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Potent and Selective Inhibition of Squalene Epoxidase by Synthetic Galloyl Esters

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n-Alkyl esters (ethyl, octyl, dodecyl, and cetyl) of gallic acid were evaluated as enzyme inhibitors of recombinant rat squalene epoxidase (SE), a rate-limiting enzyme of cholesterol biogenesis. Dodecyl (6) (IC_{50} = 0.061 μM) showed the most potent inhibition, which was far more potent than those of previously reported naturally occurring gallocatechins. Octyl gallate (5) (IC_{50} = 0.83 μM) and cetyl gallate (7) (IC_{50} = 0.59 μM) also showed good inhibition, while gallic acid (IC_{50} = 73 μM) itself was not so active. In addition, chemically synthesized galloyl ester of cholesterol (9) (IC_{50} = 3.9 μM), farnesol derivative (10) (IC_{50} = 0.57 μM), and dodecyl galloyl amide (8) (IC_{50} = 3.0 μM) were also potent inhibitors of SE. Inhibition kinetics revealed that dodecyl gallate inhibited SE in competitive (K_I = 0.033 μM) and no-time-dependent manner. The potent inhibition of the flavin monooxygenase would be caused by specific binding to the enzyme, and by scavenging reactive oxygen species required for the epoxidation reaction.

Squalene epoxidase (SE) (EC 1.14.99.7) is a nonmetallic flavoprotein monooxygenase that catalyzes the conversion of squalene to 2,3-oxidosqualene, a rate-limiting step of cholesterol biosynthesis (1). In addition to oxygen, vertebrate SE requires FAD, NADPH, a supernatant protein factor, and NADPH-cytochrome P450 reductase. SE is the only known non-cytochrome P450 enzyme that epoxidizes an unactivated alkene. The flavoprotein-mediated epoxidation is thought to proceed *via* formation of flavin C(4a)-hydroperoxide (Fig. 1). The overall reaction of the epoxidation reaction can thus be described by the following equation:

$$\text{Squalene} + O_2 + \text{NADPH} + H^+ \rightarrow$$
$$(3S)2,3\text{-Oxidosqualene} + \text{NADP}^+ + H_2O$$

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Recently, the unstable, membrane-bound enzyme with molecular mass of 64 KDa was purified, and cloned from several sources including rat (2) and human (3). Further, a truncated recombinant rat SE (Glu^{100}-His^{573}) without the N-terminal putative membrane domain has been constructed and functionally expressed in *E. coli* (4). The recombinant enzyme, which we used in our study, showed properties very similar to those of native microsomal enzyme with regard to co-factor requirement, pH dependency, and sensitivity to most of known SE enzyme inhibitors (4).

SE is thought to control the throughput from squalene to sterols in cholesterol biogenesis (1). Therefore, regulation of the level of SE *in vivo* has clinical importance and has become a potential target for design of cholesterol-lowering drugs. In principle, enzyme inhibitors for SE selectively inhibit cholesterol biosynthesis, and do not affect the synthesis of non-sterol mevalonate-derived isoprenoids (e.g. dolichol, ubiquinone, isopentenyl tRNA, and protein prenylation), which play important roles in regulation of normal cellular processes. To date, several potent and specific SE enzyme inhibitors including chemically synthesized squalene analogs and allylamine derivatives have been developed, however, there are as yet no reports of human clinical trials (5, 6).

In our previous paper (7), we reported that naturally occurring esters of gallic acid (3,4,5-trihydroxybenzoic acid) such as (−)-epigallocatechin-3-*O*-gallate (**1**) (IC_{50} = 0.69 μM), a major component of green tea polyphenols, were excellent inhibitors of SE (Scheme 1). In contrast, flavan-3-ols without gallate such as (−)-epicatechin (**2**) (IC_{50} > 1000 μM) did not show significant SE inhibition. The presence of galloyl moiety was thus suggested to be important for the SE enzyme inhibition activity.

