6.0 mL) were added cyanoethylester (417 mg, 1.41 mmol) and boronic ester (600 g, 1.18 mmol) at RT. The mixture stirred at RT for 10 min, and NEt_3 (1.15 mL, 8.19 mmol) was added. The reaction mixture was stirred

at RT for 3 h, and evaporated. The crude product was purified by silica gel column chromatography (Hexane/EtOAc=2/1) to give a yellow oil (328 mg, 51%):^{1}H-NMR (400 MHz in $CDCl_3$) δ: 1.05 (s,9H), 1.24–1.59 (m, 10H), 2.22 (q, J = 7.6 Hz, 2H), 2.63 (t, J = 6.6 Hz, 2H), 2.72 (t, J = 6.4 Hz, 2H), 3.65 (t, J = 6.6 Hz, 2H), 3.79 (q, J = 6.0 Hz, 2H), 4.31 (t, J = 6.4 Hz, 2H), 5.68 (s, 1H), 6.25 (td, J = 6.8, 16.0 Hz, 1H), 7.35–7.44 (m, 6H), 7.49 (d, J = 16.0 Hz, 1H), 7.66–7.68 (m, 4H); ^{13}C-NMR (100 MHz, $CDCl_3$) δ:18.0, 19.2, 25.6, 26.8, 28.9, 29.2, 32.4, 33.5, 37.5, 58.1, 61.8, 63.8, 63.9, 114.9, 117.0, 126.3, 127.5, 129.4, 134.1, 135.5, 140.5, 153.7, 165.2; IR (Neat): 3419, 2930, 2857, 2252, 1716 cm^{-1}.

(3Z,4E)-14-((tert-butyldiphenylsilyl)oxy)-3-(2-(2-cyanoethoxy)-2-oxoethylidene)tetradec-4-enoic acid (KH-2, 21a)

To a solution of alcohol (100 mg, 0.183 mmol) in CH_2Cl_2 (7.3 mL) was added DMP (233 mg, 0.549 mmol). The reaction mixture was stirred at RT for 0.5 h, and added sat. $Na_2S_2O_3$ aq., extracted with CH_2Cl_2, washed with brine, dried over $MgSO_4$ and gave a crude product.

To a solution of the crude product in *t*BuOH/THF/2-methyl-2-butene =3/1/1 (4.0 mL) were added $NaClO_2$ (91 mg, 1.01 mmol) and $NaH_2PO_4{\cdot}2H_2O$ (229 mg, 1.47 mmol) in H_2O (4.0 mL). The reaction mixture was stirred at RT for 0.5 h, and extracted with EtOAc, and washed with brine, dried over $MgSO_4$. The crude product was purified by silica gel column chromatography (Hexane/EtOAc=2/1) to give colorless oil (94 mg, 91%): ^{1}H-NMR (400 MHz in $CDCl_3$) δ: 1.04 (s, 9H), 1.24–1.28 (m, 6H), 1.42 (brs, 2H), 1.52–1.54 (m, 2H),2.23 (q, J = 7.2 Hz, 2H), 2.73 (t, J = 6.2 Hz, 2H), 3.39 (s, 2H), 3.65 (t, J = 6.0 Hz, 2H), 4.32 (t, J = 6.6 Hz, 2H), 5.74 (s,1H), 6.22 (td, J = 7.2, 15.6 Hz, 1H), 7.36–7.44 (m, 6H), 7.52 (d, J = 16.4 Hz, 1H), 7.68 (m, 4H); ^{13}C-NMR (100 MHz, CD_3OD) δ: 18.4, 20.1, 26.8, 27.4, 29.9, 30.1, 35.5, 41.1, 59.9, 64.9, 118.4, 127.7, 128.7, 130.8, 135.1, 136.7, 141.5.

12-(tert-butyldimethylsilyloxy)dodecan-1-ol (22a)

To a solution of 1.12-dodecanediol (5.00 g, 24.7 mmol) in CH_2Cl_2 were added imidazole (2.01 g, 29.6 mmol) and TBSCl (2.70 g, 17.3 mmol). The reaction mixture was stirred at RT for 2 h, and added sat, $NaHCO_3$ aq, extracted with CH_2Cl_2, and washed with brine, dried over $MgSO_4$. The crude product was purified by silica gel column chromatography (Hexane/EtOAc=4/1) to give colorless oil (1.38 g, 18%): ^{1}H-NMR (400 MHz in $CDCl_3$) δ: 0.05 (s, 6H), 0.89 (s, 9H), 1.22 (brs, 14H), 1.46–1.51 (m, 2H), 3.53–3.59 (m, 4H); ^{13}C-NMR (100 MHz, $CDCl_3$) δ: 18.0, 19.2, 25.7, 26.8, 29.0, 29.1, 29.2, 29.3, 29.4, 29.5, 29.6, 32.6, 33.5, 37.5, 58.1, 61.9, 64.0, 114.9, 116.9, 126.3, 127.5, 129.4, 134.1, 135.5, 140.7, 153.7, 165.2; IR (Neat): 3335, 2928, 2854 cm^{-1}.

12-(tert-butyldimethylsilyloxy)dodecanal (24a)

To a solution of oxalyl chloride (0.39 mL, 4.56 mmol) in CH_2Cl_2 (15 mL) was added DMSO (0.40 mL, 5.70 mmol) at −78 °C. and stirred for 20 min, and alcohol **22a** (600 mg, 1.90 mmol) in CH_2Cl_2 (5.0 mL) was added at −78 °C. The reaction mixture stirred at −78 °C for 20 min, and and NEt_3 (1.88 mL, 13.3 mmol) was added at −78 °C. The mixture was stirred at RT for 1 h, and quenched with sat, $NaHCO_3$ aq., extracted with CH_2Cl_2, and washed with brine, dried over $MgSO_4$. The crude product was purified by silica gel column chromatography (Hexane/EtOAc =4: 1) to give a yellow oil. (524 g, 88%): ^{1}H-NMR (400 MHz in $CDCl_3$) δ: 0.05 (s, 9H), 0.89 (s, 9H), 1.27 (brs, 14H), 1.55–1.63 (m, 4H), 2.40–2.43 (m, 2H), 3.60 (t, J = 6.0 Hz, 2H), 9.76 (m, 1H).

(E)-tert-butyldimethyl(13-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)tridec-12-enyloxy)silane (25a)

To a suspension of $CrCl_2$ (1.22 g, 9.96 mmol) and LiI (888 mg, 6.64 mmol) in THF (9.0) were added pinacol borane **11** (525 mg, 2.49 mmol) in THF (4.0 mL) and aldehyde **24a** (524 mg, 1.66 mmol) in THF (4.0 mL). The mixture was stirred at RT for 2 h, and added sat.NaHCO$_3$ aq, extracted with EtOAc, and washed with brine, dried over $MgSO_4$. The crude product was purified by silica gel column chromatography (Hexane/EtOAc=10/1) to give yellow oil (524 g, 70%): ^{1}H-NMR (400 MHz in $CDCl_3$) δ: 0.05 (s, 6H), 0.89 (s, 9H), 1.24–1.52 (m, 30H), 2.14 (q, J = 7.2 Hz, 2H), 3.59 (t, J = 6.6 Hz, 2H), 5.42 (d, J = 18.0 Hz, 1H), 6.63 (td, J = 6.4, 18.4 Hz, 1H); ^{13}C-NMR (100 MHz, $CDCl_3$) δ: 18.0, 24.4, 24.5, 25.4, 25.6, 27.8, 28.9, 29.0, 29.1, 29.2, 29.3, 29.4, 32.5, 35.5, 62.9, 82.5, 154.4; IR (Neat): 2928, 2854, 1639 cm^{-1}.

(2Z,4E)-methoxymethyl-16-(tert-butyldimethylsilyloxy)-3-(2-hydroxyethyl)hexadeca-2,4-dienoate (26a)

To a suspension of $Pd(PPh_3)_2Cl_2$ (32 mg, 0.046 mmol) in MeOH (1.0 mL) were added Segment A (72 mg, 0.25 mmol) in MeOH (1.8 mL) and boronic ester **25a** (101 mg, 0.23 mmol) in MeOH (1.8 mL) at RT. The mixture stirred at RT for 10 min, and NEt_3 (0.23 mL, 1.61 mmol) was added. The reaction mixture was stirred at RT for 3 h, and evaporated. The crude product was purified by silica gel column chromatography (Hexane/EtOAc=2/1) to give a yellow oil (80 mg, 74%): ^{1}H-NMR (400 MHz in $CDCl_3$) δ: 0.05 (s, 6H), 0.89 (s, 9H), 1.27 (s, 15H), 1.40–1.54 (m, H), 2.22 (q, J = 6.8 Hz, 2H), 2.64 (t, J = 6.6 Hz, 2H), 3.48 (s, 3H), 3.60 (t, J = 6.8 Hz, 2H), 3.78 (q, J = 5.6 Hz, 2H), 5.28 (s, 2H), 5.68 (s, 1H), 6.23 (td, J = 6.8, 16.4 Hz, 1H), 7.53 (d, J = 16.0 Hz, 1H); IR (Neat): 3398, 2928, 2854, 1716, 1635, 1597 cm^{-1}; Mass (EI) m/z 470 (M^+); HRMS calcd for $C_{26}H_{50}O_5Si$: 470.3428 found 470.3432.

(3Z,4E)-16-(tert-butyldiphenylsilyloxy)-3-(2-(methoxymethoxy)-2-oxoethylidene)hexadec-4-enoic acid (KH-10, 27a)

To a solution of alcohol **26a** (80 mg, 0.17 mmol) in CH_2Cl_2 (3.4 mL) was added DMP (288 mg, 0.68 mmol). The reaction mixture was stirred at RT for 0.5 h, and added sat. $Na_2S_2O_3$ aq., extracted with CH_2Cl_2, washed with brine, dried over $MgSO_4$ and gave a crude product.

