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Artepillin C isoprenomics: Design and synthesis of artepillin C isoprene analogues as lipid peroxidation inhibitor having low mitochondrial toxicity

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Abstract—We designed and synthesized isoprene analogues of artepillin C, a major component of Brazilian propolis, and investigated the inhibitory activity on lipid peroxidation of rat liver mitochondria (RLM) and RLM toxicity based on isoprenomics. We succeeded in the synthesis of artepillin C isoprene analogues using regioselective prenylation within the range from 22% to 53% total yield. Reactivity of artepillin C and its isoprene analogues with ABTS (2,2′-Azinobis(3-ethylbenzothiazoline-6-sulfonate)) radical cations showed only a slight difference among the molecules. The isoprene side-chain elongation analogues of artepillin C showed almost the same inhibitory activity against RLM lipid peroxidation as artepillin C. Artepillin C and its isoprene analogues had very weak RLM uncoupling activity. Moreover, artepillin C and its isoprene analogues exhibited a lower inhibitory activity against adenosine 5′-triphosphate (ATP) synthesis by about two orders of magnitude than the effective inhibitory activity against RLM lipid peroxidation. From these results we conclude that artepillin C isoprene analogues could be potent lipid peroxidation inhibitors having low mitochondrial toxicity. We also conclude that elongation of the isoprene side chain of artepillin C to increase lipophilicity had little influence on the inhibitory activity toward RLM lipid peroxidation.

1. Introduction

Mitochondria are indispensable to the maintenance of human life as the site of organization of energy production. However, it is well known that potentially toxic reactive oxygen species (ROS) are produced by the electron transport chain (ETC) in the mitochondrial inner membrane.[1] In addition, mitochondria are very susceptible to lipid peroxidation by ROS because of the presence in the mitochondrion of high concentrations of unsaturated lipids.[2] It has been pointed out that such ROS-mediated peroxidation is related to carcinogenesis and aging.[3,4] Natural anti-oxidants are present to provide protection against this. Thus, ubiquinone (coenzyme Q) is a component of the mitochondrial respiratory chain.[5] A structural feature of ubiquinone is the presence of from 1 to 12 of consecutive prenyl side chains that makes ubiquinone a highly hydrophobic structure with high affinity for membrane. In addition, a semiquinone radical of ubiquinol, as a reduced form of ubiquinone, is relatively stable so it functions as an effective antioxidant in the mitochondria.[6] Paradoxically, coenzyme Q is also involved in superoxide production by the respiratory chain.[7] The development of additional effective antioxidants targeted to the mitochondria is an important strategy for the prevention and treatment of mitochondrial dysfunction.

α-Tocopherol (vitamin E) is an endogenous antioxidant and potent lipid peroxidation inhibitor, possessing the phenolic hydroxyl group and hydrophobic alkyl side chain present in ubiquinone. Exogenous antioxidants also are known Artepillin C (Fig. 1) is a diprenyl-*p*-hydroxycinnamic acid derivative first isolated from Bac-

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charis species,[8] as a major constituent (>5%) in Brazilian propolis.[9] Artepillin C is a prenyl-containing compound that displays a variety of medicinal effects such as antibacterial,[10] antitumor,[11] apoptosis-inducing,[12] immunomodulating,[13] and antioxidative activities.[14] It is interesting that, despite being less hydrophobic than α-tocopherol, artepillin C has a three times higher inhibitory activity on lipid peroxidation than α-tocopherol.[15] Though the reason for the higher activity is unclear, this suggested to us that there exists an optimal hydrophobicity and molecular structure for the design of this class of lipid peroxidation inhibitors. In fact, drupanin, a mono-prenyl analogue of artepillin C, exhibited weaker inhibition of peroxidation of linoleic acid than did artepillin C.[14] In contrast to prenylated and non-prenylated flavonoids, an increase in the number of prenyl substituents decreased antioxidant activity in the lipid peroxidation system.[16] Therefore, we felt it was important to elucidate a relationship between the number and length of prenyl groups of isoprenoids (optimal hydrophobicity) and the potency of inhibition of lipid peroxidation based on what we have termed 'isoprenomics'[15,17] (medicinal chemistry of isoprenoid involved in structural analysis, biosynthesis, biological function, and chemotype[18]).

In this paper, we report the design and synthesis of natural and synthetic artepillin C analogues having various isoprene side chains. We then studied the inhibitory activity on rat liver mitochondria (RLM) lipid peroxidation of these analogues. In addition, we evaluated their mitochondrial toxicity with respect to RLM uncoupling activity and inhibitory activity of adenosine 5′-triphosphate (ATP) synthesis.

2. Results

2.1. Design and molecular modeling

Naturally occurring *p*-hydroxycinnamic acid analogues having isoprene side chains are limited in number. Three such compounds, including artepillin C, drupanin[19] (we have designated as 'TX-1959'), and the mono-geranyl compound[20] that we have designated 'TX-2012' have been identified. For this study, we designed additional *p*-hydroxycinnamic acid analogues having isoprene side chain such as mono-farnesyl (TX-2101), prenyl and geranyl (TX-2013), and di-geranyl (TX-2007) (Fig. 1). The highest occupied molecular orbital (HOMO) and the lowest unoccupied molecular orbital (LUMO) energy of artepillin C and these analogues were calculated to have almost the same values (Table 1).