Here in this paper, we describe that synthetic alkyl gallates are even more potent and selective inhibitors of the flavin monooxygenase. Further, we newly synthesized galloyl esters of isoprenoids and galloyl amide, which were tested for SE inhibition activities. Alkyl gallates such as n-propylgallate (E-310),

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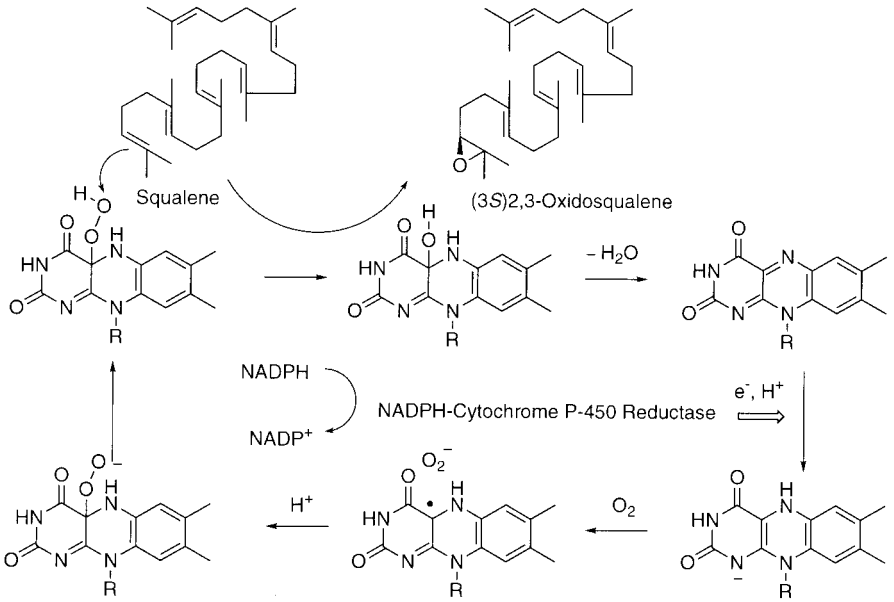
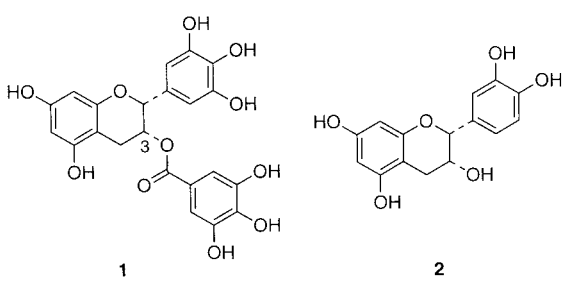


FIG. 1. Proposed mechanism of epoxidation of squalene to (*3S*)2,3-oxidosqualene by SE.

n-octylgallate (E-311), and n-dodecylgallate (E-312) are widely used as antioxidant food additives due to their scavenging activity towards reactive oxygen species. Furthermore, alkyl gallates were recently reported to induce apoptosis in tumoral cell lines and inhibit lymphocyte proliferation (8). Dodecyl gallate have been also reported to be a good inhibitors of human spleen protein tyrosine kinase (IC_{50} = 5 μM) (9).

MATERIALS AND METHODS

Chemicals. [1,25-^{14}C]Squalene (57.1 mCi/mmol) and trisnorsqualene alcohol was synthesized as described (10). Gallic acid (**3**) and its n-alkyl esters (**4–7**) were purchased from Tokyo Kasei (Tokyo, Japan). *N,N*-Dimethyl-n-dodecylamine was from Wako (Tokyo, Japan). Galloyl ester of cholesterol (**9**) was synthesized according to the published method (11). Here, the esterification of gallic acid tribenzyl ether (12) with cholesterol was carried out in the presence of 4-*N,N*-dimethylaminopyridine and dicyclohexyl carbodiimide. The tribenzyl gallate derivatives were then debenzylated by catalytic hydrogenation on palladium-charcoal. Compound **8** and **10** were respectively synthesized in the similar way from n-dodecylamine and *E,E*-farnesol.



