Ellagitannins and Hexahydroxydiphenoyl Esters as Inhibitors of Vertebrate Squalene Epoxidase

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Received February 28, 2001

Ellagitannins isolated from various plant sources as well as newly synthesized *n*-alkyl (C_1−C_{18}) esters of hexahydroxydiphenyl (HHDP) dicarboxylic acid were evaluated as enzyme inhibitors of recombinant rat squalene epoxidase, a rate-limiting enzyme of cholesterol biosynthesis. Among the ellagitannins tested, pedunculagin (IC_{50} = 2.0 μM) and eugeniin (IC_{50} = 1.6 μM), both containing (*S*)-HHDP ester group(s), showed remarkable inhibition, which was more potent than those of previously reported substrate analogue inhibitors. Furthermore, ellagic acid (IC_{50} = 2.0 μM), a bislactone formed by hydrolytic release of a HHDP group from ellagitannins, was also a good inhibitor of the enzyme. On the other hand, the synthetic HHDP esters with C_6 (IC_{50} = 0.93 μM) and C_8 alkyl side chains (IC_{50} = 0.83 μM) showed potent enzyme inhibition at the submicromolar concentration range, while esters with shorter chain lengths (C_1−C_4) and a C_{18} ester exhibited moderate inhibition (IC_{50} = 8−47 μM).

Squalene epoxidase (SE) (EC 1.14.99.7) catalyzes the conversion of squalene to (3*S*)-2,3-oxidosqualene, a rate-limiting step of cholesterol biosynthesis.[1] In addition to oxygen, vertebrate SE requires FAD, NADPH, NADPH-cytochrome P-450 reductase, and a supernatant protein factor. The nonmetallic flavin monooxygenase induces the splitting of the O−O bond, the insertion of one oxygen atom into the substrate (squalene), and reduction of the other to H_2O via formation of flavin C(4a)-hydroperoxide (Figure 1).[1] Since SE controls the throughput from squalene to sterols in cholesterol biogenesis, enzyme inhibitors of SE have been potential targets for the design of cholesterol-lowering drugs. To date, several potent and specific SE enzyme inhibitors including chemically synthesized squalene analogues and allylamine derivatives have been developed, although there are as yet no successful reports of human clinical trials.[2]

We have previously reported that gallotannins such as galloylglucoses and gallocatechins including (−)-epigallocatechin 3-*O*-gallate (EGCG) (**1**) (IC_{50} = 0.69 μM), a major component of the cholesterol-lowering green tea polyphenols, are potent and selective inhibitors of recombinant rat SE (Table 1).[3] Furthermore, *n*-dodecyl gallate (DG) (**9**) (IC_{50} = 0.061 μM), a synthetic alkyl gallate widely used as an antioxidant food additive, showed even more potent inhibition.[4] This suggests that the presence of both the galloyl moiety and the "substrate-like" hydrophobic alkyl side chain are important for potent SE inhibition. We thus hypothesized that the enzyme inhibition would be caused by specific binding of the gallate to the enzyme, possibly the dodecyl side chain occupying the substrate (squalene) binding site, and the galloyl moiety in close proximity to the FAD binding domain of the flavoenzyme, where the antioxidative gallate would trap the reactive oxygen species required for the enzyme reaction.[4] In continuation of studies in the search for potent SE inhibitors from natural sources, we tested the inhibitory activities of ellagitannins (**21**−**38**) isolated from various plant sources against recombinant rat SE enzyme. Furthermore, we synthesized chemically a series of *n*-alkyl esters of hexahydroxydiphenoyl (HHDP) dicarboxylic acid (**11**−**20**), containing an *n*-alkyl gallate dimeric structure, which were also tested for their enzyme inhibition activity. The HHDP group, the basic structural unit of ellagitannins, is derived biosynthetically from oxidative C−C coupling of galloyl groups in vivo.[5] We hypothesized that the conformationally more rigid structures of ellagitannins, as a result of biphenyl bond formation, might provide more specific interactions with the active site of the enzyme.