To a solution of the crude product in *t*BuOH/THF/2-methyl-2-butene = 3/1/1 (3.4 mL) were added $NaClO_2$ (78 mg, 0.866 mmol) and NaH_2PO_4•$2H_2O$ (187 mg, 1.20 mmol) in H_2O (3.4 mL). The reaction mixture was stirred at RT for 1.5 h, and extracted with EtOAc, and washed with brine, dried over $MgSO_4$. The crude product was purified by silica gel column chromatography (Hexane/EtOAc=2/1) to give colorless oil (50.7 mg, 62%): ^{1}H-NMR (400 MHz in $CDCl_3$) δ: 0.05 (s, 6H), 0.88 (s, 9H), 1.24–1.52 (m, 22 H), 2.22 (q, J = 7.2 Hz, 2H), 3.37 (s, 2H), 3.48 (s, 3H), 3.61 (t, J = 6.6 Hz, 2H), 5.29 (s, 2H), 5.74 (s, 1H), 6.21 (td, J = 7.2, 16.0 Hz, 1H), 7.56 (d, J = 16.0 Hz, 1H); ^{13}C-NMR (100 MHz, $CDCl_3$) δ: 18.2, 25.6, 28.3, 29.1, 29.2, 29.3, 29.4, 29.5, 32.7, 33.3, 37.4, 57.3, 61.6, 63.2. 89.7, 115.5, 126.4, 139.8, 153.2, 165.4; IR (Neat): 3325, 2928, 2854, 1716, 1635 1602 cm^{-1}; Mass (EI) m/z 484 (M^+); HRMS calcd for $C_{26}H_{48}O_6Si$: 484.3220 found 484.3218.

12-(triphenylsilyloxy)dodecan-1-ol (22b)

To a solution of 1.12-dodecanediol (5.00 g, 24.7 mmol) in CH_2Cl_2 were added imidazole (2.01 g, 29.6 mmol) and triphenylsilyl chloride (5.01 g, 17.3 mmol).The reaction mixture was stirred at RT for 1 h, and added H_2O, extracted with CH_2Cl_2, and washed with brine, dried over $MgSO_4$. The crude product was purified by silica gel column chromatography (Hexane/EtOAc=4/1) to give colorless oil (2.87 g, 25%): ^{1}H-NMR (400 MHz in $CDCl_3$) δ: 1.23–1.29 (m, 20H), 3.64 (brs, 2H), 3.78 (t, J = 9.6 Hz, 2H), 7.35–7.43 (m, 9H), 7.60–7.64 (m, 6H) ^{13}C-NMR (100 MHz, $CDCl_3$) δ:25.7, 29.2, 29.4, 29.6, 32.5, 32.7, 63.0, 63.9, 127.8, 130.0, 134.4, 135.3; IR (Neat): 3312, 2924, 2879 cm^{-1}.

12-(triphenylsilyloxy)dodecanal (24b)

To a solution of oxalyl chloride (0.45 mL, 5.21 mmol) in CH_2Cl_2 (15 mL) was added DMSO (0.46 mL, 6.51 mmol) at −78 °C and stirred for 20 min, and alcohol **22b** (1.00 g, 2.17 mmol) in CH_2Cl_2 (5.0 mL) was added at −78 °C. The reaction mixture stirred at −78 °C for 20 min, and NEt_3 (2.13 mL, 15.2 mmol) was added at −78 °C. The mixture was stirred at RT for 1 h, and quenched with sat. $NaHCO_3$ aq., extracted with CH_2Cl_2, and washed with brine, dried over $MgSO_4$. The crude product was purified by silica gel column chromatography (Hexane/EtOAc =4: 1) to give a yellow oil. (767 mg, 77%): ^{1}H-NMR (400 MHz in $CDCl_3$) δ: 1.22–1.29 (m, 16H), 1.60–1.64 (m, 4H), 2.41 (t, J = 7.4 Hz, 2H), 3.78 (t, J = 6.4 Hz, 2H), 7.40 (m, 9H), 7.62 (m, 6H), 9.76 (s, 1H).

(E)-triphenyl(13-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)tridec-12-enyloxy)silane (25b)

To a suspension of $CrCl_2$ (1.23 g 10.0 mmol) and LiI (892 mg, 6.68 mmol) in THF (9.0 mL) were added pinacol borane **11** (528 mg, 2.51 mmol) in THF (4.0 mL) and aldehyde **24b** (767 mg, 1.67 mmol) in THF (4.0 mL). The mixture was stirred at RT for 3 h, and added sat.$NaHCO_3$, extracted with EtOAc, and washed with brine, dried over $MgSO_4$. The crude product was purified by silica gel column chromatography (Hexane/EtOAc=10/1) to give yellow oil (672 mg, 69%): ^{1}H-NMR (400 MHz in $CDCl_3$) δ1.21–1.40 (m, 30H), 1.54–1.61 (m, 2H), 2.14 (q, J = 6.8 Hz, 2H), 3.78 (t, J = 6.6 Hz, 2H), 5.42 (d, J = 18.0 Hz, 1H), 6.63 (td, J = 6.4, 18.4 Hz, 1H), 7.35–7.45 (m, 9H), 7.61–7.63 (m, 6H): ^{13}C-NMR (100 MHz, $CDCl_3$) δ:25.7, 28.9, 29.2, 29.3, 29.4, 29.5, 32.5, 33.5, 37.5, 57.5, 61.9, 64.0, 89.9, 115.7, 126.5, 127.8, 129.9, 134.4, 135.3, 140.1, 153.1, 165.4; IR (Neat): 2926, 2854, 1637 cm^{-1}.

(2Z,4E)-methoxymethyl 3-(2-hydroxyethyl)-16-((triphenylsilyl)oxy)hexadeca-2,4-dienoate (26b)

To a suspension of $Pd(PPh_3)_2Cl_2$ (180 mg, 0.256 mmol) in MeOH (7.0 mL) were added Segment A (440 mg, 1.53 mmol) and boronic ester **25b** (750 mg, 1.28 mmol) at RT. The mixture stirred at RT for 10 min, and NEt_3 (1.26 mL, 8.96 mmol) was added. The reaction mixture was stirred at RT for 3 h, and evaporated. The crude product was purified by silica gel column chromatography (Hexane/EtOAc=2/1) to give a yellow oil (316 mg, 40%): ^{1}H-NMR (400 MHz in $CDCl_3$) δ: 1.22–1.28 (m, 14H), 1.43 (brs, 2H), 1.54–1.62 (m, 2H), 2.22 (q, J = 6.8 Hz, 2H), 2.63 (t, J = 6.8 Hz, 2H), 3.48 (s, 3H), 3.76–3.81 (m, 4H), 5.27 (s, 2H), 5.68 (s, 1H), 6.22 (td, J = 6.8, 16.4 Hz, 1H), 7.35–7.45 (m, 9H), 7.53 (d, J = 16.0 Hz, 1H), 7.61–7.63 (m, 6H); ^{13}C-NMR (100 MHz, $CDCl_3$) δ: 25.7, 28.9, 29.2, 29.3, 29.4, 29.5, 29.6, 32.4, 33.4, 37.6, 57.4, 61.8, 63.9, 89.8, 115.6, 126.5, 127.7, 129.8, 134.4, 135.3, 140.0, 153.1, 165.4; IR (Neat): 3489, 2928, 2854, 1718, 1635, 1597 cm^{-1}.

(3Z,4E)-3-(2-(methoxymethoxy)-2-oxoethylidene)-16-(triphenylsilyloxy)hexadec-4-enoic acid (KH-11, 27b)

To a solution of alcohol **26b** (100 mg, 0.161 mmol) in CH_2Cl_2 (3.5 mL) was added DMP (273 mg, 0.644 mmol). The reaction mixture was stirred at RT for 1 h, and added sat. $Na_2S_2O_3$ aq., extracted with CH_2Cl_2, and washed with brine, dried over $MgSO_4$ and gave a crude product.

To a solution of the crude product in *t*BuOH/THF/2-methyl-2-butene =3/1/1 (3.24 mL) were added $NaClO_2$

(73 mg, 0.81 mmol) and $NaH_2PO_4 \cdot 2H_2O$ (177 mg, 1.13 mmol) in H_2O. The reaction mixture was stirred at RT for 1 h, and extracted with EtOAc, and washed with brine, dried over $MgSO_4$. The crude product was purified by silica gel column chromatography (Hexane/EtOAc=2/1) to give yellow oil (62 mg, 61%): ^{1}H-NMR (400 MHz in $CDCl_3$) δ: 1.22–1.26 (m, 14H), 1.43 (brs, 2H), 1.54–1.62 (m, 2H), 2.21 (q, J = 6.8 Hz, 2H), 3.36 (s, 2H), 3.47 (s, 3H), 3.79 (t, J = 6.6 Hz, 2H), 5.27 (s, 2H), 5.72 (s, 1H), 6.19 (td, J = 6.8, 16.4 Hz, 1H), 7.35–7.44 (m, 9H), 7.56 (d, J = 16.0 Hz, 1H), 7.61–7.63 (m, 6H); ^{13}C-NMR (100 MHz, $CDCl_3$) δ: 25.7, 28.8, 29.2, 29.3, 29.4, 29.5, 29.6, 32.5, 33.4, 40.0, 57.6, 64.0, 90.1, 118.0, 126.3, 127.8, 129.9, 134.4, 135.4, 140.8, 147.6, 165.1, 176.1; IR (Neat):, 3228, 2928, 2854, 1717, 1635, 1602 cm^{-1}.

