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Synthesis and antiangiogenic activity of thioacetal artemisinin derivatives

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Abstract—Various thioacetal artemisinin derivatives can inhibit the angiogenesis and might be angiogenesis inhibitors. In particular, 10α-phenylthiodihydroartemisinins (**5**), 10β-benzenesulfonyl-9-*epi*-dihydroartemisinin (**11**) and 10α-mercaptodihydroartemisinin (**13**) exhibit strong growth inhibition activity against HUVEC proliferation. Compound **11** have a good inhibitiory activity upon HUVEC tube formation, and **5** and **11** show a strong inhibitory effect on angiogenesis using CAM assay at 5 μg/egg by 90%.
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1. Introduction

Angiogenesis is the formation of new vascular capillaries from preexisting host vessels by various biological stimulators.[1] In normal system, except during wound healing[2] and embryonic development,[3] an elaborate balance between positive and negative regulators tightly controls angiogenesis. On the other hands, in abnormal conditions, angiogenesis occurs in the course of tumor growth,[4] diabetic retinopathy,[5] and rheumatoid arthritis.[6] In particular, tumor angiogenesis plays a key role in the growth, invasion, and metastasis of tumors. Therefore, the control of angiogenesis may be a promising therapeutic strategy for the related diseases.

Angiogenesis is a complex process: endothelial cells (ECs) of existing blood vessels must degrade the surrounding matrix, activated, and proliferating ECs migrate into stroma of the tissues, and then form tubes. Subsequently, these new vessels maturate to form a vascular network.[7]

Strategies for regulating angiogenesis have been carried out mainly in molecular biology, such as the isolation and identification of the endogenous inhibitor,[8] as well as gene[9] and antibody therapy.[10] However, because of bioavailability, biostability, and effectiveness, it is very important to discover the antiangiogenic small molecules that might be suitable as clinical therapies.

Although angiogenesis passes through complex process, basically, ECs' function, such as the migration, differentiation, tube formation of ECs, is the most important. Therefore, in order to search for a novel small angiogenesis inhibitor, the inhibitory effects of promising molecules against the proliferation of human umbilical vein endothelial cells (HUVEC) in response to the various growth factors should be tested.

In our previous report,[11] thioacetal artemisinin derivatives (**1**) as shown in Figure 1 exhibit good growth inhibition activity against HUVEC proliferation. However, their growth inhibition activity against HUVEC certainly does not imply antiangiogenic activity in tumor angiogenic model. Therefore, based on the further screening, such as formation assay of HUVEC tube on Matrigel and Chorioallantomic membrane (CAM) assay as well as HUVEC proliferation assay, we must confirm that the selected molecules from the thioacetal artemisinin derivatives might be antiangiogenic inhibitors.

The natural sesquiterpene endoperoxide artemisinin (**2**), which was isolated from *Artemisia annua L.*,[12] has become a potential lead compound in the development

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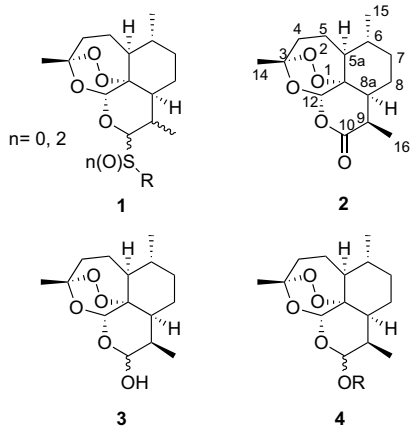


Figure 1.

of antimalarial[13] and recently anticancer agents.[14] The semisynthetic acetal-type artemisinin derivatives (**4**), ether, and ester derivatives of trioxane lactol dihydroartemisinin (**3**), were developed for their higher antimalarial efficacy and are now widely used to treat malarial patients (Fig. 1).[15]