2.2. Synthesis

As shown in Scheme 1, the preparation of the target compounds was based on regioselective *C*-prenylation of *p*-iodophenol according to our established procedure.[15,21] In the *C*-prenylation reaction, increasing the length of isoprene units tends to increase the reactivity (the yield of compounds **1**, **7**, and **13** was 58%, 62%, and 75%, respectively). The yield of **4** was relatively low (46%) due to competing *O*-prenylation. Acetylation of prenylated compounds **1**, **4**, **7**, **10**, and **13** produced compound **2**, **5**, **8**, **11**, and **14** in good yields. These underwent Mizoroki–Heck coupling with methyl acrylate in the presence of $Pd(OAc)_2$, $(o\text{-tol})_3P$, and Et_3N in dry toluene to give α, β-unsaturated carboxylates **3**, **6**, **9**, **12**, and **15** in reasonable yield, except for compound **6**. Finally, these compounds were deprotected in methanolic potassium hydroxide to give analytically pure TX-1959, TX-2007, TX-2012, TX-2013, and TX-2101 in 38%, 22%, 45%, 26%, and 53% total yield, respectively.

2.3. Antioxidant activity

The antioxidant activities of artepillin C and its isoprene analogues were evaluated by spectroscopic analysis with ABTS (2,2′-Azinobis(3-ethylbenzothiazoline-6-sulfonate)) radical cations. The IC_{50} values of artepillin C and its isoprene analogues were almost the same in the

Table 1. HOMO and LUMO energy of artepillin C and its isoprene analogues

Compound	E_{HOMO} (eV)	E_{LUMO} (eV)
Artepillin C	−8.910	−0.693
TX-1959	−8.977	−0.732
TX-2007	−8.917	−0.702
TX-2012	−9.005	−0.728
TX-2013	−8.907	−0.695
TX-2101	−9.063	−0.745

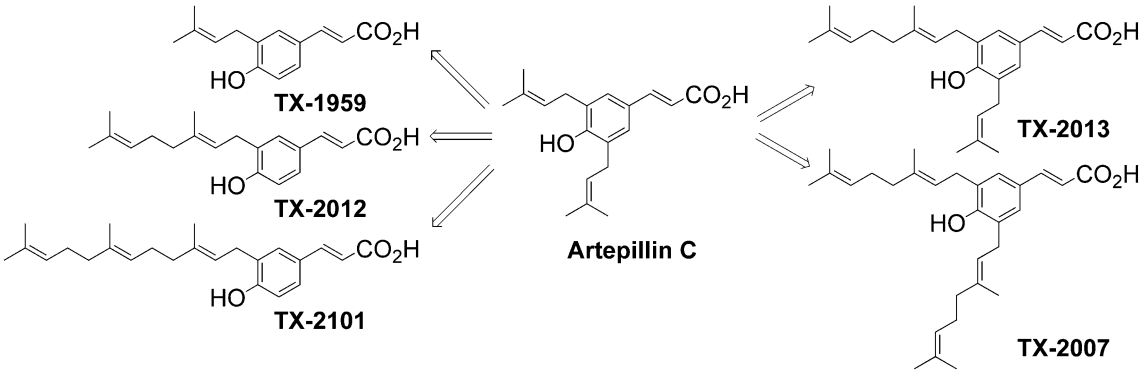
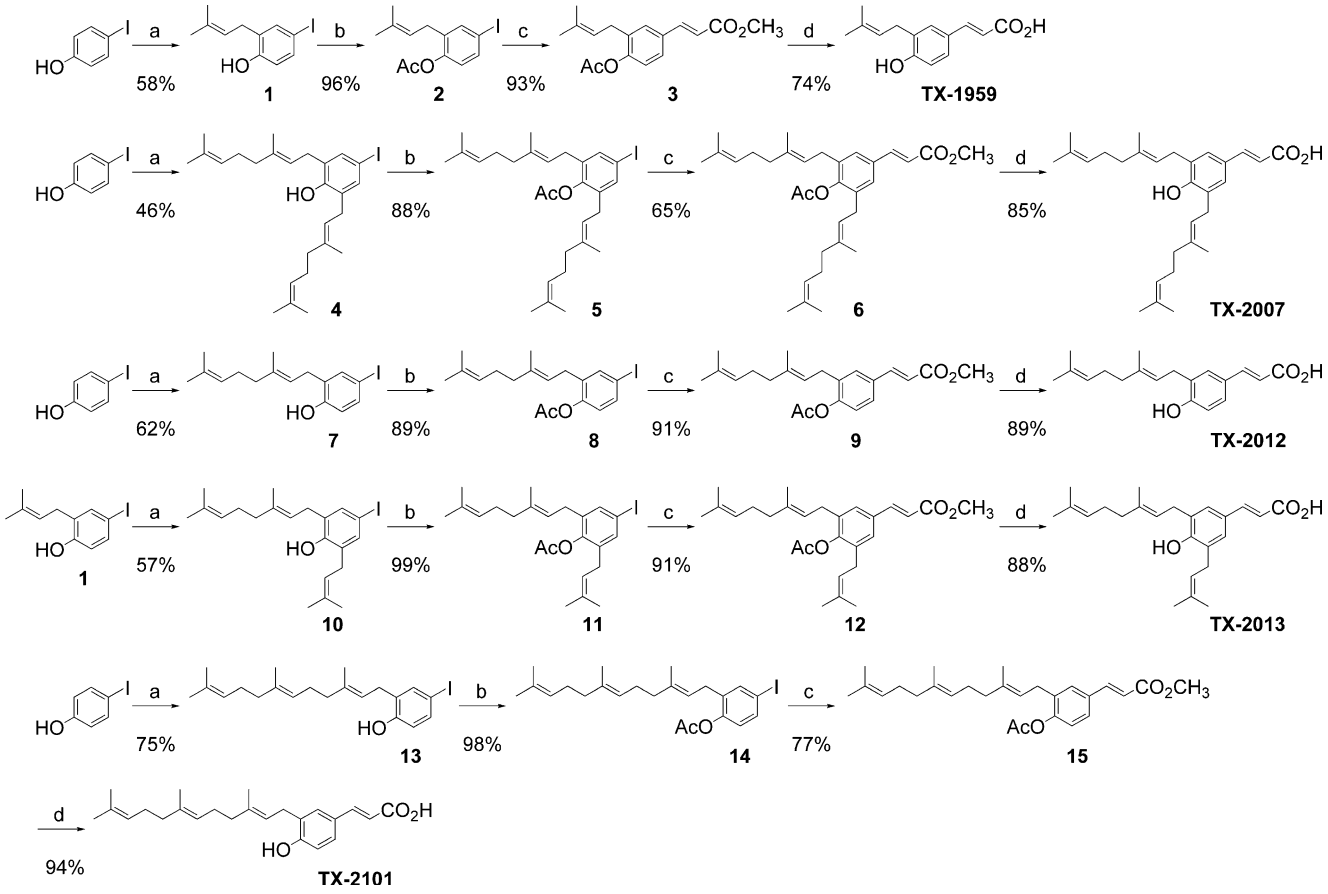