(3Z,4E)-16-hydroxy-3-(2-(methoxymethoxy)-2-oxoethylidene)hexadec-4-enoic acid (KH-13, 28)

To a solution of silyl ether **27b** (10 mg, 0.016 mmol) in THF (1.0 mL) was added TBAF (1M in THF, 32 ▫ L, 0.032 mmol) at 0 °C. The reaction mixture was stirred at 0 °C for 10 min, and quenched with sat. NH_4Cl aq., extracted with EtOAc, and washed with brine, dried over $MgSO_4$. The crude product was purified by silica gel column chromatography ($CHCl_3$/MeOH=19/1) to give yellow oil (4.3 mg, 73%): ^{1}H-NMR (400 MHz in $CDCl_3$) δ: 1.27 (brs, 14H), 1.43 (brs, 2H), 1.54–1.56 (m, 2H), 2.23 (q, J = 6.8 Hz, 2H), 3.37 (s, 2H), 3.48 (s, 3H), 3.65 (t, J = 6.4 Hz, 2H), 5.8 (s, 2H), 5.74 (s, 1H), 6.20 (td, J = 7.2, 16.0 Hz, 1H), 7.56 (d, J = 16.0 Hz, 1H); ^{13}C-NMR (100 MHz, $CDCl_3$) δ: 25.5, 28.7, 29.0, 29.1, 29.2, 29.3, 29.4, 29.5, 32.5, 33.4, 40.1, 57.6, 63.0, 90.0, 117.9, 126.3, 140.8, 148.0, 165.1, 174.8; IR (Neat): 3417, 2924, 2852, 1639 cm^{-1}.

12-(trityloxy)dodecan-1-ol (22c)

To a solution of 1.12-dodecanediol (5.00 g, 24.7 mmol) in CH_2Cl_2 were added DMAP (150 mg, 1.24 mmol) and NEt3 (5.21 mL, 37.1 mmol) and trityl chloride (6.89 g, 24.7 mmol). The reaction mixture was stirred at RT for 1 h, and added H_2O, extracted with CH_2Cl_2, and washed with brine, dried over $MgSO_4$. The crude product was purified by silica gel column chromatography (Hexane/EtOAc=4/1) to give colorless oil (4.84 g, 44%):^{1}H-NMR (400 MHz in $CDCl_3$) δ: 1.19–1.34 (m, 18H), 1.53–1.65 (m, 4H), 3.04 (t, J = 6.8 Hz, 2H), 3.64 (q, J = 5.6 Hz, 2H), 7.20–7.24 (m, 3H), 7.27–7.31 (m, 6H), 7.44 (m, 6H); ^{13}C-NMR (100 MHz, $CDCl_3$) δ:25.7, 26.1, 29.3, 29.4, 29.5, 29.6, 29.9, 32.6, 62.6, 63.5, 86.1, 126.6, 127.5, 128.5, 144.4; IR (Neat): 3346, 2926, 2852 cm^{-1}.

12-(trityloxy)dodecanal (24c)

To a solution of oxalyl chloride (0.49 mL, 5.40 mmol) in CH_2Cl_2 (18 mL) was added DMSO (0.48 mL, 6.75 mmol) at −78 °C and stirred for 20 min, and alcohol **22c** (1.00 g, 2.25 mmol) in CH_2Cl_2 (5.0 mL) was added at −78 °C. The reaction mixture stirred at −78 °C for 20 min, and NEt_3 (2.13 mL, 15.2 mmol) was added at −78 °C. The mixture was stirred at RT for 1 h, and quenched with sat, $NaHCO_3$ aq., extracted with CH_2Cl_2, and washed with brine, dried over $MgSO_4$. The crude product was purified by silica gel column chromatography (Hexane/EtOAc = 4: 1) to give a yellow oil. (847 mg, 85%): ^{1}H-NMR (400 MHz in $CDCl_3$) δ: 1.25–1.29 (m, 14H), 1.61–1.64 (m, 2H), 2.41 (t, J = 4.8 Hz, 2H), 3.78 (t, J = 6.4 Hz, 2H), 7.20–7.24 (m, 3H), 7.27–7.31 (m, 6H), 7.44 (m, 6H),9.76 (s, 1H).

(E)-4,4,5,5-tetramethyl-2-(13-(trityloxy)tridec-1-enyl)-1,3,2-dioxaborolane (25c)

To a suspension of $CrCl_2$ (1.41 g, 11.46 mmol) and LiI (1.02 g, 7.64 mmol) in THF (10 mL) were added pinacol borane **11** (603 mg, 2.86 mmol) in THF (5.0 mL) and aldehyde **24c** (846 mg, 1.91 mmol) in THF (5.0 mL). The mixture was stirred at RT for 3 h, and added sat.$NaHCO_3$ aq., extracted with EtOAc, and washed with brine, dried over $MgSO_4$. The crude product was purified by silica gel column chromatography (Hexane/EtOAc = 10/1) to give yellow oil (708 g, 65%): ^{1}H-NMR (400 MHz in $CDCl_3$) δ: 1.23–1.42 (m, 28H), 1.57–1.65 (m, 2H), 2.06 (q, J = 6.8 Hz, 2H), 2.98 (t, J = 6.8 Hz, 2H), 3.03 (t, J = 6.8 Hz, 2H), 5.38 (d, J = 18.4 Hz, 1H), 6.58 (td, J = 7.2, 17.2 Hz, 1H), 7.07-7.10 (m, 3H), 7.27–7.43 (m, 6H), 7.36–7.39 (m, 6H); ^{13}C-NMR (100 MHz, $CDCl_3$) δ: 25.2, 26.7, 28.6, 29.6, 29.7, 29.8, 29.9, 30.0, 30.1, 30.4, 36.2, 57.2, 64.0, 83.3, 86.6, 126.6, 127.1, 128.1, 128.7, 129.1, 129.8, 144.9, 155.2; IR (Neat):, 2926, 2854, 1637, 1597 cm^{-1}.

(2Z,4E)-methoxymethyl 3-(2-hydroxyethyl)-16-(trityloxy)hexadeca-2,4-dienoate (26c)

To a suspension of $Pd(PPh_3)_2Cl_2$ (167 mg, 0.238 mmol) in MeOH (6.0 mL) were added Segment A (440 mg, 1.53 mmol) in MeOH (3.0 mL) and boronic ester **25c** (750 mg, 1.28 mmol) in MeOH (3.0 mL) at RT. The mixture stirred at RT for 10 min, and NEt_3 (1.26 mL, 8.96 mmol) was added. The reaction mixture was stirred at RT for 3 h, and evaporated. The crude product was purified by silica gel column chromatography (Hexane/EtOAc=2/1) to give a yellow oil (113 mg, 16%): ^{1}H-NMR (400 MHz in $CDCl_3$) δ: 1.24–1.43 (m, 14H), 1.58–1.63 (m, 4H), 2.15 (q, J = 7.2 Hz, 2H), 2.63 (t, J = 6.4 Hz, 2H), 3.03 (t, J = 6.6 Hz, 2H), 3.48 (s, 3H), 3.78 (q, J = 6.4 Hz, 2H), 5.27 (s, 2H), 5.68 (s, 1H), 6.22 (dt, J = 7.6, 15.6 Hz, 1H), 7.20–7.24 (m, 3H), 7.27–7.31 (m, 6H), 7.44 (m, 6H), 7.53 (d, J = 16.4 Hz, 1H); ^{13}C-NMR (100 MHz, $CDCl_3$) δ: 26.2, 28.9, 29.2, 29.4, 29.5, 29.6, 30.0, 33.5, 37.5, 86.2, 89.9, 115.7, 126.5, 126.7, 127.7, 128.7, 140.2, 144.5, 153.1, 165.4; IR (Neat): 3481, 2926, 1716, 1636, 1597 cm^{-1}.

(3Z,4E)-3-(2-(methoxymethoxy)-2-oxoethylidene)-16-(trityloxy)hexadec-4-enoic acid (KH-12 27c)

To a solution of alcohol **26c** (112 mg, 0.187 mmol) in CH_2Cl_2 (4.0 mL) was added DMP (317 mg, 0.748 mmol). The reaction mixture was stirred at RT for 1 h, and added sat. $Na_2S_2O_3$ aq., extracted with CH_2Cl_2, washed with brine, dried over $MgSO_4$ and gave a crude product.

To a solution of the crude product in *t*BuOH/THF/2-methyl-2-butene = 3/1/1 (3.8 mL) were added $NaClO_2$ (85 mg, 0.94 mmol) and NaH_2PO_4•$2H_2O$ (206 mg, 1.32 mmol) in H_2O. The reaction mixture was stirred at RT for 1 h, and extracted with EtOAc, and washed with brine, dried over $MgSO_4$. The crude product was purified by silica gel column chromatography (Hexane/EtOAc=2/1) to give yellow oil (38 mg, 37%): ^{1}H-NMR (400 MHz in $CDCl_3$) δ: 1.24–1.65 (m, 18H), 2.21 (q, J = 7.2 Hz, 2H), 3.03 (t, J = 6.6 Hz, 2H), 3.38 (s,2H), 3.46 (s, 3H), 5.28 (s, 2H), 5.74 (s, 1H), 6.19 (dt, J = 7.2, 16.0 Hz, 1H), 7.20–7.24 (m, 3H), 7.27–7.30 (m, 6H), 7.44 (m, 6H), 7.56 (d, J = 16.4 Hz, 1H); ^{13}C-NMR (100 MHz, $CDCl_3$) δ: 26.2, 28.8, 29.2, 29.4, 29.5, 29.6, 30.0, 33.4, 40.0, 57.6, 63.7, 86.2, 90.0, 118.1, 126.3, 126.8, 127.6, 128.7, 140.9, 144.5, 147.6, 165.1, 175.9 03-64; IR (Neat): 3244, 2928, 2854, 1717, 1636, 1600 cm^{-1}.