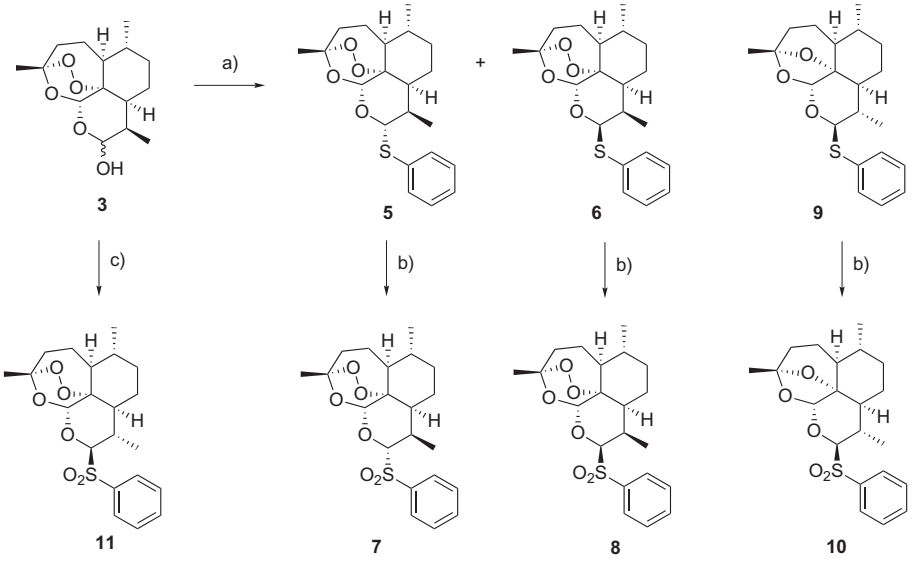
Particularly, Posner et al. reported that various sulfone endoperoxides have a highly antimalarial activity.[16] Recently, Chen et al. reported that artemisinin (**2**), dihydroartemisinin (**3**) and artesunate have the antiangiogenic activity as well as the antitumor activity on in vitro models of angiogenesis.[17]

Herein, based on the screening of formation assay of HUVEC tube on Matrigel and Chorioallantomic membrane (CAM) assay as well as growth inhibition activity against HUVEC, we report that the details of the synthesis of the thioacetal artemisinin derivatives and their antiangiogenic activity, which have proved that the thioacetal artemisinin derivatives might be angiogenic inhibitors.

2. Results and discussion

As seen in Scheme 1, separable diastereomeric mixture of 10α-(**5**) and 10β-phenylthiodihydroartemisinins (**6**) were prepared by reacting known dihydroartemisinin (**3**)[18] with thiophenol (2 equiv) under the catalysis of BF_3Et_2O (1 equiv) at room temperature during 10 min.[19] Under this condition, the reaction of *S*-acetalization of **3** was highly stereoselectively as the ratio of α:β = 10:1. The stereochemistry of *S*-phenyl thioartemisinin **5** and **6** was determined by chemical shift of H-10 and coupling constant between H-9 and H-10. Major product **5** is the α-isomer as indicated by a chemical shift (4.74 ppm) and a large coupling constant ($J = 11.0$ Hz), while minor product **6** is the β-isomer ($\delta = 5.6$ ppm and $J = 5.3$ Hz). Unlike the *O*-acetalization of dihydroartemisinin (**2**) using appropriate alcohols under the same condition, the *S*-acetalization of **3** diastereoselectively produced an α-anomer (**5**). The thioacetal products (**5** and **6**) were transformed to produce 10α-(**7**) and 10β-benzenesulfonyldihydroartemisinins (**8**), respectively, by using oxidation with H_2O_2/urea complex (UHP), trifluoroacetic anhydride (TFAA) and $NaHCO_3$ in good yields.[20] In addition, desoxy-isomer of **5** (**9**) was formed from the thioacetal reaction as the side product because of the reductive property of thiol group, and successive oxidation of **9** with H_2O_2/urea complex gave 10α-benzenesulfonyl-desoxydihydroartemisinins (**10**). It was conformed by elemental analysis that **9** and **10** are desoxy artemisinin compounds.

Dihydroartemisinin (**3**) with benzenesulfinic acid under the condition shown in Scheme 1 was directly converted 10β-benzenesulfonyl-9-*epi*-dihydroartemisinin (**11**) in 75% yield. The stereochemistry of 9-*epi*-intermediate (**11**) synthesized in this reaction was determined by ^1H and ^{13}C NMR of 9-*epi*-artemisinin series as reported by Bégué et al.[21] and comparison with its diastereomeric sulfone intermediates **7** and **8**. The chemical shift of H-10 and 16-methyl carbon at C-9 in **7** having natural



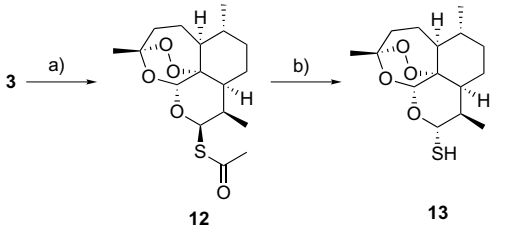
Scheme 1. Reagents and conditions: (a) Thiophenol (2 equiv), BF_3Et_2O (1 equiv), CH_2Cl_2, reflux, 10 min (95%, **5**:**6** = 10:1); (b) UHP (3 equiv), TFAA (3 equiv), $NaHCO_3$ (5 equiv), CH_3CN, −30 °C (95% for **7** and 93% for **8**); (c) Benzenesulfinic acid (2 equiv), BF_3Et_2O (1 equiv), CH_2Cl_2, rt (75%).

stereochemistry were appeared at 4.41 ppm ($J = 10.98$ Hz) and 14.0 ppm, while its epimer (**11**) were at 5.13 ppm ($J = 10.24$ Hz) and 20.0 ppm, respectively, which signals indicate all *trans* configuration and diastereomeric relationship between sulfonyl group and 16-methyl at C-9 in **7** and **11**.

