Pergamon

Bioorganic & Medicinal Chemistry Letters 10 (2000) 2525–2528

BIOORGANIC & MEDICINAL CHEMISTRY LETTERS

Inhibition of Vertebrate Squalene Epoxidase by Isoprenyl Gallates and Phenylalkyl Gallates

Ikuro Abe,* Yasuhiko Kashiwagi and Hiroshi Noguchi

University of Shizuoka, School of Pharmaceutical Sciences, 52-1 Yada, Shizuoka 422-8526, Japan

Received 7 August 2000; accepted 4 September 2000

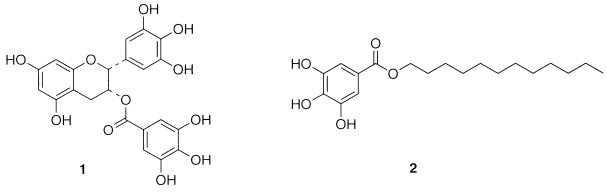
Abstract—Galloy esters with 'substrate-like' isoprenoid or phenylalkyl side chains were newly synthesized and tested for the enzyme inhibition activities toward recombinant rat squalene epoxidase. Isoprenyl gallates (**4**–**6**) showed good inhibition (IC_{50} = 1.5–5.1 μM), as potent as previously reported substrate analogues, while phenylalkyl gallates (**7**–**10**) were moderate inhibitors of the enzyme (IC_{50} = 12–61 μM).

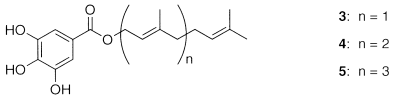
Introduction

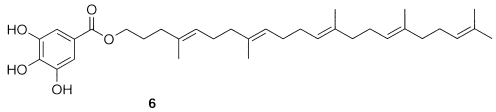
Squalene epoxidase (SE) (EC 1.14.99.7) is a non-metallic flavoprotein monooxygenase that catalyzes the conversion of squalene to 2,3-oxidosqualene, a rate-limiting step of cholesterol biosynthesis.[1] Vertebrate SE requires FAD, NADPH, a supernatant protein factor, and NADPH-cytochrome P-450 reductase. 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 be described by the following equation:

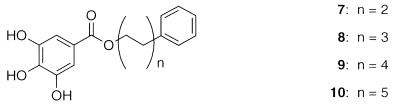
$$\text{Squalene} + O_2 + \text{NADPH} + H^+ \rightarrow (3S)\text{2,3-Oxidosqualene} + \text{NADP}^+ + H_2O$$

SE controls the throughput from squalene to sterols in cholesterol biogenesis.[1] Therefore, regulation of the level of SE in vivo 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 analogues and allylamine derivatives have been developed, however, there are as yet no reports of human clinical trials.[2,3]









In our previous papers, we reported that galloyl esters such as (−)-epigallocatechin-3-*O*-gallate (EGCG) (**1**) (IC_{50} = 0.69 μM), a major component of the cholesterol lowering green tea polyphenols, were potent and selective inhibitors of recombinant rat SE.[4–6] In particular, synthetic *n*-dodecyl gallate (DG) (**2**) (IC_{50} = 0.061 μM) showed the most potent inhibition, suggesting that the

*Corresponding author. Tel.: +81-54-264-5662; fax: +81-54-264-5662; e-mail: abei@ys7.u-shizuoka-ken.ac.jp

0960-894X/00/$ - see front matter
PII: S0960-894X(00)00526-6

presence of both galloyl moiety and the 'substrate-like' hydrophobic side chain were important for the potent SE inhibition.[7] From these observations, we postulated that the inhibition by DG would be caused by specific binding to the enzyme; possibly, the dodecyl side chain occupying the substrate (squalene) binding site, while the galloyl moiety being in close proximity to the FAD binding domain, where the antioxidative gallate would trap the reactive oxygen species required for the epoxidation reaction. In this work, in order to optimize the SE inhibition activities, several galloy esters with the 'substrate-like' isoprenoid or phenylalkyl side chain were newly synthesized and tested for the inhibition activities toward recombinant rat SE enzyme.

Materials and Methods

Chemicals. [1,25-^{14}C]Squalene (57.1 mCi/mmol)[8] and trisnorsqualene alcohol[9] were synthesized as described. Geraniol, farnesol, and geranylgeraniol were purchased from Sigma. 4-Phenyl-1-butanol, 6-phenyl-1-hexanol, 8-phenyl-1-octanol, and 10-phenyl-1-decanol were from Lancaster. For the synthesis of galloyl esters (**3**–**10**), esterification of gallic acid with the corresponding alcohol (1 equiv) was carried out under reflux for 6 h in the presence of dicyclohexyl carbodiimide (1.1 equiv) and 4-*N*,*N*-dimethylaminopyridine (0.1 equiv). Products were first purified by silica gel column chromatography, then finally by reverse-phase HPLC. Spectroscopic data for the obtained esters are as follows.