(2Z,4E)-2-cyanoethyl-14-((tert-butyldiphenylsilyl)oxy)-3-(2-hydroxyethyl)tetradeca-2,4-dienoate (20b)

To a suspension of $Pd(PPh_3)_2Cl_2$ (154 mg, 0.22 mmol) in MeOH (2.0 mL) were added cyanoethylester (385 mg, 1.31 mmol) and boronic ester (600 g, 1.09 mmol) at RT. The mixture stirred at RT for 10 min, and NEt_3 (1.10 mL, 11.0 mmol) was added. The reaction mixture was stirred at RT for 3 h, and evaporated. The crude product was purified by silica gel column chromatography (Hexane/EtOAc=2/1) to give a yellow oil (316 mg, 50%):^{1}H-NMR (400 MHz in $CDCl_3$) δ: 1.05 (s,9H), 1.24–1.44 (m, 12H), 1.52–1.57 (m, 2), 2.23 (q, J = 7.6 Hz, 2H), 2.63 (t, J = 6.6 Hz, 2H), 2.72 (t, J = 6.2 Hz, 2H), 3.65 (t, J = 6.6 Hz, 2H), 3.79 (q, J = 6.4 Hz, 2H), 4.31 (t, J = 6.4 Hz, 2H), 5.68 (s, 1H), 6.25 (td, J = 6.8, 16.0 Hz, 1H), 7.35–7.44 (m, 6H), 7.49 (d, J = 16.0 Hz, 1H), 7.66–7.68 (m, 4H); ^{13}C-NMR (100 MHz, $CDCl_3$) δ:18.0, 19.2, 25.7, 26.9, 28.8, 29.2, 29.3, 29.5, 32.5, 33.5, 39.8, 58.3, 64.0, 117.2, 126.1, 127.6, 129.5, 134.1, 135.6, 141.5, 148.2, 164.9; IR (Neat): 3419, 2930, 2857, 2252, 1716 cm^{-1}.

(3Z,4E)-14-((tert-butyldiphenylsilyl)oxy)-3-(2-(2-cyanoethoxy)-2-oxoethylidene)tetradec-4-enoic acid (KH-5, 21b)

To a solution of alcohol (100 mg, 0.173 mmol) in CH_2Cl_2 (7.0 mL) was added DMP (220 mg, 0.519 mmol). The reaction mixture was stirred at RT for 1 h, and added sat. $Na_2S_2O_3$ aq., extracted with CH_2Cl_2, washed with brine, dried over $MgSO_4$ and gave a crude product.

To a solution of the crude product in *t*BuOH/THF/2-methyl-2-butene = 3/1/1 (3.4 mL) were added $NaClO_2$ (80 mg, 0.880 mmol) and NaH_2PO_4•$2H_2O$ (192 mg, 1.23 mmol) in H_2O (3.4 mL). The reaction mixture was stirred at RT for 1 h, and extracted with EtOAc, and washed with brine, dried over $MgSO_4$. The crude product was purified by silica gel column chromatography (Hexane/EtOAc=2/1) to give colorless oil (98 mg, 96%): ^{1}H-NMR (400 MHz in $CDCl_3$) δ: 1.04 (s, 9H), 1.26 (s, 10H), 1.43 (brs, 2H), 1.51–1.57 (m, 2H), 2.23 (q, J = 6.8 Hz, 2H), 2.72 (t, J = 6.6 Hz, 2H), 3.38 (s, 2H), 3.65 (t, J = 6.6 Hz, 2H), 4.32 (t, J = 6.2 Hz, 2H), 5.73 (s,1H), 6.22 (td, J = 7.6, 16.0 Hz, 1H), 7.36–7.44 (m, 6H), 7.52 (d, J = 16.0 Hz, 1H), 7.67 (m, 4H); ^{13}C-NMR (100 MHz, CD_3OD) δ:18.4, 20.0, 26.8, 27.4, 30.0, 30.2, 30.3, 30.4, 30.6, 33.6, 34.4, 41.1, 59.9, 65.0, 118.1, 127.7, 128.7, 130.8, 135.1, 136.7, 141.5, 150.7, 166.1, 174.1; IR (Neat): 3498, 2930, 2857, 2254, 1716, 1633, 1602 cm^{-1}.

(2Z,4E)-2-cyanoethyl-16-((tert-butyldiphenylsilyl)oxy)-3-(2-hydroxyethyl)hexadeca-2,4-dienoate (20c)

To a suspension of $Pd(PPh_3)_2Cl_2$ (189 mg, 0.27 mmol) in MeOH (6.0 mL) were added cyanoethylester **19** (400 mg, 1.37 mmol) and boronic ester **15c** (600 g, 1.06 mmol) at RT. The mixture stirred at RT for 10 min, and and NEt_3 (1.04 mL, 7.42 mmol) was added. The reaction mixture was stirred at RT for 3 h, and concentrated. The crude product was purified by silica gel column chromatography (Hexane/EtOAc=2/1) to give a yellow oil (255 mg, 42%):^{1}H-NMR (400 MHz in $CDCl_3$) δ: 1.05 (s, 9H), 1.25–1.28 (m, 14H), 1.44–1.46 (m, 2H), 1.52–1.59 (m, 2H), 2.23 (q, J = 6.8 Hz, 2H), 2.63 (t, J = 6.6 Hz, 2H), 2.72 (t, J = 6.4 Hz, 2H), 3.65 (t, J = 6.6 Hz, 2H), 3.79 (q, J = 6.0 Hz, 2H), 4.31 (t, J = 6.6 Hz, 2H), 5.68 (s, 1H), 6.25 (td, J = 6.8, 16.0 Hz, 1H); ^{13}C-NMR (100 MHz, $CDCl_3$) δ: 18.0, 19.2, 25.7, 26.8, 29.0, 29.1, 29.2, 29.3, 29.4, 29.5, 29.6, 32.6, 33.5, 37.5, 58.1, 61.9, 64.0, 114.9, 116.9, 126.3, 127.5, 129.4, 134.1, 135.5, 140.7, 153.7, 165.2; IR (Neat): 3431, 2928, 2854, 2254, 1714, 1633 cm^{-1}; Mass (FAB) m/z 603 (M^+); HRMS calcd for $C_{37}H_{53}O_4NSi$: 603.3744 found 603.3749.

(3Z,4E)-14-((tert-butyldiphenylsilyl)oxy)-3-(2-(2-cyanoethoxy)-2-oxoethylidene)tetradec-4-enoic acid (KH-8, 21c)

To a solution of alcohol **20c** (65 mg, 0.11 mmol) in CH_2Cl_2 (2.2 mL) was added DMP (140 mg, 0.33 mmol). The reaction mixture was stirred at RT for 1 h, and added sat. $Na_2S_2O_3$ aq., extracted with CH_2Cl_2, washed with brine, dried over $MgSO_4$ and gave a crude product.

To a solution of the crude product in *t*BuOH/THF/2-methyl-2-butene = 3/1/1 (0.90 mL) were added $NaClO_2$ (41 mg, 0.46 mmol) and NaH_2PO_4•$2H_2O$ (99 mg, 0.64 mmol) in H_2O (0.90 mL). The reaction mixture was

stirred at RT for 1 h, and extracted with EtOAc, and washed with brine, dried over $MgSO_4$. The crude product was purified by silica gel column chromatography (Hexane/EtOAc=2/1) to give colorless oil. (45 mg, 66%): ^{1}H-NMR (400 MHz in $CDCl_3$) δ: 1.04 (s, 9H), 1.24–1.28 (s, 14H), 1.44 (brs, 2H), 1.52–1.57 (m, 2H), 2.23 (q, J = 7.6 Hz, 2H), 2.72 (t, J = 6.4 Hz, 2H), 3.38 (s, 2H), 3.65 (t, J = 6.6 Hz, 2H), 4.32 (t, J = 6.2 Hz, 2H), 5.73 (s,1H), 6.22 (td, J = 6.8, 16.8 Hz, 1H), 7.36–7.44 (m, 6H), 7.52 (d, J = 16.0 Hz, 1H), 7.67 (d, J = 8.0 Hz, 4H); ^{13}C-NMR (100 MHz, CD_3OD) δ: 18.4, 20.0, 26.8, 27.4, 29.9, 30.2, 30.3, 30.5, 30.6, 33.6, 34.4, 41.1, 59.9, 65.0, 118.1, 118.7, 127.8, 128.7, 130.8, 135.1, 136.6, 141.5, 150.8, 166.7, 174.2.; IR (Neat): 3466, 2928, 2857, 2254, 1739 cm^{-1}; Mass (FAB) m/z 618 (M^+ + 1); HRMS calcd for $C_{37}H_{52}O_5Si$: 618.3615 found 618.3620.

12-((tert-butyldiphenylsilyl)oxy)dodecanoic acid (29)

To a solution of aldehyde **14c** in *t*BuOH/THF/2-methyl-2-butene = 3/1/1 (60 mL) and H_2O (60 mL) were added $NaClO_2$ (11.3 g, 125 mmol) and NaH_2PO_4•$2H_2O$ (19.5 g, 125 mmol). The reaction mixture was stirred at RT for 1.5 h, and extracted with EtOAc, and washed with brine, dried over $MgSO_4$. The crude product was purified by silica gel column chromatography (Hexane/EtOAc=2/1) to give colorless oil (4.97 g, 87%): ^{1}H-NMR (400 MHz in $CDCl_3$) δ: 1.05 (s, 9H), 1.24–1.31 (m, 14H), 1.53–1.63 (m, 4H), 2.35 (t, *J* = 7.4 Hz, 2H), 3.65 (t, *J* = 6.6 Hz, 2H), 7.37–7.42 (m, 6H), 7.67 (m, 2H); ^{13}C-NMR (100 MHz, $CDCl_3$) δ: 19.2, 24.7, 25.7, 26.9, 29.0, 29.2, 29.3, 29.4, 29.5, 29.6, 32.5, 34.1, 64.0, 127.5, 129.4, 134.1, 135.6, 180.3; IR (Neat): 3134, 2928, 2854, 1708, 1589 cm^{-1}.