Figure 1. Structure of artepillin C and our designed artepillin C isoprene analogues based on isoprenomics.



Scheme 1. Reagents and conditions: (a) prenyl or geranyl or farnesyl bromide/NaH/toluene, rt, 1 h; (b) AcCl/DMAP/CH_2Cl_2, reflux, 4 h; (c) $CH_2CHCOOMe/Et_3N/(o\text{-tol})_3P/Pd(OAc)_2$/toluene, reflux, 20 h; (d) KOH/MeOH/H_2O, reflux, 3 h.

range of 6.84–8.37 μM. The mono-isoprene analogues such as TX-1959, TX-2012, and TX-2101 consistently showed a slightly higher reactivity than the di-isoprene analogues such as TX-2007 and TX-2013 (Table 2).

2.4. Inhibitory activity on lipid peroxidation

We next investigated the activities of the artepillin C isoprene analogues as inhibitors of RLM lipid peroxidation. ADP and Fe^{2+} were used as initiators of a radical reaction, and then IC_{50} values were estimated from O_2 consumption in the presence or absence of artepillin C isoprene analogues (Table 2). We found that the inhibitory activities of artepillin C isoprene elongation analogues TX-2013 ($IC_{50} = 0.84 \pm 0.08$) and TX-2007 ($IC_{50} = 0.83 \pm 0.09$) were nearly equal to that of artepillin C ($IC_{50} = 0.72 \pm 0.03$). In addition, the mono-prenyl analogue TX-1959 showed a weaker inhibitory activity ($IC_{50} = 37.80 \pm 3.04$) than artepillin C. The mono-geranyl analogue TX-2012 ($IC_{50} = 2.61 \pm 0.65$) and mono-farnesyl analogue TX-2101 ($IC_{50} = 2.32 \pm 0.26$) have similar inhibitory activities, and these compounds were more potent than TX-1959 ($IC_{50} = 37.80 \pm 3.04$).

Table 2. Antioxidant activity of artepillin C and its isoprene analogues

Compound	ABTS (IC_{50}, μM)	RLM lipid (IC_{50}, μM)
Artepillin C	7.81 ± 0.15	0.72 ± 0.03
TX-1959	6.92 ± 0.39	37.80 ± 3.04
TX-2007	8.21 ± 0.09	0.83 ± 0.09
TX-2012	7.33 ± 0.81	2.61 ± 0.65
TX-2013	8.37 ± 0.39	0.84 ± 0.08
TX-2101	6.84 ± 0.29	2.32 ± 0.26

Values represent means ± SD of three experiments.

2.5. Mitochondrial uncoupling activity

Phenolic compounds having a weakly acidic proton surrounded by a hydrophobic side chain act as proton uncouplers in mitochondria. An example is 3,5-di-*tert*-butyl-4-hydroxybenzylidenemalononitrile (SF6847).[22] If effective uncoupling and antioxidant activity are similar, these activities would compete so the net antioxidant activity would be weakened. Therefore, we examined the uncoupling activity of artepillin C and its analogues, all of which showed very weak or no uncoupling activity (Table 3). Interestingly, we observed an inhibition of mitochondrial ETC activities with artepillin C and its isoprene analogues at high dose (data not shown).

2.6. Inhibitory activity of ATP synthesis

To examine mitochondrial toxicity further, we investigated the activity of artepillin C and its isoprene ana-

Table 3. Mitochondrial toxicity of artepillin C and its isoprene analogues

Compound	Uncoupling activity		ATP synthesis (IC_{50}, μM)
	C_{max} (μM)	V_{max} (natomO/mg/min)	
Artepillin C	125	180	47
TX-1959	100	98	110
TX-2007	20	86	13
TX-2012	50	88	34
TX-2013	25	108	23
TX-2101	40	119	18
SF6847	0.01	280	0.007

logues as inhibitors of ATP synthesis (Table 3). As shown in Table 3, activities as inhibitors of ATP synthesis were lower than the activities as inhibitors of RLM lipid peroxidation by a factor of two orders of magnitude.

3. Discussion

In this paper, we designed and synthesized isoprene analogues of artepillin C and investigated their reactivity towards free radicals and activities as inhibitors of RLM lipid peroxidation, and as well as RLM toxicity. Our molecular orbital calculations suggest that the conversion of two-prenyl group on artepillin C into various isoprene side chains does not influence the HOMO or LUMO energies and electron distribution on artepillin C. Thus we predicted the two-prenyl groups on artepillin C do not contribute to the reactivity toward free radicals. In fact, essentially no difference in the reactivity of all isoprene analogues of artepillin C with the ABTS radical could be detected. On the other hand, we have already revealed that di-isoprene analogues, including artepillin C, have higher reactivity toward the DPPH (1,1-diphenyl-2-picrylhydrazyl) free radical than do mono-isoprene analogues.[17] These results may be explained in terms of difference in stability of the two free radicals. From these results we conclude that the reactivity of antioxidants toward free radical depends on the nature of the free radicals and the experimental conditions. Thus, care must be taken in the design of radical scavenging experiments in evaluation of the potency of antioxidants.