The reaction dihydroartemisinin (**3**) with thiolacetate under the catalysis of BF_3Et_2O shown in Scheme 2, afforded a 10β-thioacetoxydihydroartemisinin (**12**) in 84% yield, and then by the basic catalytic deacetylation, **12** was transformed to 10α-mercaptodihydroartemisinin (**13**), *S*-acetal analog of **3**. Comparing the coupling constant of H9 and H10 between **12** and **13** ($J = 5.13$ Hz for **12** and $J = 10.80$ Hz for **13**), we confirmed that the stereochemistry of C-10 position was converted from β to α caused by basic catalyst.[18]

To investigate the antiangiogenic activity of the structure activity relationship between *S*-acetal and *O*-acetal type artemisinin derivatives, we have synthesized the two α-(**14**) and β-isomer (**15**) of C-10 phenoxy dihydroartemisinin and β-artemether (**16**) using known method.[22] Desoxyartemisinin (**17**) was synthesized to compare the angiogenesis property of peroxide group (Fig. 2).

The growth inhibition effect of thioacetal-type artemisinin derivatives, known acetal type artemisinin compounds and some desoxy-artemisinin derivatives were examined on a HUVEC proliferation assay using the MTT colorimetric method.[23] The results are listed in Table 1.



Scheme 2. Reagents and conditions: (a) Thiolacetic acid (1 equiv), BF_3Et_2O (1 equiv), CH_2Cl_2, rt, 10 min (84%); (b) NaOEt (5 equiv), EtOH, rt, 1 h (62%).

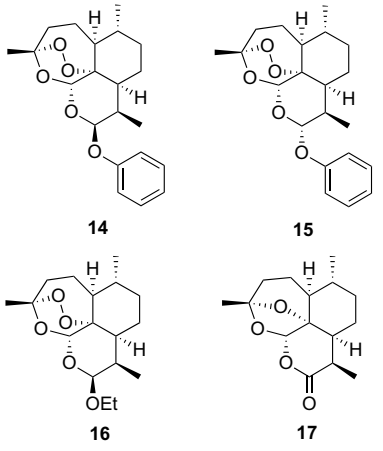


Figure 2.

The natural artemisinin (**2**) with endoperoxide ring and desoxyartemisinin (**17**) with no peroxide did not show inhibitory effect at the concentration of 50 μM, while dihydroartemisinin (**3**) was more effective than **2** in inhibiting the HUVEC growth ($IC_{50} = 8.91$ μM). As the Chen's report, we could also make sure that acetal-type derivatives might be a lead compound for use as an antiangiogenic inhibitor.

On the basis of our elementary result, we assumed that all acetal-type artemisinin derivatives (**4**) would have an inhibitory effect on HUVEC growth, but, disappointingly, 10β-(**14**) and 10α-phenoxydihydroartemisinin (**15**) with endoperoxide and aromatic phenyl group were less active than **3**.

So, as the new synthetic approach, we next synthesized a series of *S*-acetal compounds of **3** to determine the inhibitory activity on HUVEC. 10α-Phenylthiodihydroartemisinin (**5**) and 10β-phenylthiodihydroartemisinin (**6**) with endoperoxide and thiophenoxy moiety showed a very strong inhibitory activity with an $IC_{50} = 0.93$ and 5.24 μM, respectively. Among the synthetic sulfonyl compounds, such as 10α-(**7**) and 10β-benzenesulfonyldihydroartemisinin (**8**) and 10β-benzenesulfonyl-9-*epi*-dihydroartemisinin (**11**), the inhibitory potency of 9-epimer (**11**) was over 7–10 times higher than that of **7** and **8**. It was very interesting that those compounds bearing a same molecular structure but only different C-9 stereochemistry showed a lot of difference in inhibitory effect. Even if desoxy compounds (**9** and **10**) had the thioacetal functionality and β-artemether (**16**) had the endoperoxide, they have no inhibitory activity for lack of endoperoxide or aromatic functional group.

In particular, a comparison between 10α-mercaptodihydroartemisinin (**13**) and dihydroartemisinin (**3**)

Table 1. HUVEC proliferation inhibition assay results for artemisinin derivatives

Compounds	Growth inhibition IC_{50} (μM)
2	>50
3	8.91
5	0.93
6	5.24
7	12.77
8	18.21
9	>50
10	>50
11	1.74
12	4.56
13	1.29
14	33.81
15	31.03
16	>50
17	>50

IC_{50} was calculated from nonlinear regression by GraphPad Prism software ($r^2 > 0.9$).

suggests that the sulfur atom at the C-10 position was critical for inhibition activity on HUVEC. According as change oxygen to sulfur, the biological activity improved much better as seven times.