Methoxymethyl 12-((tert-butyldiphenylsilyl)oxy) dodecanoate (30)

To a solution of carboxylic acid **29** (4.97 g, 10.9 mmol) in CH_2Cl_2 were added diisopropylethylamine (5.27 g, 65.4 mmol) and MOMCl (7.04 g, 54.5 mmol) at 0 °C. The reaction mixture was stirred at RT for 1.5 h, and added sat, $NaHCO_3$ aq, extracted with CH_2Cl_2, and washed with brine, dried over $MgSO_4$. The crude product was purified by silica gel column chromatography (Hexane/EtOAc=2/1) to give colorless oil (5.42 g, 99%): ^{1}H-NMR (400 MHz in $CDCl_3$) δ: 1.05 (s, 9H), 1.25–1.28 (m, 14H), 1.54–1.55 (m, 4H), 2.36 (t, *J* = 7.6 Hz, 2H), 3.46 (s, 3H), 3.65 (t, *J* = 6.6 Hz, 2H), 5.23 (s, 2H), 7.36–7.42 (m, 6H), 7.69–7.71 (m, 2H); ^{13}C-NMR (100 MHz, $CDCl_3$) δ: 19.1, 24.7, 25.7, 26.8, 29.0, 29.2, 29.3, 29.5, 32.5, 34.2, 57.4, 63.9, 90.0, 127.5, 127.6, 129.4, 134.1, 135.2, 135.5; IR (Neat): 2929, 2856, 1739 cm^{-1}.

Methoxymethyl 12-hydroxydodecanoate (31)

To a solution of ester **30** (4.97 g, 10.9 mmol) in CH_2Cl_2 were added diisopropylethylamine (5.27 g, 65.4 mmol) and MOMCl (7.04 g, 54.5 mmol) at 0 °C. The reaction mixture was stirred at RT for 2 h, and added sat, $NaHCO_3$ aq, extracted with CH_2Cl_2, and washed with brine, dried over $MgSO_4$. The crude product was purified by silica gel column chromatography (Hexane/EtOAc=2/1) to give colorless oil (5.42 g, 99%): ^{1}H-NMR (400 MHz in $CDCl_3$) δ: 1.24–1.28 (m, 16H), 1.56–1.66 (m, 6H), 2.35 (t, *J* = 7.6 Hz, 2H), 3.46 (s, 3H), 3.63 (t, *J* = 6.6 Hz, 2H), 5.23 (s, 2H); ^{13}C-NMR (100 MHz, $CDCl_3$) δ: 24.7, 25.7, 29.0, 29.2, 29.3, 29.4, 29.5, 32.7, 34.2, 57.5, 62.9, 90.1, 173.4; IR (Neat): 3460, 2926, 2854, 1747 cm^{-1}; Mass (EI) m/z 261 (M^++1); HRMS calcd for $C_{14}H_{29}O_4$: 261.1998 found 261.2073.

Methoxymethyl 12-oxododecanoate (32)

To a solution of oxalyl chloride (0.24 mL, 2.76 mmol) in CH_2Cl_2 (10 mL) was added DMSO (0.24 mL, 3.45 mmol) at −78 °C. and stirred for 20 min, and alcohol **31** (300 mg, 1.15 mmol) in CH_2Cl_2 (6.0 mL) was added at −78 °C. The reaction mixture stirred at −78 °C for 20 min, and NEt_3 (1.21 mL, 8.63 mmol) was added at −78 °C. The mixture was stirred at RT for 1 h, and quenched with sat, $NaHCO_3$ aq., extracted with CH_2Cl_2, and washed with brine, dried over $MgSO_4$. The crude product was purified by silica gel column chromatography (Hexane/EtOAc = 4:1) to give a yellow oil. (295 mg, 99%): ^{1}H-NMR (400 MHz in $CDCl_3$) δ: 1.28 (s, 13H), 1.59–1.64 (m, 3H), 2.35 (t, *J* = 7.4 Hz, 2H), 2.42 (t, *J* = 7.4 Hz, 2H), 3.46 (s, 3H), 5.23 (s, 2H), 9.77 (s, 1H).

(E)-methoxymethyl 13-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)tridec-12-enoate (33)

To a suspension of $CrCl_2$ (840 mg 6.84 mmol) and LiI (610 mg, 4.56 mmol) in THF (4.0 mL) were added pinacol borane **11** (480 mg, 2.28 mmol) in THF (3.0 mL) and aldehyde **32** (295 mg, 1.14 mmol) in THF (3.0 mL). The mixture was stirred at RT for 2.5 h, and added sat.$NaHCO_3$, extracted with EtOAc, and washed with brine, dried over $MgSO_4$. The crude product was purified by silica gel column chromatography (Hexane/EtOAc=10/1) to give green oil (140 mg, 32%): ^{1}H-NMR (400 MHz in $CDCl_3$) δ: 1.24–1.40 (m, 26H), 1.64–1.66 (m, 2H), 2.14 (q, *J* = 6.8 Hz, 2H), 2.35 (t, *J* = 7.4 Hz, 2H), 3.46 (s, 3H), 5.23 (s, 2H), 5.42 (d, J = 18.4 Hz, 1H), 6.63 (td, J = 6.4, 18.4 Hz, 1H); ^{13}C-NMR (100 MHz, $CDCl_3$) δ: 24.7, 28.1, 29.0, 29.1, 29.2, 29.3, 29.4, 29.5, 34.3, 35.8, 57.5, 82.9, 90.1, 154.7, 173.3; IR (Neat): 2928, 2854, 1745, 1637 cm^{-1}; Mass (FAB) m/z 383 (M^++1); HRMS calcd for $C_{21}H_{40}BO_5$: 383.2969 found 383.2973.

(2Z,4E)-bis(methoxymethyl) 3-(2-hydroxyethyl) hexadeca-2,4-dienedioate (34)

To a suspension of $Pd(PPh_3)_2Cl_2$ (67 mg, 0.0952 mmol) in MeOH (1.0 mL) were added Segment A (148 mg, 0.523 mmol) and boronic ester **33** (182 mg,

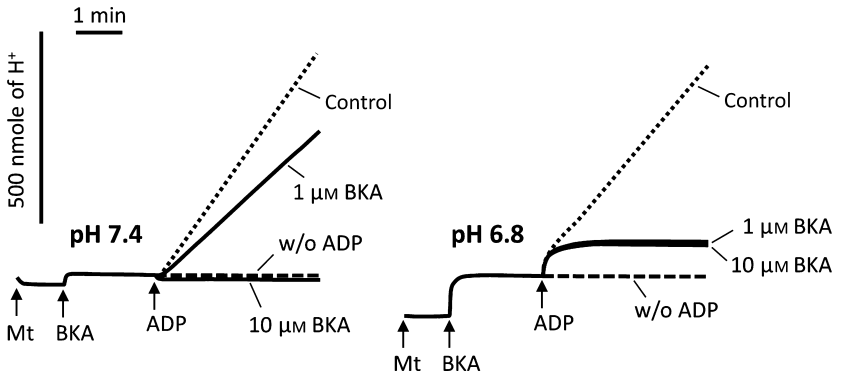


Figure 2: Experimental procedure to evaluate inhibitory effects of bongkrekic acid (BKA) analogues on mitochondrial ATP synthesis. The experimental system used to evaluate the effects of BKA analogues on the mitochondrial ATP synthesis is shown. Broken line indicates the changes in the pH of the incubation medium without the addition of ADP and BKA analogues (negative control), and dotted line indicates the changes in the pH of the incubation medium caused by the addition of ADP in the absence of BKA analogues (positive control). The left and right traces represent the typical results of the experiments at pH 7.4 and 6.8, respectively.

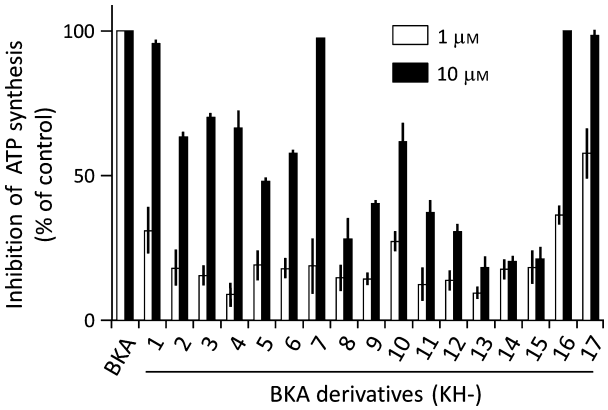


Figure 3: Inhibitory effects of bongkrekic acid (BKA) analogues on mitochondrial ATP synthesis. Inhibitory effects of individual BKA analogues at 1 μM (white column) or 10 μM (black column) on mitochondrial ATP synthesis were examined by measuring the suppression of the pH change caused by the addition of ADP at pH 6.8. The results obtained by 3 independent runs are shown as % inhibition of ATP synthesis (mean ± SD). The rate of the ATP synthesis in the absence of BKA analogues (i.e., positive control in Figure 1B) was 297 ± 46 nmol/mg/min.

0.476 mmol) at RT. The mixture stirred at RT for 10 min, and NEt_3 (1.76 mL, 12.5 mmol) was added. The reaction mixture was stirred at RT for 3 h, and evaporated. The crude product was purified by silica gel column chromatography (Hexane/EtOAc = 2/1) to give a yellow oil (195 mg, 98%): ^{1}H-NMR (400 MHz in $CDCl_3$) δ: 1.27 (brs, 12H), 1.43 (brs, 2H), 1.63–1.66 (m, 2H), 2.21 (q, J = 7.6 Hz, 2H), 2.35 (t, J = 7.8 Hz, 2H), 2.64 (t, J = 6.6 Hz, 2H), 3.46 (s, 3H), 3.48 (s, 3H), 3.79 (q, J = 6.4 Hz, 2H), 5.23 (s, 2H), 5.28 (s, 2H), 5.69 (s, 1H), 6.22 (td, J = 7.2, 16.0 Hz, 1H), 7.53 (d, J = 16.4 Hz, 1H); ^{13}C-NMR (100 MHz, CD_3OD) δ: 24.8, 28.9, 29.0, 29.1, 29.2, 29.3, 29.4, 29.5, 33.4, 34.3, 37.5, 57.5, 61.9, 89.7, 90.1, 115.7, 126.5, 140.1, 153.1, 165.4, 173.4; IR (Neat): 3444, 2926, 2854, 1732, 1716, 1635, 1597 cm^{-1}; Mass (EI) m/z 414 (M^+); HRMS calcd for $C_{22}H_{38}O_7$: 414.2618 found 414.2612.