It is well known that mitochondria are susceptible to lipid peroxidation by reactive free radicals because of the presence in the mitochondrion of a high concentration of unsaturated lipid.[2] Consequently, RLM are widely used as a preparation to evaluate the activities of inhibitors of lipid peroxidation. In this study, we have shown that artepillin C, TX-2007, and TX-2013 have essentially the same activities as inhibitors of RLM lipid peroxidation. This result suggests that isoprene chain elongation of artepillin C has no effect on activity with respect to inhibition of RLM lipid peroxidation. However, the inhibition of RLM lipid peroxidation by mono-prenyl analogue TX-1959 was greatly decreased compared to artepillin C, probably because of its lower hydrophobicity ($\log P = 3.53$). Interestingly, the potency of inhibition of RLM lipid peroxidation was increased by the replacement of the prenyl side chain of TX-1959 with the geranyl side chain (TX-2012), whereas the activity was not changed in the replacement of the geranyl side chain of TX-2012 with the farnesyl side chain (TX-2101). In general, we have shown that a more hydrophobic antioxidant has a higher inhibitory activity toward lipid peroxidation,[23] although we suggested that the relationship may be applicable to a limited range of hydrophobicity. In the case of the artepillin C isoprene analogues, the upper limit of the log*P* value of artepillin C (5.66) or TX-2012 (5.60) may be taken for the hydrophobicity–activity relationship.

The relatively hydrophobic artepillin C isoprene analogues act as potent RLM lipid peroxidation inhibitors. It seemed possible that these molecules could also affect mitochondrial functions such as mitochondrial uncoupling or ATP synthesis. Despite this expectation, mitochondrial uncoupling activities of artepillin C isoprene analogues were lower compared to their activities as inhibitors of lipid peroxidation. Artepillin C ($pK_a = 9.64$) is much less acidic than the potent mitochondrial uncoupler SF6847 ($pK_a = 6.86$). Thus, the phenolic hydroxyl group of artepillin C is hardly dissociated under physiological pH. For this reason, we felt that artepillin C and its isoprene analogues would not function as protonophore uncouplers on the mitochondrial membrane. However, the question of inhibition of mitochondrial ETC activities with artepillin C and its isoprene analogues at high dose remains open. On the other hand, inhibition of ATP synthesis by artepillin C and its isoprene analogues was observed. However, the IC_{50} values for inhibition of ATP synthesis were higher by about one to two orders of magnitude than those for inhibition of lipid peroxidation. Interestingly, we found a good correlation ($r = 0.973$) between the magnitude of hydrophobic parameter log *P* values and the potency of inhibition of ATP synthesis (Fig. 2). Therefore, we propose that artepillin C and its isoprene analogues may be a useful inhibitor of lipid peroxidation in particular because of its low mitochondrial toxicity.

Excluding post-modification products, at present only three *p*-hydroxycinnamic acid derivatives having isoprene side chains have been isolated from natural sources, namely artepillin C (two prenyl side chain), drupanin (one prenyl side chain), and mono-geranyl 'TX2012'. It is interesting to note that naturally occurring small molecules having isoprene side chains longer than the geranyl group, such as the farnesyl group or larger, have not found except for tocotrienol (vitamin E), vitamin K_2, and ubiquinone, all required by a living organism. Based on this, we have suggested that these highly hydrophobic isoprene side chains became mandatory for these molecules as the assumed important biological functions in lipid membrane. For example, Ras proteins are guanine nucleotide-binding proteins that undergo post-translational modifications,[24] such as farnesylation, that lead to their localization tin the plasma membrane. Furthermore, natural products having isoprene side chains isolated from plants seem limited to those possessing the prenyl side chain. With respect to this difference, we proposed that an optimal hydrophobicity of essential bio-

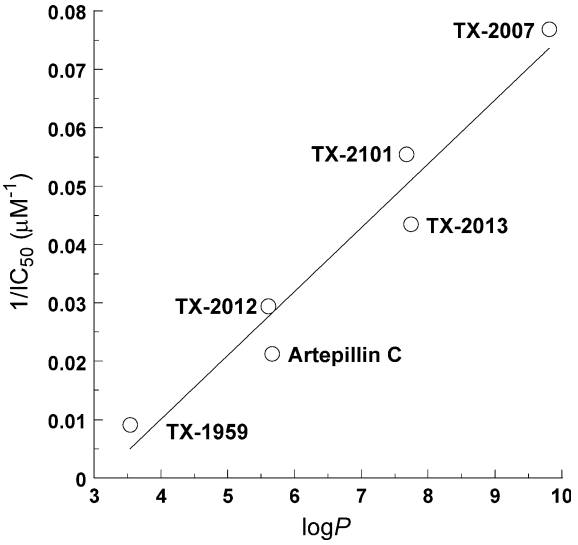


Figure 2. Hydrophobic parameter log *P* versus inhibitory activity of ATP synthesis ($1/IC_{50}$).

logical molecules exists. Thus, such natural products are prenylated to protect them from being too strongly bound to membrane but they have sufficient hydrophobicity to express membrane associated biological activities. If this hypothesis were true, novel membrane-bound biologically active small molecules might be designed by artificial modification of various plant materials with farnesyl side chains.