SCHEME 1

Compound **8:** ^{1}H NMR (400 MHz, $CDCl_3$): δ 6.83 (s, 2H, H-1, H-5), 3.36 (br, 2H), 1.58 (br, 6H), 1.26 (br, 14H), 0.88, (t, J = 6.8 Hz, 3H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 168.2, 144.6 (C-2, C-4), 135.1 (C-3), 125.7 (C-6), 106.6 (C-1, C-5), 40.1, 31.9, 29.6, 29.6, 29.6, 29.5, 29.5, 29.3, 29.3, 26.0, 22.6, 14.0. MS (FAB/NBA, pos.): m/z (%) = 338 (100) $[MH]^+$, 154 (41), 136 (31). HRMS (FAB/NBA, pos.) for $C_{19}H_{32}NO_4$: calcd 338.4674, found 338.4689.

Compound **9**: ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.15 (s, 2H, H-1, H-5), 5.40 (dd, J = 3.6, 0.8 Hz, 1H), 2.42 (brd, J = 7.2 Hz, 1H), 1.06 (s, 3H), 0.92 (s, 3H), 0.87 (d, J = 6.8 Hz, 6H), 0.69 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 166.4, 144.0 (C-2, C-4), 139.8, 136.6 (C-3), 122.7, 122.2 (C-6), 109.3 (C-1, C-5), 74.6, 56.7, 56.2, 50.1, 42.4, 39.8, 39.5, 38.2, 37.0, 36.7, 36.2, 35.8, 32.0, 32.0, 28.2, 28.0, 27.9, 24.3, 23.8, 22.8, 22.5, 21.1, 19.3, 18.7, 11.9. MS (FAB/NBA, pos.): m/z (%) = 561 (53) $[MNa]^+$, 370 (100). HRMS (FAB/NBA, pos.) for $C_{34}H_{50}O_5Na$: calcd 561.7578, found 561.7603.

Compound **10**: ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.23 (s, 2H, H-1, H-5), 4.30 (brm, 2H), 0.94–1.76 (m, 17 H), 0.86 (d, J = 6.4 Hz, 6H), 0.85 (d, J = 6.8 Hz, 6H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 166.7, 143.4 (C-2, C-4), 135.3 (C-3), 122.3 (C-6), 110.0 (C-1, C-5), 63.7, 39.4, 37.4, 37.4, 37.4, 37.3, 35.7, 35.6, 32.8, 30.0, 28.0, 24.8, 24.4, 22.7, 22.6, 19.7, 19.7, 19.6, 19.6. MS (FAB/NBA, pos.): m/z (%) = 381 (40) $[MH]^+$, 307 (20), 171 (39), 154 (100), 136 (100). HRMS (FAB/NBA, pos.) for $C_{22}H_{37}O_5$: calcd 381.5328, found 381.5344.

Enzymes. A truncated 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 as described (4). The purified recombinant enzyme showed an apparent K_M = 3.8 μM and k_{cat} = 4.1 min^{-1} for squalene. Rat NADPH-cytochrome P450 reductase was prepared as described (4). Recombinant human superoxide dismutase was purchased from Wako (Tokyo, Japan).

Enzyme inhibition assay. Inhibitors were dissolved in 2 μL of ethanol and preincubated for 10 min at 37°C in the assay mixture containing 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 P450 reductase (0.05 U), 1 mM NADPH, 0.1 mM FAD, 0.1% Triton X-100, and