So when assuming the HUVEC proliferation inhibitory effect listed in Table 1, it was postulated that the thioacetal dihydroartemisinin derivatives bearing sulfur acetal linkage at C-10 position and aromatic functional group as well as endoperoxide ring showed significantly efficient activity.

Table 2. HUVEC tube formation inhibition assay results for thioacetal artemisinin derivatives

Compounds	Inhibition percentage at 10 μg/mL (%)
2	0
3	19
5	20
6	16
7	41
8	52
9	0
10	32
11	74
12	0
13	7
14	3
15	16
16	48
17	0

The inhibition percentages were determined with a image analysis software, Image-Pro plus version 3.0.

Next, the activity to suppress the growth factor induced tube formation by HUVEC on Matrigel was assessed at the concentration of 10 μg/mL.[24] As shown in Table 2, artemisinin (**2**), dihydroartemisinin (**3**) and some desoxy artemisinin derivatives (**9**, **10**, and **17**) showed no tube formation inhibitory activity or very weak activity.

10α-Phenylthiodihydroartemisinin (**5**) and 10β-phenylthiodihydroartemisinin (**6**) with high growth inhibitory activity upon HUVEC showed a weak activity, but 10α-(**7**) and 10β-benzenesulfonyldihydroartemisinin (**8**) with relatively weak growth inhibition activity than **5** and **6** had a moderate activity. Particularly, 10β-benzenesulfonyl-9-*epi*-dihydroartemisinin (**11**) with potent growth inhibition activity effectively inhibited the tube formation by 74% at the concentration of 10 μg/mL (Fig. 3).

When compared to thioacetal compounds (**5** and **6**) and sulfonyl derivaitves (**7**, **8**, and **11**), sulfone substitution at the C-10 position of tricyclic artemisinin improved the tube formation inhibitory activity.

Interestingly, 10β-benzenesulfonyl-9-*epi*-desoxyartemisinin (**10**) and β-artemether (**16**), which have no inhibitory activity against HUVEC growth, exhibited moderate tube formation inhibitory activity by 32% and 48%, respectively. So, detail study on the structure activity relationship about various functional groups is needed.

Third, in vivo inhibitory effect of thioacetal artemisinin derivatives on angiogenesis was examined using CAM assay at the concentration of 5 μg/egg.[25] As shown in

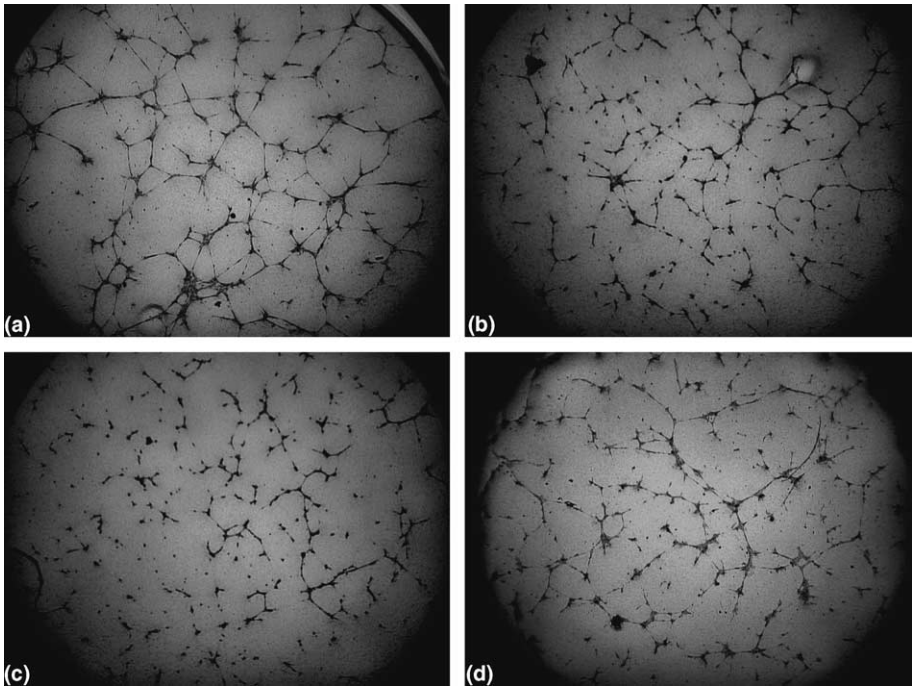


Figure 3. Effect of selected thioacetal artemisinin derivatives on tube formation by human umbilical vein endothelial cells (HUVEC) on Matrigel. (a) Control, (b) compound **8** (10 μg/mL), (c) compound **11** (10 μg/mL), (d) compound **16** (10 μg/mL).