(3Z,4E)-16-(methoxymethoxy)-3-(2-(methoxymethoxy)-2-oxoethylidene)-16-oxohexadec-4-enoic acid (KH-14, 35)

To a solution of alcohol **34** (100 mg, 0.24 mmol) in CH_2Cl_2 (5.0 mL) was added DMP (407 mg, 0.96 mmol). The reaction mixture was stirred at RT for 1 h, and added sat. $Na_2S_2O_3$ aq., extracted with CH_2Cl_2, washed with brine, dried over $MgSO_4$ and gave a crude product.

To a solution of the crude product in *t*BuOH/THF/2-methyl-2-butene =3/1/1 (5.0 mL) were added $NaClO_2$ (109 mg, 1.20 mmol) and NaH_2PO_4•$2H_2O$ (262 mg, 1.68 mmol) in H_2O (5.0 mL). The reaction mixture was stirred at RT for 1 h, and extracted with EtOAc, and washed with brine, dried over $MgSO_4$. The crude product was purified by silica gel column chromatography (Hexane/EtOAc=2/1) to give colorless oil (38 mg, 37%): ^{1}H-NMR (400 MHz in $CDCl_3$) δ: 1.24–1.30 (m, 14H), 1.43 (brs, 2H), 1.63–1.66 (m, 2H), 2.22 (q, J = 7.6 Hz, 2H), 2.36 (t, J = 7.4 Hz, 2H), 3.38 (s, 2H), 3.47 (s, 3H), 3.48 (s, 3H), 5.24 (s, 2H), 5.28 (s, 2H), 5.74 (s, 1H), 6.21 (td, J = 7.6, 15.6 Hz, 1H), 7.56 (d, J = 16.0 Hz, 1H); ^{13}C-NMR (100 MHz, CD_3OD) δ: 25.9, 29.9, 30.1, 30.2, 30.3, 30.4, 30.5, 30.6, 34.4, 35.1, 41.0, 57.7, 90.9, 91.2, 118.6, 127.8, 141.4, 150.7, 166.7, 174.1, 174.9; IR (Neat): 3083, 2933, 2928, 2854, 1737, 1717, 1635 cm^{-1}; Mass (FAB) m/z 429 (M^++1); HRMS calcd for $C_{22}H_{37}O_8$: 429.2488 found 429.2498.

Preparation of mitochondria from rat liver

Mitochondria were prepared from the liver of 8-week-old male Wistar rats by the differential centrifugation procedure

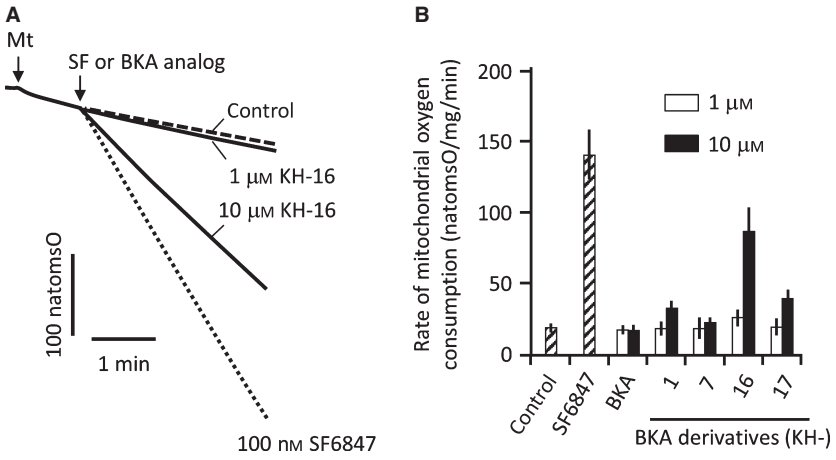


Figure 4: Evaluation of the permeabilization effects of the four bongkrekic acid (BKA) analogues on the mitochondrial inner membrane. Panel A presents the experimental system used for evaluation of permeabilization of the mitochondrial inner membrane. The very slow oxygen consumption observed in the absence of added chemicals (broken line) and the rapid oxygen consumption caused by the addition of SF6847 (dotted line) indicated the mitochondrial inner membrane showing lowest and highest permeability, respectively. Panel B shows the effects of the BKA analogues on the rate of mitochondrial oxygen consumption. The rates of oxygen consumption observed in the absence of chemicals or in the presence of SF6847 are shown as hatched columns. Those observed in the presence of BKA or its analogues at 1 and 10 μM are shown with white and black columns, respectively. The bars represent the SD values of individual experiments.

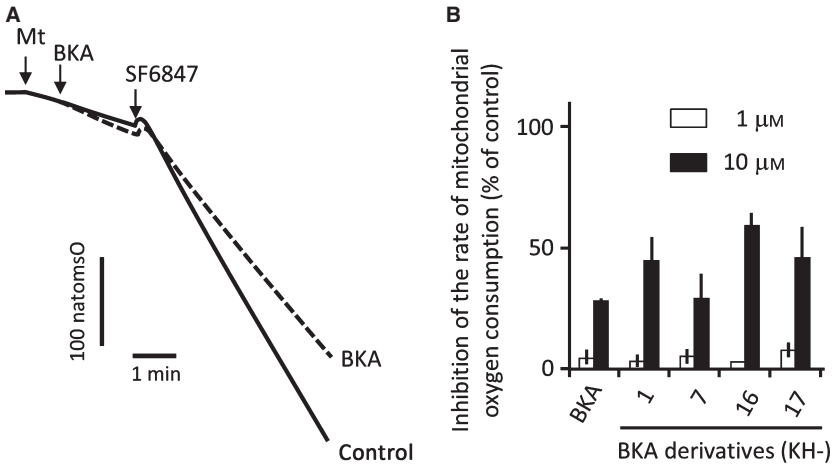


Figure 5: Evaluation of the inhibitory effects of bongkrekic acid (BKA) analogues on the mitochondrial electron transport system. Panel A outlines the experiment used to evaluate the inhibitory effects of BKA on the mitochondrial electron transport system. The solid line shows the result obtained in the absence of BKA; and the broken line, that in the presence of 10 μM BKA. Panel B shows the inhibitory effect of BKA and its analogues on the SF6847-stimulated oxygen consumption. The results observed in the presence of BKA and its analogues at 1 or 10 μM are shown with white and black columns, respectively. The bars represent the SD values of individual experiments. The maximum rate of oxygen consumption observed in the absence of BKA or its analogue (trace 'control' in panel A) was 141 $\pm$ 17 natomsO/mg/min.

reported previously (13). Final mitochondrial pellets were suspended in aliquots of medium containing 250 mM sucrose and 2 mM Tris-Cl at pH 7.4. The resulting suspension was used as a stock solution of mitochondria and kept on ice during the experiments. Its protein concentration was determined by the biuret method using bovine serum albumin as a standard.

Evaluation of the inhibitory effects of BKA analogues on mitochondrial ATP synthesis

The velocity of ATP synthesis in the mitochondrial suspension was measured by calculating the change in the pH of the incubation medium (13,14). Briefly, mitochondria were suspended in the appropriate amount of medium (3 mM potassium phosphate buffer, pH 7.4 or 6.8, containing

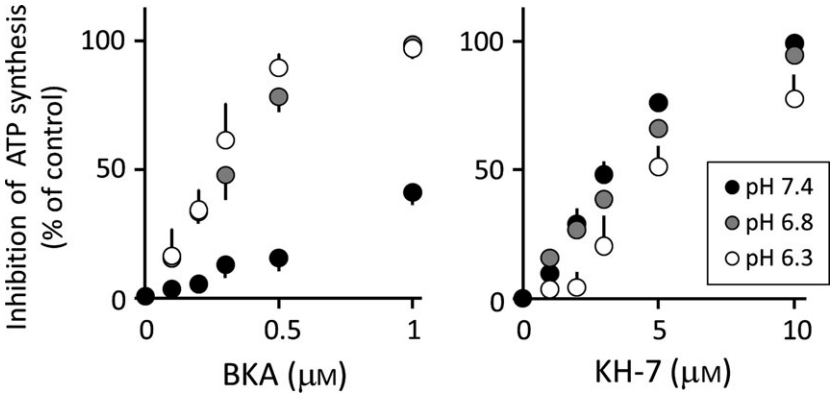


Figure 6: Comparison of the pH dependency of the inhibitory effects of bongkrekic acid (BKA) and KH-7 on ATP synthesis. Inhibitory effects on the mitochondrial ATP synthesis of BKA and KH-7 at various concentrations in three incubation media of pH 6.3, 6.8, and 7.4 were examined by measuring the pH of the incubation medium as stated in Figure 1. Rates of ATP synthesis in the absence of BKA or KH-7 (i.e., control) at pH 6.3, 6.8, and 7.4 were 174 ± 22, 297 ± 46, and 384 ± 36 nmol/mg/min, respectively.

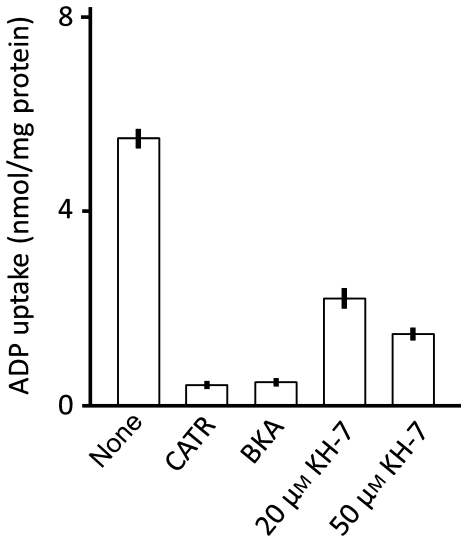


Figure 7: Direct inhibitory effects of KH-7 on the mitochondrial ADP/ATP carrier. For evaluation of the direct effects of KH-7 on the mitochondrial ADP/ATP carrier, uptake of ADP was measured by using [^{3}H]ADP as a tracer. ADP uptakes observed in the absence (none) and presence of an inhibitor (CATR or BKA at 10 μM) were used as positive and negative controls, respectively. For details of experiments, see the Methods section.