4. Conclusion

In this report, we report the design and synthesis of novel artepillin C isoprene analogues, including natural and synthetic molecules, based on isoprenomics. We further demonstrate that these isoprene analogues may function as potent lipid peroxidation inhibitors having low mitochondrial toxicity. We also show that elongation of isoprene side chain of artepillin C has little influence on potency of inhibition RLM lipid peroxidation but leads to an increase in RLM toxicity.

5. Experimental

5.1. General procedures

^{1}H NMR spectra were recorded on a JEOL JNM-EX400 spectrometer (400 MHz) with tetramethylsilane as the internal standard. Chemical shifts are reported in ppm. Coupling constants are reported in Hz. Mass spectra were measured on a Shimadzu GC-MS QP-1000 mass spectrometer using an electron ionization (EI) method. All melting points were determined by Yanagimoto micro melting point apparatus (MP-S3 model) and are uncorrected. Elemental analyses were performed with a Yanako CHN recorder MT-5. Reactions were monitored by analytical thin-layer chromatography (TLC) with use of Merck silica gel $60F_{254}$ glass plates. Column chromatography was performed on Kanto Chemical silica gel 60N (230–400 mesh). All the chemicals were purchased from Wako Pure Chemical Industries, Ltd (Osaka, Japan), Kanto Chemical Co., Inc. (Tokyo, Japan), Tokyo Chemical Industry Co., Ltd (Tokyo, Japan), and Sigma–Aldrich Japan (Tokyo, Japan).

5.2. Molecular modeling methods

The semi-empirical MO calculation was applied by the AM1 methods of Stewart and the program MOPAC2000 (Fujitsu, Japan).[25] The orbital energy (eV) of the HOMO and LUMO was calculated.

5.3. General procedure for C-prenylation: synthesis of 1, 4, 7, 10, and 13

p-Iodophenol was dissolved in dry toluene, and then NaH (1.1 mol equiv) was added. After the reaction mixture was stirred at room temperature for 5 min, prenyl or geranyl or farnesyl bromide (1.0 or 2.0 per mol of *p*-iodophenol) was added. After the reaction mixture was stirred at room temperature for 1 h, the mixture was poured into ice water and then acidified with 2 M CH_3COOH. The mixture was extracted with Et_2O and washed with saturated aqueous $NaHCO_3$ followed by saturated aqueous NaCl. The Et_2O layer was dried (anhydrous $MgSO_4$) and evaporated under reduced pressure. Residues were purified by silica gel column chromatography with hexanes and Et_2O to give the product as a colorless oil.

5.3.1. 4-Iodo-2-(3-methyl-2-butenyl)phenol (1). Yield 58%. ^{1}H NMR ($CDCl_3$) δ 7.35 (m, 2H), 6.55 (d, 1H, J = 8.4 Hz), 5.92 (s, 1H), 5.26 (t, 1H, J = 7.2 Hz), 3.27 (d, 2H, J = 7.2 Hz), 1.75 (s, 6H).

5.3.2. 2,6-Di[(2*E*)-3,7-dimethyl-2,6-octadienyl]-4-iodophenol (4). Yield 46%. ^{1}H NMR ($CDCl_3$) δ 7.26 (s, 2H), 5.37 (s, 1H), 5.25 (td, 2H, J = 7.3 Hz, 1.2 Hz), 5.07 (m, 2H), 3.29 (d, 4H, J = 7.3 Hz), 2.09 (m, 8H), 1.73 (s, 6H), 1.69 (s, 6H), 1.60 (s, 6H).

5.3.3. 2-[(2*E*)-3,7-Dimethyl-2,6-octadienyl]-4-iodophenol (7). Yield 62%. ^{1}H NMR ($CDCl_3$) δ 7.38 (m, 2H), 6.58 (dd, 1H, J = 6.3 Hz, 2.7 Hz), 5.27 (td, 1H, J = 7.1 Hz, 1.2 Hz), 5.16 (s, 1H), 5.06 (m, 1H), 3.30 (d, 2H, J = 7.1 Hz), 2.10 (m, 4H), 1.76 (s, 3H), 1.69 (s, 3H), 1.60 (s, 3H); EIMS *m/e*: 356 (M^+).

5.3.4. 2-[(2*E*)-3,7-Dimethyl-2,6-octadienyl]-4-iodo-6-(3-methyl-2-butenyl)phenol (10). Yield 57%. ^{1}H NMR ($CDCl_3$) δ 7.26 (s, 2H), 5.38 (s, 1H), 5.26 (t, 2H, J = 7.2 Hz), 5.07 (t, 1H, J = 6.8 Hz), 3.28 (t, 4H, J = 6.6 Hz), 2.11 (m, 4H), 1.77 (s, 3H), 1.74 (s, 6H), 1.69 (s, 3H), 1.60 (s, 3H); EIMS *m/e*: 424 (M^+).

5.3.5. 4-Iodo-2-[(2*E*,6*E*)-3,7,11-trimethyl-2,6,10-dodecatrienyl]phenol (13). Yield 75%. ^{1}H NMR ($CDCl_3$) δ 7.38 (m, 2H), 6.57 (dd, 1H, J = 8.8 Hz, 1.7 Hz), 5.27 (t, 1H, J = 7.2 Hz), 5.14 (s, 1H), 5.08 (t, 2H, J = 6.1 Hz), 3.31 (d, 2H, J = 7.2 Hz), 2.05 (m, 8H), 1.76 (s, 3H), 1.67 (s, 3H), 1.60 (s, 6H); EIMS *m/e*: 424 (M^+).

5.4. General procedure for acetylation: synthesis of 2, 5, 8, 11, and 14

Prenylated *p*-iodophenol (**1**, **4**, **7**, **10**, **13**) and DMAP (1.2 mol equiv) were dissolved in dry CH_2Cl_2, and then AcCl (2.0 mol equiv) was added dropwise. The mixture was refluxed for 4 h, the mixture was cooled to room temperature and evaporated. The residue was chromatographed over silica gel eluated by hexanes/Et_2O (10:1) to afford acetylated compound as colorless oil.