Table 3. Inhibitory effect on angiogenesis using CAM assay at 5 μg/egg

Compounds	Inhibited eggs/tested eggs	Inhibition percentage (%)
5	18/20	90
6	11/16	69
7	6/16	38
8	6/15	40
11	16/18	89

Table 3, selected thioacetal molecules (**5** and **6**) and 9-*epi*-sulfonyl artemisinin **11** strongly inhibited the formation of new blood vessel on CAM. Particularly, **5** and **11** have a potent activity by 90%. The other analogues (**7** and **8**) were mildly potent.

In conclusion, with the various screening methods, such as growth inhibition activity against HUVEC, formation assay of HUVEC tube on Matrigel and Chorioallantomic membrane (CAM) assay, we concluded that the thioacetal deoxoartemisinin derivatives can inhibit the angiogenesis and might be angiogenesis inhibitors.

3. Experimental

3.1. General

Melting points were determined on Gallenkamp apparatus and were not corrected. Specific rotations were determined on optical activity AA-5 polarimeter at the given temperatures. IR spectra were recorded as thin films for solid and neat state for liquid on Mattson FTIR spectrometer. ^{1}H and ^{13}C NMR spectra recorded on Jeol Lambda 300 spectrometer at 300 and 75 MHz, respectively, in the indicated solvent using TMS as internal standard. Chemical shifts are expressed in ppm (d) and coupling constants (J) in Hz. Mass spectrum were determined on HP 5973 MSD system. Thin-layer chromatography (TLC) has been performed on precoated Merck silica gel 60 F254 plates. Elemental analysis was carried out on CE instruments EA1110 elemental analyzer. All other reagents were commercially available.

3.2. 10α-Phenylthiodihydroartemisinin (5) and 10β-phenylthiodihydroartemisinin (6)

Thiophenol (698 mg, 6.34 mmol) and BF_3Et_2O (500 mg, 3.52 mmol) were added to a stirred solution of dihydroartemisinin **2** (1 g, 3.52 mmol) in CH_2Cl_2 (50 mL) at room temperature. The solution was stirred for 10 min, after which it was diluted with CH_2Cl_2 (100 mL), washed with sat-$NaHCO_3$ and brine. The organic layer was separated, dried with $MgSO_4$, filtered and evaporated to dryness. The crude product was purified by flash chromatography (hexane:EtOAc 15:1) to give 1.13 g (85%) of **5** and 140 mg (11%) of **6** for **5**: white crystal, mp 115–117 °C; $[\alpha]_D^{20}$ +108.3 (c 0.120, $CHCl_3$); IR (KBr pellet) ν_{max} 2922, 1592, 1384, 1042 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.68 (2H, dd, $J = 8.3$, 1.8 Hz, Ph), 7.26 (3H, m, Ph), 5.35 (1H, s, H-12), 4.73 (1H, d, $J = 11.0$ Hz, H-10), 2.57 (1H, m, H-9), 2.37 (1H, td, $J = 14.5$, 4.0 Hz, H-4α), 1.47 (3H, s, H-14), 0.95 (3H, d, $J = 6.2$ Hz, H-16), 0.90 (3H, d, $J = 7.1$ Hz, H-15) ppm; ^{13}C NMR (75 MHz, $CDCl_3$) δ 132.9, 132.5, 128.6, 127.3, 104.3, 92.2, 83.5, 80.3, 51.7, 46.0, 37.4, 36.2, 34.1, 31.1, 26.0, 24.8, 21.4, 20.2, 15.1 ppm; GC/MSD (m/z) retention time 23.4 (min) 376 (M^+), 330, 316, 287, 267, 225, 207, 138 (100); Anal. Calcd for $C_{21}H_{28}O_4S$: C, 66.99; H, 7.50; S, 8.52. Found: C, 67.34; H, 7.73; S, 8.17; for **6**: white crystal, mp 98–99 °C; $[\alpha]_D^{20}$ +276.7 (c 0.300, $CHCl_3$); IR (KBr pellet) ν_{max} 2926, 1551, 1384, 1253, 1097, 1021 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.54 (2H, dd, $J = 8.6$, 1.5 Hz, Ph), 7.26 (3H, m, Ph), 5.74 (1H, s, H-12), 5.57 (1H, d, $J = 5.3$ Hz, H-10), 3.12 (1H, m, H-9), 2.39 (1H, td, $J = 14.3$, 3.8 Hz, H-4α), 1.44 (3H, s, H-14), 1.05 (3H, d, $J = 7.3$ Hz, H-16), 0.98 (3H, d, $J = 6.4$ Hz, H-15) ppm; ^{13}C NMR (75 MHz, $CDCl_3$) δ 136.9, 130.9, 128.8, 126.7, 104.2, 90.2, 88.3, 81.0, 52.6, 45.1, 37.2, 36.4, 34.4, 32.7, 26.0, 24.6, 24.3, 20.3, 15.0 ppm; GC/MSD (m/z) retention time 23.8 (min) 376 (M^+), 316, 287, 267, 225, 207, 138 (100); Anal. Calcd for $C_{21}H_{28}O_4S$: C, 66.99; H, 7.50; S, 8.52. Found: C, 67.54; H, 7.73; S, 8.18.