Geranyl gallate (3). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.23 (2H, s), 5.43 (1H, t, J = 7.2 Hz), 5.09 (2H, d, J = 7.2 Hz), 4.77 (2H, d, J = 6.8 Hz), 2.10–2.04 (4H, m), 1.74 (3H, s), 1.68 (3H, s), 1.60 (3H, s). HRMS (FAB/glycerol, negative) for $C_{17}H_{21}O_5$: calcd 305.1389, found 305.1383.

Farnesyl gallate (4). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.23 (2H, s), 5.43 (1H, t, J = 6.4 Hz), 5.09 (2H, m), 4.77 (2H, d, J = 6.8 Hz), 2.12–1.96 (8H, m), 1.74 (3H, s), 1.68 (3H, s), 1.60 (6H, s). HRMS (FAB/glycerol, negative) for $C_{22}H_{29}O_5$: calcd 373.2015, found 373.2010.

Geranylgeranyl gallate (5). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.23 (2H, s), 5.43 (1H, t, J = 6.8 Hz), 5.12–5.09 (3H, m), 4.78 (2H, d, J = 6.8 Hz), 2.11–1.98 (12H, m), 1.75 (3H, s), 1.68 (3H, s), 1.60 (9H, s). HRMS (FAB/glycerol, negative) for $C_{27}H_{37}O_5$: calcd 441.2641, found 441.2641.

Trisnorsqualene gallate (6). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.22 (2H, s), 5.15–5.10 (5H, m), 4.24 (2H, t, J = 8 Hz), 2.08–1.99 (18H, m), 1.83 (2H, m), 1.68 (3H, s), 1.62–1.60 (15H, m). HRMS (FAB/glycerol, negative) for $C_{34}H_{49}O_5$: calcd 537.3583, found 537.3577.

Phenylbutyl gallate (7). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.26 (2H, s), 7.21–7.18 (5H, m), 4.26 (2H, m), 2.65 (2H, t, J = 7.2 Hz), 1.70 (2H, m), 1.62 (2H, m). HRMS (FAB/glycerol, negative) for $C_{17}H_{17}O_5$: calcd 301.1076, found 301.1080.

Phenylhexyl gallate (8). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.24 (2H, s), 7.17–7.15 (5H, m), 4.23 (2H, m), 2.60 (2H, t, J = 8 Hz), 1.71 (2H, m), 1.63 (2H, m), 1.43–1.37 (4H, m). HRMS (FAB/glycerol, negative) for $C_{19}H_{21}O_5$: calcd 329.1389, found 329.1384.

Phenyloctyl gallate (9). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.24 (2H, s), 7.17–7.15 (5H, m), 4.24 (2H, m), 2.59 (2H, t, J = 7.8 Hz), 1.71 (2H, m), 1.60 (2H, m), 1.34–1.28 (8H,