Results and Discussion

Ellagitannins (**21**−**37**), evaluated for enzyme inhibition, were classified into two groups based on the location of the diphenoyl group in the glucose moiety: (i) 2,3- and/or 4,6-substituted and (ii) 2,4- and/or 3,6-substituted ellagitannins. The former tannins usually contain (*S*)-HHDP group(s), while the diphenoyl group of the latter possesses the *R*-configuration. Among the ellagitannins tested, pedunculagin (**22**) (IC_{50} = 2.0 μM) and eugeniin (**23**) (IC_{50} = 1.6 μM), both containing the 4,6-(*S*)-HHDP ester group, showed good inhibition toward recombinant rat SE (Table 1). The inhibition activities were more potent than those of known vertebrate SE inhibitors (substrate analogues): trisnorsqualene alcohol (IC_{50} = 4 μM for pig SE),[6a] trisnorsqualene cyclopropylamine (IC_{50} = 2 μM for pig SE),[6b] and trisnorsqualene difluoromethylidene (IC_{50} = 5.4 μM for rat SE).[6c]

Introduction of a galloyl group at C-1 of glucose in **22** and replacement of the 4,6-(*S*)-HHDP group of **22** with a gallagyl (tetraphenoyl) group both decreased the activity as seen in **24** and **25**. The inhibitory activities of *C*-glucosyl ellagitannins (**34** and **35**), which also contained a 4,6-(*S*)-HHDP moiety, were much less potent. In contrast, among the 2,4- or 3,6-substituted ellagitannins, corilagin (**27**), geraniin (**28**), and chebulagic acid (**33**), which contain a 1(β)-*O*-galloyl-3,6-(*R*)-HHDP glucose moiety as a common structural feature, also showed good inhibition against the enzyme, with IC_{50} values ranging from 3.2 to 4.1 μM. However, they were slightly less potent than **22** and **23**.

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10.1021/np010100y CCC: $20.00
Published on Web 07/07/2001

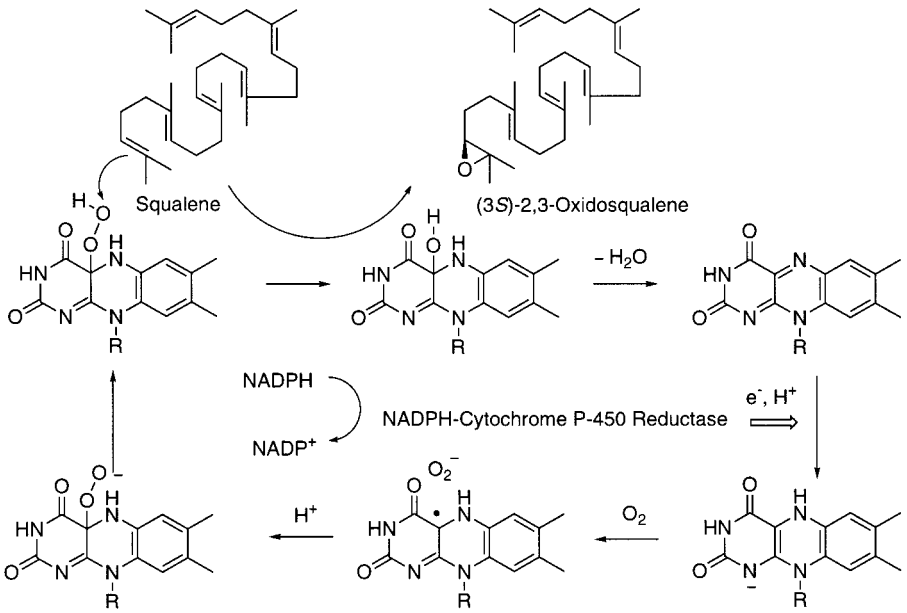


Figure 1. Proposed mechanism of epoxidation of squalene to (3*S*)-2,3-oxidosqualene by SE.

Table 1. Inhibition of Recombinant Rat Squalene Epoxidase by Ellagitannins and Hexahydroxydiphenoyl Esters