3.3. 10α-Benzenesulfonyldihydroartemisinin (7)

Trifluoroacetic anhydride (1.13 mL, 7.97 mmol) was added to a stirred suspension of ureahydrogenperoxide (750 mg, 7.97 mmol) in acetonitrile (50 mL) for 10 min at room temperature. The solution was added dropwise to a stirred suspension of **5** (1 g 2.66 mmol) and $NaHCO_3$ (1.11 g 13.3 mmol) in acetonitrile (50 mL) at −40 °C for 10 min. The suspension was stirred for 20 min, after the solution was quenched with water (100 mL), extracted with EtOAc (3 × 50 mL) and washed with brine (50 mL). The organic layer was separated, dried with $MgSO_4$, filtered, and evaporated to dryness. The crude product was purified by flash chromatography (hexane:EtOAc 5:1) to give a desired product **7** (1.03 g, 95%). For **7**; white crystal, mp 135–137 °C; $[\alpha]_D^{20}$ +62.5 (c 0.160, $CHCl_3$); IR (KBr pellet) ν_{max} 3051, 2927, 1551, 1383, 1252, 1096, 1012 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.98 (2H, d, $J = 7.9$ Hz, Ph), 7.63 (1H, t, $J = 7.1$ Hz, Ph), 7.50 (2H, t, $J = 7.9$ Hz, Ph), 5.26 (1H, s, H-12), 4.42 (1H, d, $J = 11.0$ Hz, H-10), 2.42 (1H, m), 2.26 (1H, td, $J = 14.5$, 4.0 Hz, H-4α), 1.35 (3H, s, H-14), 1.12 (3H, d, $J = 7.0$ Hz, H-16), 0.90 (3H, d, $J = 6.0$ Hz, H-15) ppm; ^{13}C NMR (75 MHz, $CDCl_3$) δ 135.9, 133.8, 130.0, 128.4, 104.3, 91.9, 90.7, 79.6, 51.2, 46.5, 37.3, 35.9, 33.9, 28.3, 25.6, 24.6, 21.3, 20.0, 14.0 ppm; GC/MSD (m/z) retention time 25.8 (min) 408 (M^+), 392, 364, 327, 251 (100), 233, 205; Anal. Calcd for $C_{21}H_{28}O_6S$: C, 61.74; H, 6.91; S, 7.85. Found: C, 61.81; H, 7.01; S, 7.40.

3.4. 10β-Benzenesulfonyldihydroartemisinin (8)

Same method as shown in the synthesis of **7**. Yield 93%. For **8**; white crystal, mp 94–96 °C; $[\alpha]_D^{20}$ +145.8 (c 0.240, $CHCl_3$); IR (KBr pellet) ν_{max} 3054, 2986, 1422, 1384, 1265, 1048 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.94 (2H, d, $J = 6.9$ Hz, Ph), 7.60 (1H, t, $J = 7.2$ Hz, Ph),

7.52 (2H, t, $J = 7.5$ Hz, Ph), 5.98 (1H, s, H-12), 5.01 (1H, d, $J = 6.3$ Hz, H-10), 3.17 (1H, m), 2.28 (1H, td, $J = 14.4$, 3.9 Hz, H-4α), 1.35 (3H, d, $J = 7.7$ Hz, H-14), 1.18 (3H, s, H-16), 0.94 (3H, d, $J = 4.5$ Hz, H-15) ppm; ^{13}C NMR (75 MHz, $CDCl_3$) δ 133.4, 129.1, 128.8, 128.7, 103.8, 92.0, 90.3, 80.9, 52.1, 43.8, 37.0, 36.1, 34.1, 31.6, 25.5, 24.5, 23.4, 20.1, 13.2 ppm; GC/MSD (m/z) retention time 26.5 (min) 408 (M^+), 392, 364, 327, 251 (100), 233, 205; Anal. Calcd for $C_{21}H_{28}O_6S$: C, 61.74; H, 6.91; S, 7.85. Found: C, 62.02; H, 7.26; S, 7.55.