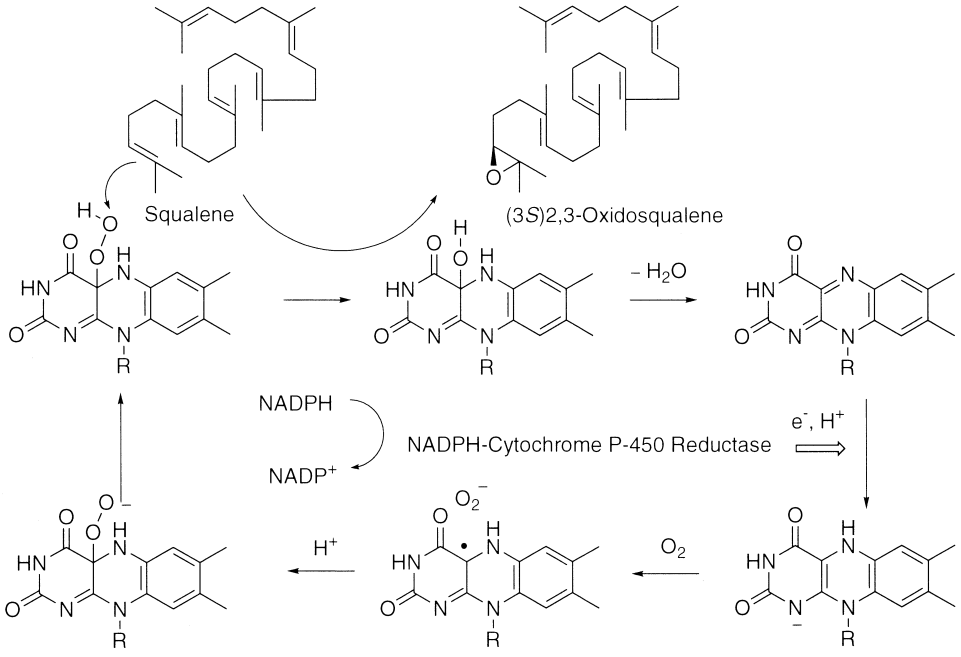


Figure 1. Proposed mechanism of epoxidation of squalene to (*3S*)2,3-oxidosqualene by SE.

m). HRMS (FAB/glycerol, negative) for $C_{21}H_{25}O_5$: calcd 357.1702, found 357.1700.

Phenyldecyl gallate (10). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.23 (2H, s), 7.16–7.14 (5H, m), 4.19 (2H, m), 2.57 (2H, t, J = 8 Hz), 1.65 (2H, m), 1.58 (2H, m), 1.28–1.24 (12H, m). HRMS (FAB/glycerol, negative) for $C_{23}H_{29}O_5$: calcd 385.2015, found 385.2015.

Enzymes. As described previously, 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.[10] The purified recombinant enzyme, with an apparent K_M = 3.8 μM and k_{cat} = 4.1 min^{-1} for squalene, showed properties very similar to those of native microsomal enzyme with regard to co-factor requirement, pH dependency, and sensitivity to most known SE enzyme inhibitors.

Enzyme assay. Gallates 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 preadsorbed 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). All experiments were carried out in duplicate. The IC_{50} values were determined by non-linear regression analysis of % control versus semi-log concentration. Dose–inhibition curves were generated with eight sample concentrations ranging from 0.1 μM to 100 μM. This time, DG (**2**) (IC_{50} = 0.1±0.02 μM) was employed as a positive control.

Results and Discussion

Galloy esters with isoprenoid side chain (**3**–**6**) or phenylalkyl side chain (**7**–**10**) were chemically synthesized by conventional esterification methods, and tested for the inhibition activities toward recombinant rat SE enzyme. Isoprenyl units were incorporated since our previous work suggested that the presence of both galloyl moiety and the 'substrate (squalene)-like' hydrophobic side chain were important for the potent SE inhibition.[7] On the other hand, the phenyl alkyl side chain was chosen since we expected π–π interactions with aromatic amino acid residues located at the active site of the enzyme.[1]

Inhibition assay results are summarized in Figure 2. Isoprenyl gallates (C_{15}, C_{20}, and C_{27}) were found to be good inhibitors of SE (IC_{50} = 1.5–5.1 μM). Among them, farnesyl (C_{15}) gallate (**4**) showed the most potent inhibition (IC_{50} = 1.5 μM), which was more potent than those of known vertebrate SE inhibitors (substrate analogues); trisnorsqualene alcohol (IC_{50} = 4 μM for pig SE),[9] trisnorsqualene cyclopropylamine (IC_{50} = 2 μM for pig SE),[11] and trisnorsqualene difluoromethylidene (IC_{50} = 5.4 μM for rat SE).[12] Geranyl (C_{10}) gallate and phenylalkyl (C_{10}–C_{16}) gallates just showed moderate inhibition (IC_{50} = 12–61 μM).

None of the compounds showed more potent inhibition than the previously reported DG (**2**) (IC_{50} = 0.061 μM). Incorporation of neither the 'substrate-like' isoprenyl units nor aromatic ring significantly enhanced the inhibition activities. Apparently, more flexible and/or less bulky dodecyl (C_{12}) side chains fit much better into the active site of the enzyme. As proposed in our previous paper,[7] the galloyl moiety would bind in close proximity to the FAD binding domain, while the hydrophobic side chain occupies the substrate binding site of the flavin monooxygenase. The SE enzyme reaction has been thought to proceed via formation of flavin