compound	IC_{50} value (mean ± SEM) (μM)
(−)-epigallocatechin-3-*O*-gallate (EGCG) (**1**)	0.69 ± 0.07[a]
theasinensin A (**2**)	0.13 ± 0.02[a]
procyanidin B-2 3,3′-di-*O*-gallate (**3**)	0.54 ± 0.06[b]
1,2,6-tri-*O*-galloyl-β-D-glucose (**4**)	0.63 ± 0.07[b]
1,2,3,4,6-penta-*O*-galloyl-β-D-glucose (**5**)	0.47 ± 0.05
gallic acid (**6**)	73 ± 8[c]
ethylgallate (**7**)	4.2 ± 0.5[c]
n-octylgallate (**8**)	0.83 ± 0.1[c]
n-dodecylgallate (**9**)	0.061 ± 0.007[c]
n-cetylgallate (**10**)	0.59 ± 0.07[c]
dimethyl 2,2′,3,3′,4,4′-hexahydroxybiphenyl-6,6′-dicarboxylate (**11**)	16.4 ± 1.5
diethyl 2,2′,3,3′,4,4′-hexahydroxybiphenyl-6,6′-dicarboxylate (**12**)	13.3 ± 1.5
n-dibutyl 2,2′,3,3′,4,4′-hexahydroxybiphenyl-6,6′-dicarboxylate (**13**)	7.9 ± 0.8
n-dihexyl 2,2′,3,3′,4,4′-hexahydroxybiphenyl-6,6′-dicarboxylate (**14**)	0.93 ± 0.1
n-dioctyl 2,2′,3,3′,4,4′-hexahydroxybiphenyl-6,6′-dicarboxylate (**15**)	0.83 ± 0.1
n-didecyl 2,2′,3,3′,4,4′-hexahydroxybiphenyl-6,6′-dicarboxylate (**16**)	1.1 ± 0.2
n-didodecyl 2,2′,3,3′,4,4′-hexahydroxybiphenyl-6,6′-dicarboxylate (**17**)	1.3 ± 0.2
n-ditetradecyl 2,2′,3,3′,4,4′-hexahydroxybiphenyl-6,6′-dicarboxylate (**18**)	1.5 ± 0.2
n-dihexadecyl 2,2′,3,3′,4,4′-hexahydroxybiphenyl-6,6′-dicarboxylate (**19**)	2.5 ± 0.3
n-dioctadecyl 2,2′,3,3′,4,4′-hexahydroxybiphenyl-6,6′-dicarboxylate (**20**)	47 ± 5
2,3-(*S*)-hexahydroxydiphenoyl-D-glucose (**21**)	32 ± 4
pedunculagin (**22**)	2.0 ± 0.5
eugeniin (**23**)	1.6 ± 0.3
1(β)-*O*-galloylpedunculagin (**24**)	7.0 ± 0.8
punicalagin (**25**)	40 ± 4
punicalin (**26**)	20 ± 3
corilagin (**27**)	4.0 ± 0.5
geraniin (**28**)	4.1 ± 0.4
furosin (**29**)	10 ± 1
mallotinic acid (**30**)	8.0 ± 0.9
mallotusinic acid (**31**)	8.0 ± 0.9
chebulinic acid (**32**)	12 ± 2
chebulagic acid (**33**)	3.2 ± 0.4
castalagin (**34**)	30 ± 3
casuarinin (**35**)	32 ± 4
sanguiin H-6 (**36**)	12 ± 2
phyllyraeoidin (**37**)	100 ± 10
ellagic acid (**38**)	2.0 ± 0.5

[a] Reported in ref 3a. [b] Ref 3b. [c] Ref 4a.

Furthermore, replacement of the 3,6-(*R*)-HHDP group of **27** and **28** with a valoneoyl group yielded less potent compounds as found in **30** and **31**, respectively. The compound also became less potent as observed in **32**, when the 3,6-(*R*)-HHDP group of **33** was replaced with galloyl groups. Moreover, ellagitannins with high molecular weight, e.g., the dimeric sanguiin H-6 (**36**) (IC_{50} = 12 μM) and phyllyraeoidin (**37**) (IC_{50} = 100 μM), showed moderate inhibition.

In general, ellagitannins are not as inhibitory as the previously tested gallotannins (**1**−**5**) (Table 1). These results suggest that the inhibitory effects of the naturally