3.5. 10β-Phenylthio-desoxy-9-*epi*-dihydroartemisinin (9)

Thiophenol (350 mg, 3.17 mmol) and BF_3Et_2O (250 mg, 1.25 mmol) were added to a stirred solution of dihydroartemisinin **2** (500 mg, 1.25 mmol) in CH_2Cl_2 (30 mL) at room temperature. The solution was stirred for 12 h, after which it was diluted with CH_2Cl_2 (50 mL), washed with satd $NaHCO_3$ and brine. The organic layer was separated, dried with $MgSO_4$, filtered and evaporated to dryness. The crude product was purified by flash chromatography (hexane:EtOAc = 20:1) to give a desoxy product **9** (321 mg, 50%). For **9**; white crystal, mp 102–104 °C; $[\alpha]_D^{20}$ −62.5 (c 0.064, $CHCl_3$); IR (KBr pellet) ν_{max} 3049, 2927, 1552, 1384, 1252, 1209, 1097, 1039 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.52 (2H, d, $J = 8.5$ Hz, Ph), 7.23 (1H, t, $J = 8.0$ Hz, Ph), 7.18 (2H, t, $J = 7.3$ Hz, Ph), 5.45 (1H, s, H-12), 5.11 (1H, d, $J = 10.5$ Hz, H-10), 1.49 (3H, s, H-14), 1.20 (3H, d, $J = 7.0$ Hz, H-16), 0.89 (3H, d, $J = 5.7$ Hz, H-15) ppm; ^{13}C NMR (75 MHz, $CDCl_3$) δ 136.2, 128.7, 128.6, 126.0, 107.2, 97.5, 82.7, 82.4, 45.2, 43.7, 39.3, 35.1, 34.3, 32.3, 23.5, 22.0, 20.8, 18.6 ppm; GC/MSD (m/z) retention time 22.3 (min) 360 (M^+), 331, 314, 299, 281, 269, 251 (100); Anal. Calcd for $C_{21}H_{28}O_3S$: C, 69.96; H, 7.83; S, 8.89. Found: C, 68.69; H, 7.77; S, 8.91.

3.6. 10β-Benzenesulfonyl-desoxy-9-*epi*-dihydroartemisinin (10)

Same method as shown in the synthesis of **7**. Yield 84%. For **10**; white crystal, mp 129–131 °C; $[\alpha]_D^{20}$ −86.5 (c 0.104, $CHCl_3$); IR (KBr pellet) ν_{max} 2927, 1551, 1384, 1251, 1101, 1010 cm^{-1}; ^{1}H NMR(300 MHz, $CDCl_3$) δ 7.93 (2H, d, $J = 7.1$ Hz, Ph), 7.64 (1H, t, $J = 8.1$ Hz, Ph), 7.55 (2H, t, $J = 6.3$ Hz, Ph), 5.30 (1H, s, H-12), 4.60 (1H, d, $J = 9.9$ Hz, H-10), 1.40 (3H, s, H-14), 1.39 (3H, d, $J = 6.5$ Hz, H-16), 0.86 (3H, d, $J = 5.4$ Hz, H-15) ppm; ^{13}C NMR (75 MHz, $CDCl_3$) δ 136.9, 133.7, 129.3, 128.7, 107.6, 96.7, 88.4, 82.7, 44.8, 44.7, 35.1, 34.1, 33.9, 32.9, 31.7, 23.6, 21.9, 21.6, 18.5 ppm; GC/MSD (m/z) retention time 26.8 (min) 392 (M^+), 374, 346, 331, 301, 283 (100); Anal. Calcd for $C_{21}H_{28}O_5S$: C, 64.26; H, 7.19; S, 8.17. Found: C, 63.96; H, 7.08; S, 8.34.

3.7. 10β-Benzenesulfonyl-9-*epi*-dihydroartemisinin (11)