5.4.1. 4-Iodo-2-(3-methyl-2-butenyl)phenyl acetate (2). Yield 96%. ^{1}H NMR ($CDCl_3$) δ 7.52 (m, 2H), 6.77 (d, 1H, J = 8.0 Hz), 5.17 (t, 1H, J = 6.8 Hz), 3.17 (d, 2H, J = 6.8 Hz), 2.29 (s, 3H), 1.76 (s, 6H).

5.4.2. 2,6-Di[(2*E*)-3,7-dimethyl-2,6-octadienyl]-4-iodophenyl acetate (5). Yield 88%. ^{1}H NMR ($CDCl_3$) δ 7.37 (s, 2H), 5.19 (t, 2H, J = 7.1 Hz), 5.09 (m, 2H), 3.14 (d, 4H, J = 7.1 Hz), 2.29 (s, 3H), 2.06 (m, 8H), 1.69 (s, 6H), 1.66 (s, 6H), 1.60 (s, 6H); EIMS *m*/*e*: 491 ([M−Ac]$^+$).

5.4.3. 2-[(2*E*)-3,7-Dimethyl-2,6-octadienyl]-4-iodophenyl acetate (8). Yield 89%. ^{1}H NMR ($CDCl_3$) δ 7.50 (m, 2H), 6.77 (d, 1H, J = 8.3 Hz), 5.19 (td, 1H, J = 7.3 Hz, 1.1 Hz), 5.10 (t, 1H, J = 6.7 Hz), 3.19 (d, 2H, J = 7.3 Hz), 2.30 (s, 3H), 2.07 (m, 4H), 1.69 (s, 3H), 1.67 (s, 3H), 1.61 (s, 3H); EIMS *m*/*e*: 398 (M$^+$).

5.4.4. 2-[(2*E*)-3,7-Dimethyl-2,6-octadienyl]-4-iodo-6-(3-methyl-2-butenyl)phenyl acetate (11). Yield 99%. ^{1}H NMR ($CDCl_3$) δ 7.37 (s, 2H), 5.17 (m, 2H), 5.10 (m, 1H), 3.13 (t, 4H, J = 6.3 Hz), 2.30 (s, 3H), 2.08 (m, 4H), 1.74 (s, 3H), 1.69 (s, 3H), 1.67 (s, 3H), 1.66 (s, 3H), 1.61 (s, 3H); EIMS *m*/*e*: 466 (M$^+$).

5.4.5. 4-Iodo-2-[(2*E*,6*E*)-3,7,11-trimethyl-2,6,10-dodecatrienyl]phenyl acetate (14). Yield 98%. ^{1}H NMR ($CDCl_3$) δ 7.52 (m, 2H), 6.77 (d, 1H, J = 8.3 Hz), 5.19 (td, 1H, J = 7.3 Hz, 1.2 Hz), 5.10 (m, 2H), 3.19 (d, 2H, J = 7.3 Hz), 2.29 (s, 3H), 2.05 (m, 8H), 1.76 (s, 3H), 1.67 (s, 3H), 1.60 (s, 6H); EIMS *m*/*e*: 466 (M$^+$).

5.5. General procedure for Mizoroki–Heck coupling: synthesis of 3, 6, 9, 12, 15

A solution of acetylated compound (**2**, **5**, **8**, **11**, and **14**), methyl acrylate (5.0 mol equiv), Et_3N (2.0 mol equiv), (*o*-tol)$_3$P (10% mol equiv), and $Pd(OAc)_2$ (5% mol equiv) in dry toluene was refluxed for 20 h. The mixture was cooled to room temperature, diluted with Et_2O, and filtered through celite. The filtrate was evaporated under reduced pressure. Residues were purified by column chromatography on silica gel (hexanes/Et_2O) to give α,β-unsaturated carboxylate as a light yellow oil.

5.5.1. Methyl (*E*)-3-[4-(acetyloxy)-3-(3-methyl-2-butenyl)phenyl]-2-propenoate (3). Yield 93%. ^{1}H NMR ($CDCl_3$) δ 7.65 (d, 1H, J = 15.6 Hz), 7.28 (m, 2H), 7.05 (d, 1H, J = 8.8 Hz), 6.38 (d, 1H, J = 15.6 Hz), 5.21 (t, 1H, J = 6.8 Hz), 3.80 (s, 3H), 3.24 (d, 2H, J = 6.8 Hz), 2.32 (s, 3H), 1.76 (s, 3H), 1.72 (s, 3H).

5.5.2. Methyl (*E*)-3-4-(acetyloxy)-3,5-di[(2*E*)-3,7-dimethyl-2,6-octadienyl]phenyl-2-propenoate (6). Yield 65%. ^{1}H NMR ($CDCl_3$) δ 7.62 (d, 1H, J = 16.0 Hz), 7.24 (s, 2H), 6.34 (d, 1H, J = 16.0 Hz), 5.23 (t, 2H, J = 7.1 Hz), 5.10 (m, 2H), 3.79 (s, 3H), 3.21 (d, 4H, J = 7.1 Hz), 2.32 (s, 3H), 2.08 (m, 8H), 1.68 (s, 12H), 1.60 (s, 6H); EIMS *m*/*e*: 450 ([M−Ac]$^+$).