Chart 1

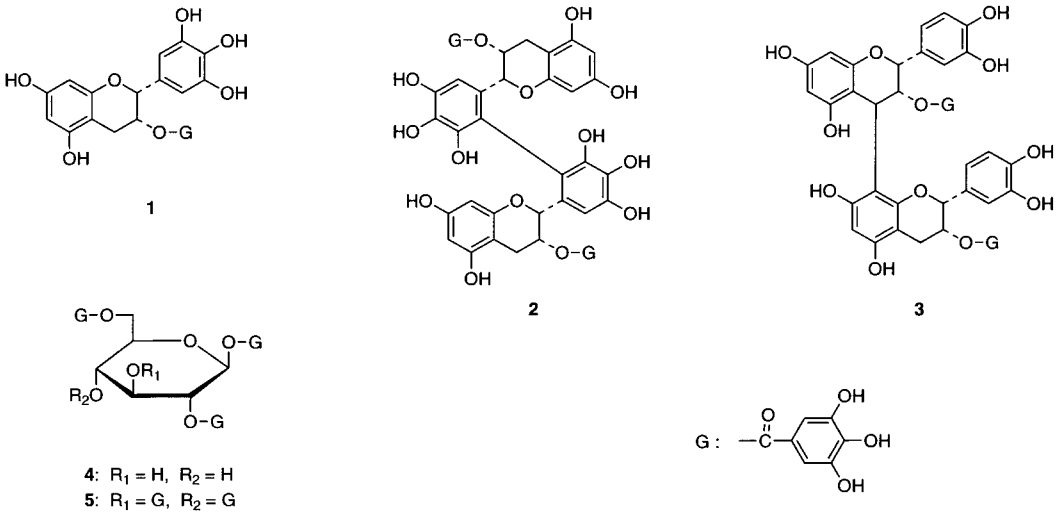
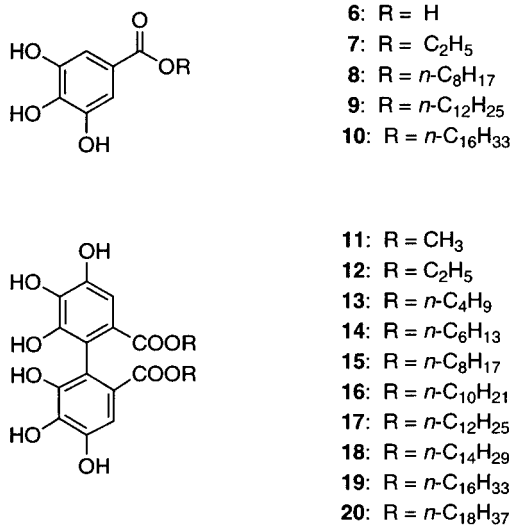


Chart 2



occurring HDDP esters and their derivatives, namely, dehydrohexahydroxydiphenoyl (DHHDP), valoneoyl, chebuloyl, and gallagyl groups, which are biosynthetically derived by oxidative coupling of galloyl esters,[5] may be weaker than those of the original galloyl esters. The conformationally more rigid HHDP-bearing ellagitannins appear to have less affinity to the active site of the enzyme.

For ellagitannins, a variety of biological activities, including high levels of cytotoxic[7] and antiviral activity[8] in the micromolar to nanomolar concentration ranges, have been reported. The above-described remarkable inhibition activities against SE, the rate-limiting enzyme of cholesterol biosynthesis, add another potential beneficial features of ellagitannins. In addition, it should be noted here that the inhibitory activity of ellagic acid (**38**) (IC_{50} = 2.0 μM), a bislactone formed by hydrolytic release of the HHDP group from ellagitannins, was far more potent than that of gallic acid (**6**) (IC_{50} = 73 μM), which is produced by hydrolytic release of gallotannins *in vivo*.