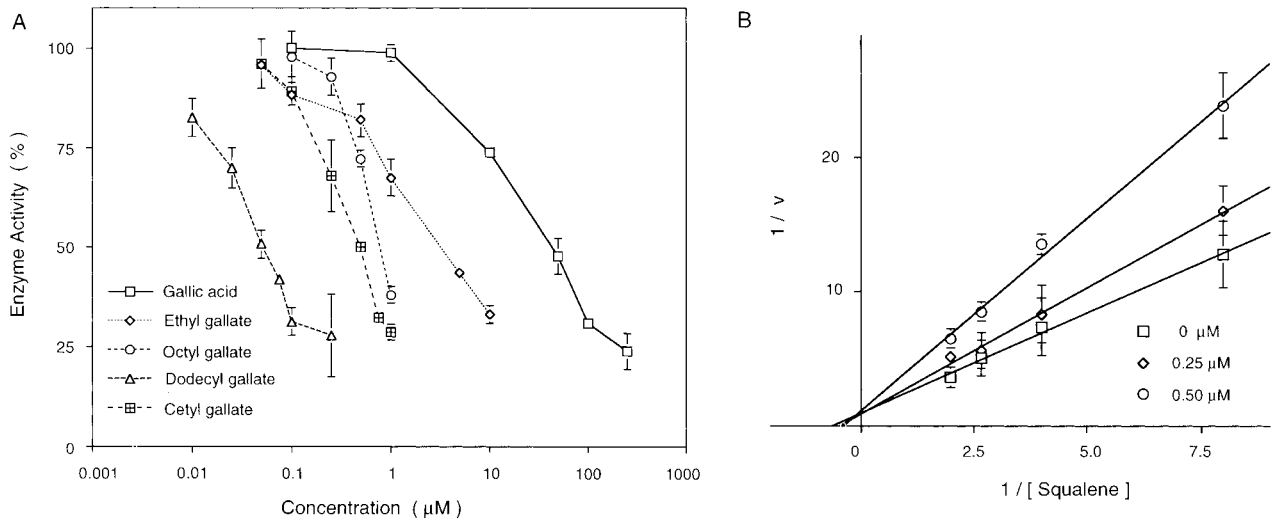


FIG. 2. (A) Inhibition activities of gallic acid (**3**) and its n-alkyl esters (**4–7**) toward recombinant rat SE. (B) Lineweaver–Burk analysis for SE inhibition by DG (**6**).

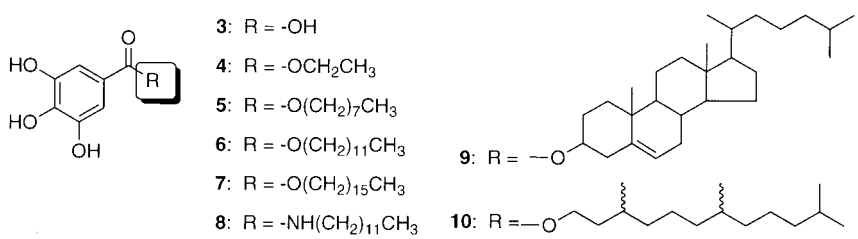
[1,25-^{14}C]squalene (5 μM, 2 $\times$ 10^4 dpm). Here, in the assay mixture, the supernatant protein factor was replaced by Triton X-100. After incubation at 37°C for 60 min, the enzyme reaction was quenched by addition of 200 μL of 10% KOH in methanol, and 10 μL of 0.1% cold carrier squalene and oxidosqualene in ethanol. The lipids were extracted with 400 μL of CH_2Cl_2, and separated by TLC (Whatman silica gel 60A with preadsorband strip) which was developed with 5% ethyl acetate in hexane. The Rf 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).

Inhibition kinetics. The K_I values were determined using the same condition as described above. The experiments were carried out in duplicate using three concentrations of inhibitor (0, 0.25, 0.50 μM). For each inhibitor concentration, substrate was added to give four substrate concentrations: 0.125, 0.25, 0.375, and 0.50 μM. For the analysis of the time dependency of inhibition, the experiments were carried out in duplicate using four concentrations of inhibitor: (0, $\frac{1}{2}$ $\times$ [IC_{50}], 1 $\times$ [IC_{50}], 2 $\times$ [IC_{50}]). The SE enzyme (10 μg) was incubated with the inhibitors in total volume of 200 μL, while aliquots (20 μL) of the inhibited SE solution were removed at time intervals of 0, 10, 20, and 30 min, and added to 180 μL of reaction mixture containing 20 mM Tris-HCl, pH 7.4, NADPH-cytochrome P450 reductase (0.05 U), 1 mM NADPH, 0.1 mM FAD, 0.1% Triton X-100, and [1,25-^{14}C]squalene (5 μM, 2 $\times$ 10^4 dpm). The diluted enzyme solutions were then incubated at 37°C for 60 min and analyzed as described. The log (percentage of remaining activity) was plotted against time to determine the k_{inact} value.