5.5.3. Methyl (*E*)-3-4-(acetyloxy)-3-[(2*E*)-3,7-dimethyl-2,6-octadienyl]phenyl-2-propenoate (9). Yield 91%. ^{1}H NMR ($CDCl_3$) δ 7.65 (d, 1H, J = 16.1 Hz), 7.39 (m, 2H), 7.05 (dd, 1H, J = 7.6 Hz, 1.0 Hz), 6.37 (d, 1H, J = 16.1 Hz), 5.23 (td, 1H, J = 7.3 Hz, 1.2 Hz), 5.10 (td, 1H, J = 6.6 Hz, 1.2 Hz), 3.80 (s, 3H), 3.25 (d, 2H, J = 7.3 Hz), 2.32 (s, 3H), 2.08 (m, 4H), 1.70 (s, 3H), 1.68 (s, 3H), 1.60 (s, 3H); EIMS *m*/*e*: 356 (M$^+$).

5.5.4. Methyl (*E*)-3-{4-(acetyloxy)-3-[(2*E*)-3,7-dimethyl-2,6-octadienyl]-5-(3-methyl-2-butenyl)phenyl}-2-propenoate (12). Yield 91%. ^{1}H NMR ($CDCl_3$) δ 7.62 (d, 1H, J = 16.0 Hz), 7.23 (s, 2H), 6.35 (d, 1H, J = 16.0 Hz), 5.23 (m, 2H), 5.11 (t, 1H, J = 6.8 Hz), 3.80 (s, 3H), 3.20 (t, 4H, J = 6.3 Hz), 2.32 (s, 3H), 2.09 (m, 4H), 1.76 (s, 3H), 1.69 (s, 6H), 1.68 (s, 3H), 1.61 (s, 3H); EIMS *m*/*e*: 424 (M$^+$).

5.5.5. Methyl (*E*)-3-4-(acetyloxy)-3-[(2*E*,6*E*)-3,7,11-trimethyl-2,6,10-dodecatrienyl]phenyl-2-propenoate (15). Yield 77%. ^{1}H NMR ($CDCl_3$) δ 7.65 (d, 1H, J = 16.1 Hz), 7.39 (m, 2H), 7.05 (dd, 1H, J = 7.3 Hz, 1.7 Hz), 6.37 (d, 1H, J = 16.1 Hz), 5.23 (td, 1H, J = 7.3 Hz, 1.2 Hz), 5.10 (m, 2H), 3.80 (s, 3H), 3.26 (d, 2H, J = 7.3 Hz), 2.31 (s, 3H), 2.03 (m, 8H), 1.70 (s, 3H), 1.67 (s, 3H), 1.60 (s, 3H), 1.59 (s, 3H); EIMS *m*/*e*: 424 (M$^+$).

5.6. General procedure for deprotection: synthesis of TX-1959, TX-2007, TX-2012, TX-2013, and TX-2101

KOH (5% aqueous solution) was added to a solution of the α, β-unsaturated carboxylate (**3**, **6**, **9**, **12**, and **15**) in MeOH. The mixture was refluxed for 3 h, cooled to 0 °C, and acidified with 1 N HCl. The MeOH was evaporated under reduced pressure, and the aqueous residue was extracted with Et_2O. Extracts were washed with saturated aqueous $NaHCO_3$ and followed saturated aqueous NaCl. The Et_2O layer was dried (anhydrous $MgSO_4$) and evaporated under reduced pressure. Residues were purified by column chromatography on silica gel (CH_2Cl_2/MeOH) to give artepillin C isoprene analogues.

5.6.1. TX-1959 (drupanin[19]). (*E*)-3-[4-Hydroxy-3-(3-methyl-2-butenyl)phenyl]-2-propenoic acid. White solid. Yield 74%. ^{1}H NMR ($CDCl_3$) δ 7.71 (d, 1H, J = 15.6 Hz), 7.34 (m, 2H), 6.82 (d, 1H, J = 8.4 Hz), 6.30 (d, 1H, J = 15.6 Hz), 5.31 (t, 1H, J = 6.8 Hz), 3.37 (d, 2H, J = 6.8 Hz), 1.79 (s, 6H). Anal. Calcd for $C_{14}H_{16}O_3$: C, 72.39; H, 6.94. Found: C, 72.14; H, 6.86.

5.6.2. TX-2007. (*E*)-3-{3,5-Di[(2*E*)-3,7-dimethyl-2,6-octadienyl]-4-hydroxyphenyl}-2-propenoic acid. Light yellow oil. Yield 85%. ^{1}H NMR ($CDCl_3$) δ 7.70 (d,

1H, J = 15.9 Hz), 7.21 (s, 2H), 6.28 (d, 1H, J = 15.9 Hz), 5.74 (br s, 1H), 5.32 (t, 2H, J = 6.4 Hz), 5.09 (t, 2H, J = 6.8 Hz), 3.36 (d, 4H, J = 7.0 Hz), 2.11 (m, 8H), 1.77 (s, 6H), 1.69 (s, 6H), 1.61 (s, 6H); EIMS *m/e*: 436 (M^+). Anal. Calcd for $C_{29}H_{40}O_3$: C, 79.77; H, 9.23. Found: C, 79.93; H, 9.43.

5.6.3. TX-2012. (*E*)-3-{3-[(2*E*)-3,7-Dimethyl-2,6-octadienyl]-4-hydroxyphenyl}-2-propenoic acid. Light yellow oil. Yield 89%. ^{1}H NMR ($CDCl_3$) δ 7.72 (d, 1H, J = 15.9 Hz), 7.33 (m, 2H), 6.83 (d, 1H, J = 8.1 Hz), 6.30 (d, 1H, J = 15.9 Hz), 5.32 (td, 1H, J = 7.2 Hz, 1.2 Hz), 5.07 (t, 1H, J = 6.6 Hz), 3.38 (d, 2H, J = 7.2 Hz), 2.12 (m, 4H), 1.78 (s, 3H), 1.69 (s, 3H), 1.60 (s, 3H); EIMS *m/e*: 300 (M^+). Anal. Calcd for $C_{19}H_{24}O_3$: C, 75.97; H, 8.05. Found: C, 75.67; H, 8.06.