200 mM sucrose, 20 mM KCl, 3 mM $MgCl_2$) to give a protein concentration of 0.7 mg/mL. After the addition of 10 mM succinate (plus 1.25 μg/mL rotenone) as a respiratory substrate, ATP synthesis was started by the addition of 200 μM ADP. The incubation time-dependent change in the pH of the mitochondrial suspension was monitored by pH electrode model PCE108CW (Tokokagaku, Tokyo, Japan) and recorded at 25 °C. The changes in pH were calibrated by the addition of 100 μM oxalic acid.

Evaluation of the membrane permeabilization effects of BKA analogues, and inhibitory effects of these analogues on the mitochondrial electron transport system

The membrane permeabilization effects of the BKA analogues, and their inhibitory effects on the mitochondrial electron transport system were determined by measuring the rate of oxygen consumption by mitochondria. For this purpose, mitochondria were suspended in the above-mentioned medium (pH 6.8), supplemented with 5 mM succinate (plus 1.25 μg/mL rotenone) as a respiratory substrate. After the addition of a certain amount of individual BKA analogues, time-dependent changes in the oxygen concentration of the mitochondrial suspension were monitored at 25 °C by a Clark-type oxygen electrode (Yellow Springs Instrument, model 5331, YSI Life Sciences, Yellow Springs, OH, USA).

Evaluation of the inhibitory effects of KH-7 on the mitochondrial ADP uptake

To evaluate the direct action of KH-7 on the mitochondrial ADP/ATP carrier, we measured the uptake of [^{3}H]ADP into mitochondria as described previously (15). Briefly, mitochondria (1 mg protein) were suspended in the above-mentioned medium (0.5 mL, pH 7.4, not supplemented with the respiratory substrate) and incubated at 25 °C for 2 min in the presence or absence of certain chemicals (BKA, CATR or KH-7). Then, a 10-μL aliquot of 2 mM ADP solution containing [^{3}H]ADP (specific radioactivity of 185 MBq/mmol ADP) was added. After an incubation for 10 seconds, the reaction was terminated by the addition of a sufficient amount of CATR to make its final concentration of 10 μM; and mitochondria were then pelleted by centrifugation at 5500 × *g* for 1 min. After complete removal of the supernatant, the mitochondrial pellet was solubilized with 200 μL of 1% SDS solution. The radioactivity in 50 μL of the solubilized mitochondrial solution was counted in an Aloka liquid scintillation counter, model LSC-3500, Hitachi Aloka Medical, Ltd., Tokyo, Japan.

Results

Bongkrekic acid analogues used in the present study

Bongkrekic acid has 3 carboxylic acids connected by a branched chain of partially unsaturated fatty acid. This structure suggested to us that the anionic functional

groups such as carboxylate would be essential for the binding of BKA to biomolecules and that the fatty acid chain would control the spatial configuration of these functional groups. Hence, we designed a general structure for the BKA analogues used in the present study (Figure 1A), one consisting of two parts, left and right. As shown in Table 1, the left part contained carboxylate(s) and/or an ester (R^1) of acid-labile methoxymethyl ester or base-labile cyanoethyl ester, a (*Z,E*)-conjugated diene, and a lipophilic fatty acid chain of various length (n = 6, 8, 10, and 16); and the right part, a functional group of hydrophilic carboxy (COOH), methoxymethyl ester (methoxymethyloxycarbonyl group), hydroxy (OH) group, highly hydrophilic (lipophilic) trialkylsiloxy groups (Si^tBuPh_2, Si^tBuPh_2, $SiPh_3$), or alkyl ether (CPh_3). We prepared a total of 17 BKA analogues and examined their effects on the mitochondrial ADP/ATP carrier.

Inhibitory effects of these 17 BKA analogues on mitochondrial ATP synthesis

First, we roughly tested the inhibitory effects of these 17 BKA analogues on the mitochondrial ADP/ATP carrier by measuring their effects on the ATP synthesis in isolated mitochondria. If a certain one of these chemicals showed an inhibitory effect on the mitochondrial ADP/ATP carrier, import of ADP into the mitochondrial matrix would be suppressed; and, hence, ATP synthesis would be inhibited. Because H^+ is consumed during ATP synthesis, as shown below,

$$ADP + Pi + H^+ \rightarrow /ATP + H_2O$$

ATP synthesis in mitochondria can be determined by measuring the pH of the incubation medium (14). In addition, the inhibitory effect of BKA, the parental compound of the chemicals used, on the mitochondrial ATP synthesis is known to be sensitive to the pH of the incubation medium (16). Thus, the actions of the test chemicals were evaluated under two pH conditions, those of 7.4 and 6.8. As shown in Figure 2, the addition of ADP to the mitochondria suspended in medium containing inorganic phosphate (Pi) caused alkalinization of the incubation medium, thus reflecting ATP synthesis, regardless of the differences in pH of the incubation medium. In the medium of pH 7.4, BKA at 1 μM had a partial inhibitory effect on the alkalinization of the incubation medium, but that at 10 μM suppressed it perfectly. On the contrary, in the medium of pH 6.8, BKA was effective in suppressing the alkalinization of the incubation medium even at 1 μM. As these results supported the reported pH-dependent action of BKA on mitochondrial ATP synthesis, that is, stronger effects at a slightly acidic pH than at neutral pH (16), this experimental system was concluded to be suitable for evaluation of the inhibitory effects of BKA analogues on mitochondrial ATP synthesis; and so we adopted it for evaluating the action of the 17 BKA analogues. Because the inhibitory effect of BKA on the mitochondrial ATP synthesis was more remarkable at pH 6.8 than at pH 7.4, we tested the analogue action at pH 6.8.

As shown in Figure 3, the inhibitory effects of the BKA analogues on mitochondrial ATP synthesis were much more moderate than that effect of BKA, and no analogues showed perfect inhibition at 1 μM, as seen with BKA. However, the actions of 4 of the analogues, that is, KH-1, KH-7, KH-16 and KH-17, were relatively remarkable; and these compounds at 10 μM completely suppressed the alkalinization of the incubation medium. Therefore, we focused on these four BKA analogues in subsequent experiments.

As stated above, an increase in the pH of the incubation medium reflects inhibition of ATP synthesis. In the case of BKA at low concentrations, its inhibitory effect on ATP synthesis could be simply attributable to its inhibitory action on the ADP/ATP carrier, because it does not show remarkable side effects on other mitochondrial functions**.** However, when we evaluate the effects of new chemicals on mitochondrial ATP synthesis, we must pay adequate attention to their possible side effects that may influence this synthesis. Thus, we next tested whether these analogues had any (i) permeabilization effects on the mitochondrial inner membrane, or (ii) inhibitory effects on the mitochondrial electron transport system, because these actions are major side effects that would influence mitochondrial ATP synthesis. These effects could be evaluated by measuring the oxygen consumption by mitochondria.

Side effects of BKA analogues affecting ATP synthesis

We first evaluated the permeabilization effects of BKA analogues on the mitochondrial inner membrane. As shown in Figure 4A, when mitochondria were suspended in the medium containing a respiratory substrate (trace 'control'), gradual oxygen consumption, to compensate for the H^+ leakage through the mitochondrial inner membrane, was observed. The addition of 100 nM SF6847, a most effective protonophore (17,18), remarkably accelerated the mitochondrial oxygen consumption. Using these two conditions as the membrane states showing the lowest and the highest H^+ permeabilities, respectively, we examined the permeabilization effects of the 4 BKA analogues on the mitochondrial inner membrane. As shown in Figure 4B, KH-16 at 10 μM markedly increased the rate of mitochondrial oxygen consumption, and KH-1 and KH-17 showed weak acceleration effects when tested at the same concentration. KH-7 also slightly accelerated the mitochondrial oxygen consumption at 10 μM, but this effect was almost negligible.

We next evaluated the inhibitory effects of BKA analogues on the mitochondrial electron transport system. This effect was evaluated by measuring how the rate of oxygen consumption stimulated by SF6847 would be inhibited by the individual BKA analogues. For this purpose, mitochondria were pretreated with BKA or with KH-1, KH-7, KH-16 or KH-17 at 1 or 10 μM for 1 min, and then 100 nM SF6847 was added. If BKA analogues had an

inhibitory effect on the mitochondrial electron transport system, then the rate of oxygen consumption effected by the addition of SF6847 would be decreased. A typical result obtained with 10 μM BKA is shown in Figure 5A; and a summary of the results obtained with the 4 BKA analogues, in Figure 5B. BKA itself showed moderate inhibitory effects on the mitochondrial electron transport system at 10 μM BKA. KH-7 also showed moderate inhibitory effects at 10 μM, almost to the same extent as observed with BKA; but the other 3 BKA analogues, KH-1, KH-16, and KH-17, showed much stronger effects than BKA. However, these effects were almost negligible at the 1 μM concentration. Based on these results, the inhibitory effects of KH-1, KH-16, and KH-17 on the mitochondrial ATP synthesis were concluded to be not simply attributable to the results of the specific inhibition of mitochondrial ADP/ATP carrier; but KH-7 was concluded to specifically inhibit mitochondrial ADP/ATP carrier without showing remarkable side effects on the other mitochondrial functions.

pH dependency of the inhibitory effect of BKA and its analogues on mitochondrial ATP synthesis

We next examined the pH dependency of the inhibitory effect of BKA and KH-7 on mitochondrial ATP synthesis. As shown in Figure 6 (see also Figure 2, left traces), BKA showed a strong pH-dependent inhibitory effect on the ATP synthesis, and it showed strong inhibition, with it being stronger at a slightly acidic pH than at pH 7.4. On the contrary, KH-7 showed a weak and opposite pH dependency; that is, it was most effective at neutral pH (7.4) and rather weaker at the slightly acidic pH. Therefore, KH-7 was concluded to have lost its parental pH dependency. Possibly, the third carboxyl group present in the parental BKA but lacking in KH-7 was responsible for the pH dependency of the inhibitory action of BKA.