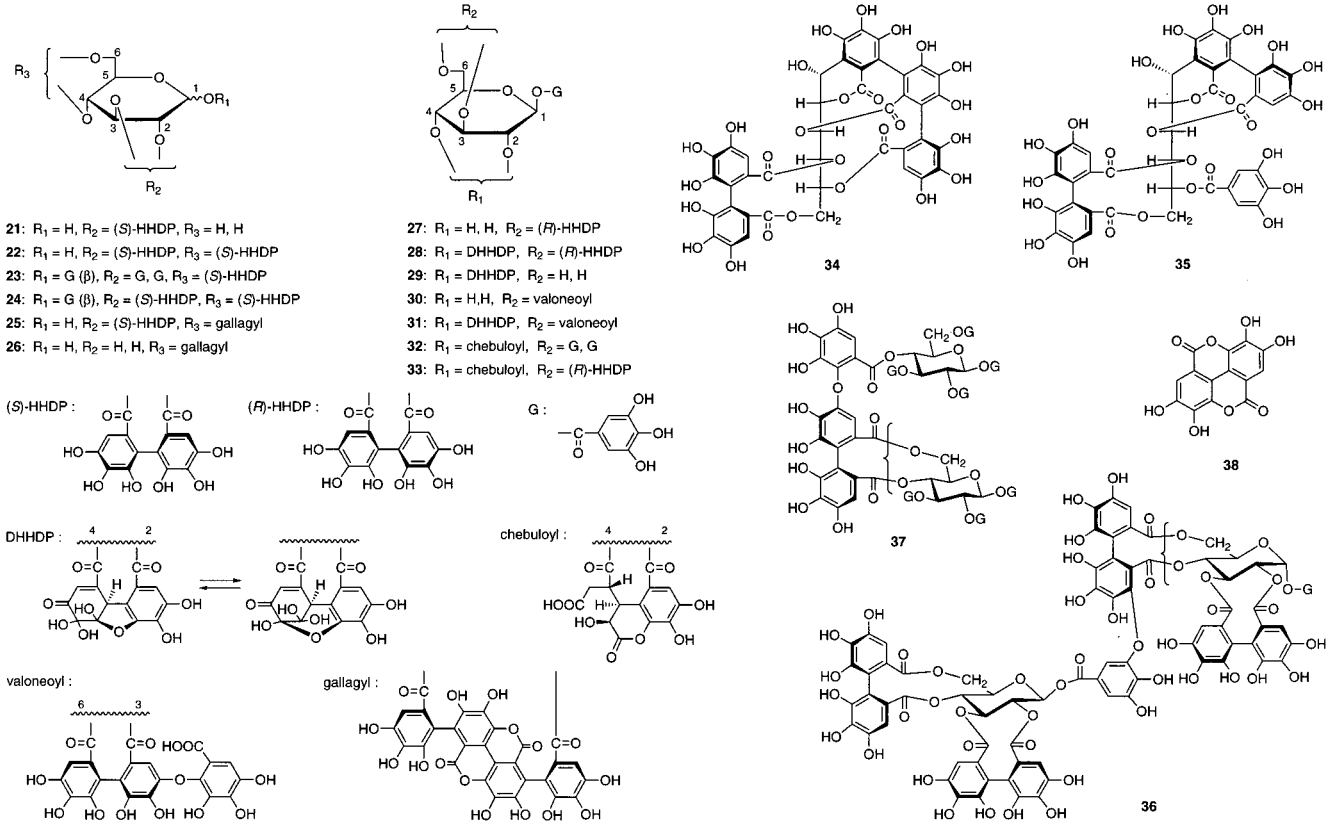
The HHDP group, originating from oxidative C−C coupling of two galloyl groups, has been regarded as playing an important role in the resultant biological activities of ellagitannins. In fact, a series of synthetic HHDP derivatives prepared as simple analogues of ellagitannins demonstrated potent and selective inhibition of protein kinase C[9] and HIV replication.[10] On the basis of these observations, *n*-alkyl HHDP esters (**11**−**20**), containing a dimeric structure of *n*-alkyl gallates, were synthesized chemically from ellagic acid (**38**) according to a method described before[9b] and tested for enzyme inhibition. Thus, hexabenzyldiphenyl dicarboxylic acid obtained from ellagic acid was converted to an acid chloride, which was further reacted with various chain length *n*-alkyl alcohols. Debenzylation of the products by catalytic hydrogenation afforded the HHDP esters as racemic mixtures of (*R*)- and (*S*)-atoropisomers.

The HHDP esters (**11**−**20**) showed potent enzyme inhibition according to the alkyl chain length (Table 1). Maximum inhibition activities were obtained with the C_6 (**14**) (IC_{50} = 0.93 μM) and the C_8 ester (**15**) (IC_{50} = 0.83 μM) at the submicromolar concentration level and were far more potent than those of naturally occurring ellagitannins, as described above. However, derivatives with a shorter chain length (C_1−C_4) and the C_{18} ester showed only moderate inhibition (IC_{50} = 8−47 μM). These results for the *n*-alkyl HHDP esters were similar to those obtained for *n*-alkyl gallates. However, the HDDP esters showed less potent inhibition activities than the corresponding galloyl esters (**7**−**10**): C_2 (IC_{50} = 4.2 μM), C_8 (IC_{50} = 0.83 μM), C_{12} (IC_{50} = 0.061 μM), and C_{16} (IC_{50} = 0.59 μM).[4a] It appears that conformationally more flexible and less bulky galloyl esters better fit into the active site of the enzyme than the more rigid and hindered HHDP esters. In contrast, it has been reported that, in the case of protein kinase C, the HHDP esters showed more potent enzyme inhibition than the corresponding digalloyl esters.[9] To further optimize the inhibition activities, the design and synthesis of new HHDP and galloyl derivatives are now in progress in our laboratories.