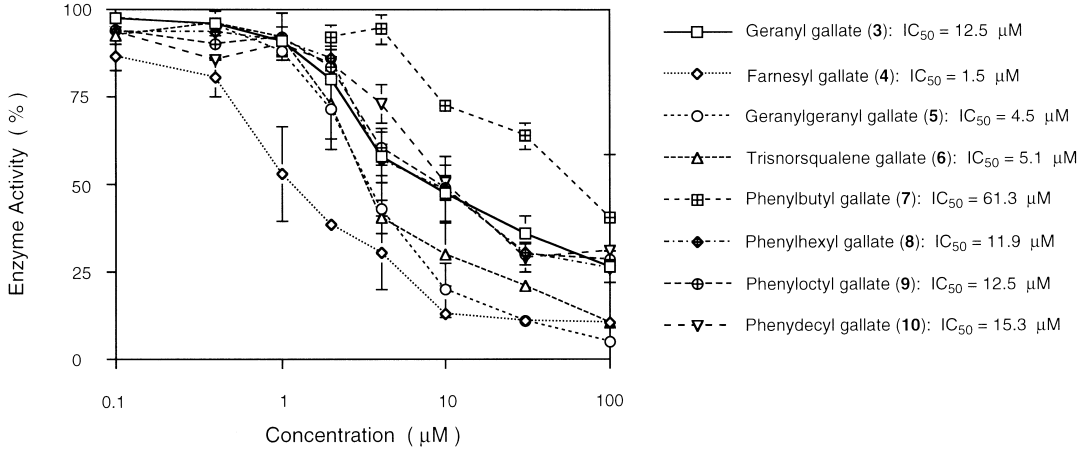


Figure 2. Inhibition activities of galloyl esters (**3**–**10**) toward recombinant rat SE.

C(4a)-hydroperoxide intermediate, which would be trapped by the antioxidative galloyl moiety, leading to the inactivation of the enzyme.

In order to test this hypothesis, since as yet no three-dimensional crystal structure has been reported for SE, a molecular modeling study is now in progress with the crystal structure of bacterial *p*-hydroxybenzoate hydroxylase, the best characterized flavin monooxygenase which shows ca. 20% amino acid sequence identity to that of rat SE. This flavoenzyme also suffered potent inhibition by the gallates.[13] Finally, as we have recently reported, galloyl esters are known to be potent inducers of apoptosis in human monoblastic leukemia U937 cells.[14] The apoptosis inducing activities of the newly synthesized gallates will be reported in due course.

Acknowledgements

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 indebted to Mr. Kenji Matsuura (JEOL) for HR-MS measurements. This work was supported in part by a Grant-in-Aid for Scientific Research on Priority Areas (A) from the Ministry of Education, Sciences, Sports and Culture Japan (no. 12045254 to I.A.).

References and Notes

1. Abe, I.; Prestwich, G. D. Squalene epoxidase and oxidosqualene:lanosterol cyclase. Key enzymes in cholesterol biosynthesis. In *Comprehensive Natural Products Chemistry*; Barton, D. H. R., Nakanishi, K., Eds.; Pergamon: Oxford, 1999; Vol. 2, Chapter 10, pp 267–298.
2. Abe, I.; Tomesch, J. C.; Wattanasin, S.; Prestwich, G. D. *Nat. Prod. Rep*. **1994**, *11*, 279.
3. Abe, I.; Prestwich, G. D. *Drug Discovery Today* **1998**, *3*, 389.
4. Abe, I.; Seki, T.; Umehara, K.; Miyase, T.; Noguchi, H.; Sakakibara, J.; Ono, T. *Biochem. Biophys. Res. Commun*. **2000**, *268*, 767.
5. Abe, I.; Seki, T.; Noguchi, H.; Hara, Y. *Jpn. J. Food Chem*. **2000**, *7*, 47.
6. Abe, I.; Seki, T.; Noguchi, H.; Kashiwada, Y. *Planta Medica* **2000**, in press.
7. Abe, I.; Seki, T.; Noguchi, H. *Biochem. Biophys. Res. Commun*. **2000**, *270*, 137.
8. Sen, S. E.; Prestwich, G. D. *J. Med. Chem*. **1989**, *32*, 2152.
9. Sen, S. E.; Prestwich, G. D. *J. Am. Chem. Soc*. **1989**, *111*, 1508.
10. Nagumo, A.; Kamei, T.; Sakakibara, J.; Ono, T. *J. Lipid Res*. **1995**, *36*, 1489.
11. Sen, S. E.; Prestwich, G. D. *J. Am. Chem. Soc*. **1989**, *111*, 8761.
12. Moore, W. R.; Schatzman, G. L.; Jarvi, E. T.; Gross, R. S.; McCarthy, J. R. *J. Am. Chem. Soc*. **1992**, *114*, 360.
13. Abe, I.; Kashiwagi, K.; Noguchi, H. *FEBS Lett*. **2000**, in press.
14. Saeki, K.; Yuo, A.; Isemura, M.; Abe, I.; Seki, T.; Noguchi, H. *Biol. Pharm. Bull*. **2000**, in press.