Evaluation of the direct inhibitory effects of KH-7 on the mitochondrial ADP/ATP carrier

Finally, we tested the direct action of KH-7 on the mitochondrial ADP/ATP carrier by measuring the uptake of [^{3}H]ADP. KH-7 at lower concentrations such as 1–10 μM was not remarkably effective, but clear suppression of ADP uptake was observed at 20 or 50 μM, as shown in Figure 7. It should be emphasized that the ADP uptake via the ADP/ATP carrier observed in the present experimental condition was not influenced by the factors such as an increase in the H^+ permeability caused by SF6847. Therefore, a moderate but specific inhibitory effect of KH-7 on the mitochondrial ADP/ATP carrier was clearly demonstrated.

Discussion

To examine whether a certain compound has an inhibitory effect on the mitochondrial ADP/ATP carrier, we can simply evaluate its direct action on the carrier, as demonstrated in the last part of the Results section. However, it is also very important to know whether it shows remarkable side effects on other mitochondrial functions aside from the ADP/ATP carrier, especially when we test the compound on the whole mitochondria rather than on the isolated ADP/ATP carrier. Therefore, in the present study, we first examined the effects of BKA analogues on mitochondrial ATP synthesis and on other properties of mitochondria. Among the 17 analogues tested, 4 of them, that is, KH-1, KH-7, KH-16, and KH-17, showed inhibitory effects on mitochondrial ATP synthesis. Of these, the inhibitory effect of KH-16 on mitochondrial ATP synthesis could hardly be attributed to inhibition of ADP/ATP carrier, because it showed remarkable side effects on the mitochondrial membrane permeability and respiratory chain. When we focused on the remaining 3 analogues (KH-1, KH-7, and KH-17), it became evident that all of them had a common left part of their structure, the part consisting of two carboxyl groups. This moiety may have been essential for their inhibitory effects on ADP/ATP carrier, because substitution of R1 position of KH-7 with MOM group (i.e., KH-9) caused a loss of the inhibitory effects of KH-7.

Interestingly, moreover, KH-17 had 3 carboxyl groups, like the parental BKA. It should be also emphasized that the length of the alkyl chain connecting left and right parts of KH-17 was essentially the same as that of the parental BKA, as schematically shown in our recent paper (12). Therefore, the presence of a third carboxyl group at right part of the molecule, even at a distance from the two carboxyl groups in the left part, similar to that of BKA, was a 'non-sufficient condition' for strong inhibitory effects against ADP/ATP carrier. When we compared the structures of BKA and KH-17, BKA had 3 additional methyl groups and one OMe group. Inclusion of these side chains would be expected to have made the molecule much more hydrophobic. If we assume that the higher hydrophobicity of BKA, especially at the right side of the molecule, was necessary for the stronger inhibitory effect on the ADP/ATP carrier, the strongest inhibitory effect of HK-7 among the 17 BKA analogues observed in the present study becomes explainable, because it has the same left side structure as that of BKA, and has a sterically bulky hydrophobic group, the *tert*-butyl diphenylsilyl (TBDPS) group, as the right part of the structure. However, it should be mentioned that other analogues sharing the same right and left parts of the molecule as those of KH-7, but having different alkyl chain lengths, such as KH-1 and KH-4, showed weak or no inhibitory effects on the mitochondrial ADP/ATP carrier. A possible explanation for the different activities of these analogues is that the presence of this TBDPS group at an appropriate distance from the left part of the structure containing the two carboxyl groups was important for the inhibitory action on the ADP/ATP carrier. To validate these interpretations, further structure–activity relationship studies, using analogues lacking

the TBDPS group as a negative control, would seem to be required.

One of the remarkable characteristics observed for KH-7 was its pH-independent inhibitory action toward the ADP/ATP carrier. We would like to consider why KH-7 showed such a unique property. As clearly mentioned in the past study (19), for the inhibition of the ADP/ATP carrier, BKA must be translocated to the matrix side of the mitochondrial inner membrane; and for efficient translocation across the mitochondrial inner membrane, the carboxyl groups of the BKA molecule should not be dissociated (i.e., only the neutral form of BKA can be easily translocated to the matrix side of the mitochondrial inner membrane). Therefore, loss of pH dependence in the inhibitory action on the ADP/ATP carrier observed with KH-7 could be attributable to the absence of the carboxyl group in its right part. In addition, lack of this third carboxyl group in its right part may also have significantly weakened the inhibitory action of KH-7 toward the ADP/ATP carrier. To examine these possibilities, a further study on the structure/activity relationship of BKA analogues, such as that examining the BKA molecule just lacking its right carboxyl group or that evaluating the KH-7 derivative having the right carboxyl group, would seem to be necessary.

In the present study, we successfully identified KH-7 as a new analogue of BKA. Although the parental BKA molecule is hard to synthesize and shows strong pH dependence in its action, synthesis of KH-7 was much easier (12); and this analogue did not show strong pH dependence. Although KH-7 showed a much weaker inhibitory effect on the mitochondrial ADP/ATP carrier than BKA, its action was specific for the carrier. Thus, KH-7 could be a nice tool to understand the catalytic mechanism of the mitochondrial ADP/ATP carrier.

Presently, we only focused on the inhibitory effects of BKA analogues on its primary target protein, that is, the mitochondrial ADP/ATP carrier. However, BKA is also thought to work as an inhibitor of mitochondrion-dependent apoptosis, because it largely suppresses the mitochondrial permeability transition (20), which causes the release of mitochondrial cytochrome c (a trigger of apoptosis), into cytosol. Thus, studies on BKA analogues are important for possible development of therapeutics for the treatment of certain mitochondrion-dependent diseases.

Acknowledgments

This study was supported by a research fund from the Network Joint Research Center for Materials and Devices, and by a grant from Science and Technology Research Promotion Program for Agriculture, Forestry, Fisheries, and Food Industry. We thank Prof. A. Kano (Kyushu University) for helpful discussions.

Conflict of Interest

The authors have no financial/commercial conflicts of interest to be declared.

References

1. Palmieri F. (2013) The mitochondrial transporter family SLC25: identification, properties and physiopathology. Mol Aspect Med;34:465–484.
2. Clémençon B., Babot M., Trézéguet V. (2013) The mitochondrial ADP/ATP carrier (SLC25 family): pathological implications of its dysfunction. Mol Aspect Med;34:485–493.
3. Klingenberg M. (2008) The ADP and ATP transport in mitochondria and its carrier. Biochim Biophys Acta;1778:1978–2021.
4. Nury H., Dahout-Gonzalez C., Trézéguet V., Lauquin G.J., Brandolin G., Pebay-Peyroula E. (2006) Relations between structure and function of the mitochondrial ADP/ATP carrier. Annu Rev Biochem;75:713–741.
5. Bruni A., Luciani S., Contessa A.R. (1964) Inhibition by atractyloside of the binding of adenine nucleotides of rat liver mitochondria. Nature;201:1219–1220.
6. Duée E.D., Vignais P.V. (1965) Exchange between extra- and intramitochondrial adenine nucleotides. Biochim Biophys Acta;107:184–188.
7. Pfaff E., Klingenberg M., Heldt H.W. (1965) Unspecific permeation and specific exchange of adenine nucleotides in liver mitochondria. Biochim Biophys Acta;104:312–315.
8. Aquila H., Misra D., Eulitz M., Klingenberg M. (1982) Complete amino acid sequence of the ADP/ATP carrier from beef heart mitochondria. Hoppe-Seyler's J Physiol Chem;363:345–349.
9. Pebay-Peyroula E., Dahout-Gonzalez C., Kahn R., Trézéguet V., Lauquin G.J.M., Brandolin G. (2003) Structure of mitochondrial ADP/ATP carrier in complex with carboxyatractyloside. Nature;426:39–44.
10. Stubbs M. (1981) Inhibitors of the adenine nucleotide translocase. In: Erecińska M., Wilson D.F., editors. Inhibitors of Mitochondrial Functions. Oxford: Pergamon Press; p. 283–304.
11. Sato Y., Aso Y., Shindo M. (2009) Efficient synthesis of bongkrekic acid. Three-component convergent strategy. Tetrahedron Lett;50:4164–4166.
12. Okuda K., Hasui K., Abe M., Matsumoto K., Shindo M. (2012) Molecular design, synthesis, and evaluation of novel potent apoptosis inhibitors inspired from bongkrekic acid. Chem Res Toxicol;25:2253–2260.
13. Terada H., Shima O., Yoshida K., Shinohara Y. (1990) Effects of the local anesthetic bupivacaine on oxidative phosphorylation in mitochondria. Change from

decoupling to uncoupling by formation of a leakage type ion pathway specific for H^+ in cooperation with hydrophobic anions. J Biol Chem;265:7837–7842.
14. Nishimura M., Ito T., Chance B. (1962) Studies on bacterial photophosphorylation. III. A sensitive and rapid method of determination of photophosphorylation. Biochim Biophys Acta;59:177–182.
15. Shinohara Y., Nagamune H., Terada H. (1987) The hydrophobic cationic cyanine dye inhibits oxidative phosphorylation by inhibiting ADP transport, not by electrophoretic transfer, into mitochondria. Biochem Biophys Res Commun;148:1081–1086.
16. Kemp A. Jr, Out T.A., Guiot H.F., Souverijn J.H. (1970) The effect of adenine nucleotides and pH on the inhibition of oxidative phosphorylation by bongkrekic acid. Biochim Biophys Acta;223:460–462.
17. Terada H. (1981) The interaction of highly active uncouplers with mitochondria. Biochim Biophys Acta;639:225–242.
18. Terada H. (1990) Uncouplers of oxidative phosphorylation. Environ Health Perspect;87:213–218.
19. Klingenberg M., Appel M., Babel W., Aquila H. (1983) The binding of bongkrekate to mitochondria. Eur J Biochem;131:647–654.
20. Zoccarato F., Rugolo M., Siliprandi D., Siliprandi N. (1981) Correlated effluxes of adenine nucleotides, Mg^{2+} and Ca^{2+} induced in rat-liver mitochondria by external Ca^{2+} and phosphate. Eur J Biochem;114:195–199.