Experimental Section

Chemicals. Ellagitannins were isolated from the following plants and purified as reported previously: 2,3-(*S*)-hexahydroxydiphenoyl-D-glucose (**21**), pedunculagin (**22**), eugeniin (**23**), 1(β)-*O*-galloylpedunculagin (**24**), and sanguiin H-6 (**36**) (*Sanguisorba officinalis*);[11a] corilagin (**27**), furosin (**29**), mallotinic acid (**30**), and mallotusinic acid (**31**) (*Mallotus japonicus* and *M. philippinensis*);[11b] castalagin (**34**) and casuarinin (**35**) (*Castanea mollissima*);[11c] punicalagin (**25**) and punicalin (**26**) (*Punica granatum*);[11d] chebulinic acid (**32**) and chebulagic acid (**33**) (*Terminalia chebula*);[11e] phyllyraeoidin (**37**) (*Quercus phillyraeoides*).[11f] Ellagic acid (**38**) and geraniin (**28**) were purchased from Wako (Tokyo, Japan). The HHDP esters were

Chart 3



synthesized chemically from ellagic acid (**38**) according to a method previously described.[9b]

Spectroscopic data for the newly synthesized compounds **13**−**20** are as follows.

***n*-Dibutyl 2,2′,3,3′,4,4′-hexahydroxybiphenyl-6,6′-dicarboxylate (13):** colorless needles (from H_2O); mp 185−187 °C; ^{1}H NMR (400 MHz, acetone-d_6 + D_2O) δ 0.83 (6H, t, J = 7.5 Hz, *n*-butyl CH_3), 1.17, 1.32 (each 4H, m, *n*-butyl CH_2), 3.87 (4H, t, J = 6.5 Hz, *n*-butyl CH_2−O−), 7.16 (2H, s, H-5′); ^{13}C NMR (100 MHz, acetone-d_6 + D_2O) δ 13.98 (CH_3), 19.86, 31.29 (CH_2), 64.32 (CH_2−O−), 110.71 (C-5, 5′), 118.73 (C-1, 1′), 123.04 (C-6, 6′), 137.48 (C-3, 3′), 144.40 (C-4, 4′), 144.63 (C-2, 2′), 167.57 (COO); FABMS *m*/*z* 451 (M + H)$^+$; HRFABMS (positive) *m*/*z* 451.1616 (calcd for $C_{22}H_{27}O_{10}$, 451.1604).

***n*-Dihexyl 2,2′,3,3′,4,4′-hexahydroxybiphenyl-6,6′-dicarboxylate (14):** colorless needles (from H_2O); mp 170−172 °C; ^{1}H NMR (400 MHz, acetone-d_6 + D_2O) δ 0.87 (6H, t, J = 7 Hz, *n*-hexyl CH_3), 1.13−1.36 (16H, m, *n*-hexyl CH_2), 3.86 (4H, t, J = 6.5 Hz, *n*-hexyl CH_2−O−), 7.16 (2H, s, H-5′); ^{13}C NMR (100 MHz, acetone-d_6 + D_2O) δ 14.31 (CH_3), 23.15, 26.44, 29.19, 32.24 (CH_2), 64.63 (CH_2−O−), 110.71 (C-5, 5′), 118.77 (C-1, 1′), 123.02 (C-6, 6′), 137.49 (C-3, 3′), 144.38 (C-4, 4′), 144.63 (C-2, 2′), 167.65 (COO); FABMS *m*/*z* 507 (M + H)$^+$; HRFABMS (positive) *m*/*z* 507.2227 (calcd for $C_{26}H_{35}O_{10}$, 507.2230).

***n*-Dioctyl 2,2′,3,3′,4,4′-hexahydroxybiphenyl-6,6′-dicarboxylate (15).** colorless needles (from acetone−H_2O); mp 171−172 °C; ^{1}H NMR (400 MHz, acetone-d_6 + D_2O) δ 0.88 (6H, t, J = 7 Hz, *n*-octyl CH_3), 1.13−1.38 (24H, m, *n*-octyl CH_2), 3.86 (4H, m, *n*-octyl CH_2−O−), 7.16 (2H, s, H-5′); ^{13}C NMR (100 MHz, acetone-d_6 + D_2O) δ 14.33 (CH_3), 23.28, 26.77, 29.25, 29.92, 30.01, 32.59 (CH_2), 64.61 (CH_2−O−), 110.74 (C-5, 5′), 118.72 (C-1, 1′), 123.06 (C-6, 6′), 137.48 (C-3, 3′), 144.38 (C-4, 4′), 144.63 (C-2, 2′), 167.59 (COO); FABMS *m*/*z* 563 (M + H)$^+$; HRFABMS (positive) *m*/*z* 563.2848 (calcd for $C_{30}H_{43}O_{10}$, 563.2856).