RESULTS AND DISCUSSION

Dodecyl gallate (DG) (**6**) (IC_{50} = 0.061 μM) showed the most potent inhibition toward recombinant rat SE (Fig. 2A), which was 10 times more potent than that of previously reported naturally occurring (−)-epigallocatechin-3-*O*-gallate (**1**) (IC_{50} = 0.69 μM), and 1,000 times more potent than that of gallic acid (**3**) (IC_{50} = 73 μM) itself (Scheme 2). Octyl gallate (**5**) (IC_{50} = 0.83 μM) and cetyl gallate (**7**) (IC_{50} = 0.59 μM) also showed good enzyme inhibition. In addition, chemically synthesized galloyl ester of cholesterol (**9**) (IC_{50} = 3.9 μM), farnesol derivative (**10**) (IC_{50} = 0.57 μM), and dodecyl galloyl amide (**8**) (IC_{50} = 3.0 μM) were also potent inhibitors of SE, while *N*,*N*-dimethyl-n-dodecylamine (IC_{50} = 39 μM) showed poor enzyme inhibition. Thus, it was demonstrated that the presence of both galloyl moiety and the "substrate-like" hydrophobic alkyl side chain is important for the potent SE inhibition. Inhibition kinetics revealed that DG inhibited SE in competitive manner (K_I = 0.033 μM) (Fig. 2B) and showed no-time dependency ($k_{inact} < 0.01$ min^{-1}).

The inhibition activities of the galloyl esters were far more potent than those of known vertebrate SE inhib-



SCHEME 2

itors such as chemically synthesized squalene analogs; trisnorsqualene alcohol (IC_{50} = 4 μM for pig SE; IC_{50} = 7.9 μM for rat recombinant SE in our assay system) (13), trisnorsqualene cyclopropylamine (IC_{50} = 2 μM for pig SE) (14), and trisnorsqualene difluoromethylidene (IC_{50} = 5.4 μM for rat SE) (15), but not so potent as allylamine derivatives including NB-598 (IC_{50} = 0.75 nM for human HepG2 SE) (16).

The non-metallic flavoprotein-mediated epoxidation has been proposed to proceed *via* formation of flavin C(4a)-hydroperoxide intermediate (Fig. 1). The potent inhibition of the flavin monooxygenase by DG would be caused by specific binding to the enzyme; possibly, dodecyl side chain occupies the squalene binding site, while the galloyl moiety in close proximity of the FAD binding domain, where gallate would trap reactive oxygen species required for the enzyme reaction. Alkyl gallates such as n-propylgallate (E-310), n-octylgallate (E-311), and DG (E-312) are widely used as antioxidant food additives for preventing the rancidity of fats due to their scavenging activity towards reactive oxygen species. The reactivity of gallate with active oxygen species including superoxide anion has been well studied and reported to be one of the highest (17). Interestingly, presence of human recombinant superoxide dismutase (up to 15 unit) in the assay mixture did not affect the SE enzyme activity at all. Furthermore, as described previously, antioxidative vitamins; α-tocophenol and L-ascorbic acid, did not inhibit SE at 1000 μM concentration, suggesting that, in these cases, there is no specific binding to the enzyme involved.

It is likely that the antioxidative DG would also inhibit other oxygenase reactions in the cholesterol biogenesis such as oxidative removal of methyl groups of lanosterol catalyzed by cytochrome P450 systems. However, rat recombinant lanosterol 14α-demethylase (CYP51) (IC_{50} = ca. 100 μM) and NADPH-cytochrome P450 reductase (IC_{50} > 100 μM) did not suffer significant enzyme inhibition (Y. Aoyama, personal communication). Thus, the submicromolar level SE enzyme inhibition by DG (IC_{50} = 0.061 μM) was highly selective. Further analysis of the enzyme inhibition mechanism are now in progress in our laboratories. Finally, a series of alkyl gallates including DG were reported to be potent inducer of apoptosis in tumoral cell lines (8). The apoptosis inducing activities of newly synthesized galloyl esters of cholesterol (**9**) farnesol derivative (**10**), and dodecyl galloyl amide (**8**), are also under investigation.

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