5.6.4. TX-2013. (*E*)-3-{3-[(2*E*)-3,7-Dimethyl-2,6-octadienyl]-4-hydroxy-5-(3-methyl-2-butenyl)phenyl}-2-propenoic acid. Light yellow oil. Yield 88%. ^{1}H NMR ($CDCl_3$) δ 7.70 (d, 1H, J = 15.9 Hz), 7.20 (s, 2H), 6.28 (d, 1H, J = 15.9 Hz), 5.73 (brs, 1H), 5.31 (t, 2H, J = 7.1 Hz), 5.08 (m, 1H), 3.36 (t, 4H, J = 7.1 Hz), 2.11 (m, 4H), 1.79 (s, 3H), 1.77 (s, 6H), 1.69 (s, 3H), 1.61 (s, 3H); EIMS *m/e*: 368 (M^+). Anal. Calcd for $C_{24}H_{32}O_3$: C, 78.22; H, 8.75. Found: C, 78.03; H, 8.59.

5.6.5. TX-2101. (*E*)-3-{4-Hydroxy-3-[(2*E*,6*E*)-3,7,11-trimethyl-2,6,10-dodecatrienyl]phenyl}-2-propenoic acid. Light yellow oil. Yield 94%. ^{1}H NMR ($CDCl_3$) δ 7.72 (d, 1H, J = 15.9 Hz), 7.33 (m, 2H), 6.82 (d, 1H, J = 8.3 Hz), 6.30 (d, 1H, J = 15.9 Hz), 5.32 (td, 1H, J = 7.1 Hz, 1.1 Hz), 5.08 (m, 2H), 3.38 (d, 2H, J = 7.1 Hz), 2.06 (m, 8H), 1.79 (s, 3H), 1.67 (s, 3H), 1.60 (s, 6H); EIMS *m/e*: 368 (M^+). Anal. Calcd for $C_{24}H_{32}O_3$: C, 78.22; H, 8.75. Found: C, 78.05; H, 8.73.

5.7. Antioxidant activity

Reactivity of artepillin C isoprene analogues with ABTS (2,2′-Azinobis(3-ethylbenzothiazoline-6-sulfonate)) radical cations was measured according to the method of Re et al.[26] A stock solution of ABTS radical cations was prepared one day before the assay by 5 ml of 7 mM ABTS and 2.45 mM potassium persulfate, followed by storage in dark at room temperature. The stock solution of ABTS radical cations was diluted with water or ethanol to an absorbance of about 0.70 at 734 nm. The decolorization assay was started by mixing 2 ml of the diluted ABTS solution with 20 μl of a test compound solution in a cuvette. Test compounds were dissolved with ethanol. At 6 min after mixing, the absorbance was measured at 734 nm. In a control experiment, a test compound solution was replaced with ethanol, and the radical scavenging activity of the test compound was expressed as IC_{50} value.

5.8. Preparation of rat liver mitochondria (RLM)

RLM were isolated from male Wistar rats (140–220 g) as reported by Myers and Slater.[27] The mitochondrial protein content was determined by the biuret method using bovine serum albumin as a standard.

5.9. Inhibition activity of RLM lipid peroxidation

Lipid peroxidation in RLM was performed at 25 °C and monitored as oxygen consumption with a Clark-type oxygen electrode in a total volume of 2.53 ml. Mitochondria were added at concentrations of 0.7 mg protein/ml. Peroxidation was started by addition of final concentrations of 1 mM ADP and 100 μM $FeSO_4$ in medium consisting of 175 mM KCl and 10 mM Tris–HCl buffer, pH 7.4. The amount of O_2 consumed during peroxidation was calculated assuming that the saturation concentration of O_2 at 25 °C is 258 μM. The percent inhibition with drug on RLM lipid peroxidation was calculated by the following equation:

$$\text{Inhibition}(\%) = \{1 - (R_p t/t_{inh}) \times k\} \times 100\%;$$
$$k = t_{inh0}/R_{p0}t_0,$$

where R_p, rate of lipid peroxidation (nmol O/min); R_{p0}, rate of lipid peroxidation of control (nmol O/min); t, total time (min); t_0, total time of control (min); t_{inh}, induction time (min); t_{inh0}, induction time of control (min). The mean effective concentration of antioxidant tested required for its 50% inhibition was defined as IC_{50} in the RLM lipid peroxidation reaction.

5.10. Mitochondrial uncoupling activity

The respiration of mitochondria was monitored polarographically with a Clark-type oxygen electrode. The incubation medium consisted of 200 mM sucrose, 2 mM $MgCl_2$, and 1 mM EDTA in 10 mM potassium phosphate buffer (pH 7.4). Mitochondria were added at 0.7 mg protein/ml in a total volume of 2.53 ml succinate (10 mM, plus rotenone at 1 μg/mg protein) was the respiratory substrate. The activity was determined by measuring changes in the rate of state 4 respiration upon addition of a test compound.

5.11. Inhibitory activity of ATP synthesis

ATP synthesis in mitochondria was determined as reported by Nishimura et al.[28] by measuring the increase in pH of the medium associated with ATP synthesis at 25 °C in medium consisting of 200 mM sucrose, 20 mM KCl, 3 mM $MgCl_2$, and 3 mM potassium phosphate buffer, pH 7.4, in a total volume of 1.68 ml. The reaction was started by adding 160 μM ADP to the mitochondrial suspension (0.7 mg protein/ml) energized with 5 mM succinate (plus rotenone at 1 μg/mg protein). The pH change was monitored using a pH meter.

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