***n*-Didecyl 2,2′,3,3′,4,4′-hexahydroxybiphenyl-6,6′-dicarboxylate (16).** colorless needles (from acetone−H_2O); mp 166−168 °C; ^{1}H NMR (400 MHz, acetone-d_6 + D_2O) δ 0.88 (6H, t, J = 7 Hz, *n*-decyl CH_3), 1.12−1.40 (32H, m, *n*-decyl CH_2), 3.86 (4H, m, *n*-decyl CH_2−O−), 7.16 (2H, s, H-5′); ^{13}C NMR (100 MHz, acetone-d_6 + D_2O) δ 14.36 (CH_3), 23.35, 26.85, 29.35, 30.10, 30.13, 30.33, 30.40, 32.68 (CH_2), 64.68 (CH_2−O−), 110.92 (C-5, 5′), 118.73 (C-1, 1′), 123.24 (C-6, 6′), 137.61 (C-3, 3′), 144.48 (C-4, 4′), 144.77 (C-2, 2′), 167.59 (COO); FABMS *m*/*z* 619 (M + H)$^+$; HRFABMS (positive) *m*/*z* 619.3492 (calcd for $C_{34}H_{51}O_{10}$, 619.3482).

***n*-Didodecyl 2,2′,3,3′,4,4′-hexahydroxybiphenyl-6,6′-dicarboxylate (17).** colorless needles (from acetone−H_2O); mp 161−163 °C; ^{1}H NMR (400 MHz, acetone-d_6 + D_2O) δ 0.88 (6H, t, J = 7 Hz, *n*-dodecyl CH_3), 1.14−1.38 (40H, m, *n*-dodecyl CH_2), 3.87 (4H, m, *n*-dodecyl CH_2−O−), 7.16 (2H, s, H-5′); ^{13}C NMR (100 MHz, acetone-d_6 + D_2O) δ 14.36 (CH_3), 23.35, 26.85, 29.33, 30.12, 30.14, 30.28, 30.34, 30.46, 32.67 (CH_2), 64.66 (CH_2−O−), 110.85 (C-5, 5′), 118.75 (C-1, 1′), 123.14 (C-6, 6′), 137.57 (C-3, 3′), 144.45 (C-4, 4′), 144.72 (C-2, 2′), 167.58 (COO); FABMS *m*/*z* 675 (M + H)$^+$; HRFABMS (positive) *m*/*z* 675.4110 (calcd for $C_{38}H_{59}O_{10}$, 675.4108).

***n*-Ditetradecyl 2,2′,3,3′,4,4′-hexahydroxybiphenyl-6,6′-dicarboxylate (18).** colorless needles (from acetone−H_2O); mp 157−158 °C; ^{1}H NMR (400 MHz, acetone-d_6 + D_2O) δ 0.88 (6H, t, J = 7 Hz, *n*-tetradecyl CH_3), 1.14−1.40 (48H, m, *n*-tetradecyl CH_2), 3.87 (4H, m, *n*-tetradecyl CH_2−O−), 7.16 (2H, s, H-5′); ^{13}C NMR (100 MHz, acetone-d_6 + D_2O) δ 14.32 (CH_3), 23.32, 26.84, 29.34, 30.05, 30.08, 30.33, 30.44, 32.65 (CH_2), 64.65 (CH_2−O−), 110.89 (C-5, 5′), 118.70 (C-1, 1′), 123.20 (C-6, 6′), 137.60 (C-3, 3′), 144.47 (C-4, 4′), 144.75 (C-2, 2′), 167.56 (COO); FABMS *m*/*z* 731 (M + H)$^+$; HRFABMS (positive) *m*/*z* 731.4738 (calcd for $C_{42}H_{67}O_{10}$, 731.4735).

***n*-Dihexadecyl 2,2′,3,3′,4,4′-hexahydroxybiphenyl-6,6′-dicarboxylate (19).** colorless needles (from acetone-H_2O); mp 152−154 °C; ^{1}H NMR (400 MHz, acetone-d_6 + D_2O) δ 0.88 (6H, t, J = 7 Hz, *n*-hexadecyl CH_3), 1.14−1.40 (56H, m, *n*-hexadecyl CH_2), 3.87 (4H, m, *n*-hexadecyl CH_2−O−), 7.16 (2H, s, H-5′); ^{13}C NMR (100 MHz, acetone-d_6 + D_2O) δ 14.32 (CH_3), 23.31, 26.84, 29.34, 30.05, 30.06, 30.13, 30.33, 30.44, 32.6 (CH_2), 64.7 (CH_2−O−), 110.9 (C-5, 5′), 118.7 (C-1, 1′), 123.2 (C-6, 6′), 137.6 (C-3, 3′), 144.5 (C-4, 4′), 144.8 (C-2, 2′), 167.6 (COO); FABMS

m/z 787 $(M + H)^+$; HRFABMS (positive) *m/z* 787.5391 (calcd for $C_{46}H_{75}O_{10}$, 787.5361).

***n*-Dioctadecyl 2,2′,3,3′,4,4′-hexahydroxybiphenyl-6,6′-dicarboxylate (20).** colorless needles (from acetone-H_2O); mp 137−144 °C; ^{1}H NMR (400 MHz, acetone-d_6 + D_2O) δ 0.88 (6H, t, J = 7 Hz, *n*-octadecyl CH_3), 1.14−1.40 (64H, m, *n*-octadecyl CH_2), 3.87 (4H, m, *n*-octadecyl CH_2−O−), 7.16 (2H, s, H-5′); ^{13}C NMR (100 MHz, acetone-d_6 + D_2O) δ 14.31 (CH_3), 23.28, 26.81, 29.28, 30.03, 30.10, 30.31, 30.33, 30.38, 30.43, 32.6 (CH_2), 64.61 (CH_2−O−), 110.76 (C-5, 5′), 118.73 (C-1, 1′), 123.06 (C-6, 6′), 137.47 (C-3, 3′), 144.37 (C-4, 4′), 144.63 (C-2, 2′), 167.57 (COO); FABMS *m/z* 843 $(M + H)^+$; HRFABMS (positive) *m/z* 843.6002 (calcd for $C_{50}H_{83}O_{10}$, 843.5986).

Enzyme Inhibition Assay. As described previously,[3,4] a recombinant rat SE (Glu^{100}-His^{573}) without the N-terminal putative membrane domain and with an additional hexahistidine tag at the C-terminal was expressed in *E. coli* and purified by Ni-NTA agarose and Blue Sepharose CL-6B columns.[34] The purified recombinant enzyme exhibited an apparent K_M = 3.8 μM and k_{cat} = 4.1 min^{-1} for squalene. Test compounds were dissolved in 2 μL of ethanol containing 0.5% Triton X-100. The assay mixture contained in a total volume of 200 μL of 20 mM Tris-HCl, pH 7.4, the recombinant rat SE (1.5 μg/mL), NADPH-cytochrome P-450 reductase (0.05 U), 1 mM NADPH, 0.1 mM FAD, 0.1% (w/v) Triton X-100, and [1,-25-^{14}C]squalene (5 μM, 2 × 10^4 dpm). After incubation at 37 °C for 1 h, the enzyme reaction was quenched by addition of 200 μL of 10% KOH in methanol and 10 μL of 0.1% (w/v) cold carrier squalene and oxidosqualene in ethanol. The lipids were extracted with 400 μL of CH_2Cl_2 and separated by TLC (Whatman silica gel 60A with preadsorbent strip), which was developed with 5% ethyl acetate in hexane. The R_f values were 0.84 for squalene and 0.54 for oxidosqualene. Radioactivities were analyzed by radio-TLC scanning (Bioscan Imaging Scanner System 200, IBM with Autochanger 4000). All experiments were carried out in duplicate. The IC_{50} values were determined by nonlinear regression analysis of % control versus semilog concentration. Dose-inhibition curves were generated with eight sample concentrations ranging from 0.1 to 100 μM. In the assay, DG (**9**) was employed as a positive control.

Acknowledgment. We thank Drs. Teruo Ono and Jun Sakakibara (Niigata University School of Medicine) for the recombinant rat SE clone used in this study. We are grateful to Drs. K. Mihashi and T. Fujioka (Fukuoka University, School of Pharmaceutical Sciences) for HRMS measurements. This work was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Sciences, Sports and Culture (to I.A. and Y.K.), a research grant from Kowa Life Science Foundation (to I.A.), and the Promotion and Mutual Aid Corporation for Private Schools of Japan (to Y.K.).

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NP010100Y