Benzenesulfinic acid (500 mg, 3.5 mmol) and BF_3Et_2O (250 mg, 1.76 mmol) were added to a stirred solution of dihydroartemisinin **2** (500 mg, 1.76 mmol) in CH_2Cl_2 (30 mL) at room temperature. The solution was stirred for 10 min, after which it was diluted with CH_2Cl_2 (50 mL), washed with sat $NaHCO_3$ and brine. The organic layer was separated, dried with $MgSO_4$, filtered, and evaporated to dryness. The crude product was purified by flash chromatography (hexane:EtOAc = 5:1) to give a product **11** (1.07 g, 75%). For **11**; white crystal, mp 107–108 °C; $[\alpha]_D^{20}$ +71.4 (c 0.210, $CHCl_3$); IR (KBr pellet) ν_{max} 2926, 1552, 1384, 1252, 1210, 1101, 1020 cm^{-1} ; ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.99 (2H, d, $J = 7.0$ Hz, Ph), 7.64 (1H, t, $J = 7.3$ Hz, Ph), 7.54 (2H, t, $J = 7.7$ Hz, Ph), 5.41 (1H, s, H-12), 5.14 (1H, d, $J = 10.3$ Hz, H-10), 1.43 (3H, d, $J = 7.0$ Hz, H-16), 1.05 (3H, s, H-14), 0.92 (3H, d, $J = 5.7$ Hz, H-15) ppm; ^{13}C NMR (75 MHz, $CDCl_3$) δ 137.5, 133.4, 129.2, 128.5, 102.3, 90.6, 89.7, 81.9, 50.7, 48.5, 37.2, 35.9, 34.8, 33.8, 30.9, 25.1, 24.6, 20.7, 19.7 ppm; GC/MSD (m/z) retention time 27.2 (min) 408 (M^+), 390, 375, 350, 221, 163 (100); Anal. Calcd for $C_{21}H_{28}O_6S$: C, 61.74; H, 6.91; S, 7.85. Found: C, 61.57; H, 7.25; S, 7.65.

3.8. 10β-Thioacetoxydihydroartemisinin (12)

Thioacetic acid (320 mg, 4.2 mmol) and BF_3Et_2O (300 mg, 2.11 mmol) were added to a stirred solution of dihydroartemisinin **2** (600 mg, 2.11 mmol) in CH_2Cl_2 (30 mL) at room temperature. The solution was stirred for 10 min, after which it was diluted with CH_2Cl_2 (50 mL), washed with satd $NaHCO_3$ and brine. The organic layer was separated, dried with $MgSO_4$, filtered, and evaporated to dryness. The crude product was purified by flash chromatography (hexane:EtOAc = 5:1) to give 606 mg (84%) of thioacetoxy product **14**. For **14**; white crystal, mp 114–119 °C; $[\alpha]_D^{20}$ +305.6 (c 0.180, $CHCl_3$); IR (KBr pellet) ν_{max} 2933, 2873, 1707, 1550, 1382, 1252, 1058, 1009 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.12 (1H, d, $J = 5.1$ Hz, H-10), 5.33 (1H, s, H-12), 3.17 (1H, m, H-9), 2.37 (3H, s, –SCOCH$_3$), 2.41–2.31 (1H, m, H-4α), 1.35 (3H, s, H-14), 0.87 (3H, d, $J = 6.0$ Hz, H-16), 0.88 (3H, d, $J = 7.4$ Hz, H-15) ppm; ^{13}C NMR (75 MHz, $CDCl_3$) δ 193.20, 104.37, 89.34, 82.69, 80.46, 52.40, 44.80, 37.25, 36.12, 34.21, 31.25, 31.19, 25.90, 24.48, 23.54, 20.19, 14.19 ppm; GC/MSD (m/z) retention time 21.3 (min) 324 (M^+−16), 296, 282, 263, 237, 221, 180, 162 (100); Anal. Calcd for $C_{17}H_{26}O_5S$: C, 59.62; H, 7.65; S, 9.36. Found: C, 60.29; H, 7.37; S, 9.48.

3.9. 10α-Mercaptodihydroartemisinin (13)

Sodium ethoxide (300 mg 4.41 mmol) was added to a stirred solution of **14** (300 mg 0.88 mmol) in ethanol (20 mL). The suspension was stirred for 1 day at room temperature. The solution was then added to aqueous HCl (10 mL, 1 M), extracted with EtOAc (3 × 30 mL) and washed with brine. The organic layer was separated, dried with $MgSO_4$, filtered, and evaporated to dryness. The crude product was purified by flash chromatography (hexane:EtOAc = 5:2) to give a mercapto product **15** (163 mg, 62%). For 15; colorless oil; $[\alpha]_D^{20}$ −183.9 (c 0.018, $CHCl_3$); IR (neat) ν_{max} 2929, 2873, 1550, 1379,

1242, 1036 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ 5.29 (1H, s, H-12), 4.73 (1H, d, $J = 10.8$ Hz, H-10), 2.71 (1H, m, H-9), 2.36 (1H, td, $J = 14.3$, 3.8 Hz, H-4α), 1.41(3H, s, H-14), 0.99 (3H, d, $J = 7.1$ Hz, H-16), 0.94 (3H, d, $J = 6.0$ Hz, H-15) ppm; ^{13}C NMR (75 MHz, $CDCl_3$) δ 104.17, 92.57, 86.98, 80.51, 51.82, 46.12, 37.34, 36.28, 33.96, 31.88, 26.07, 24.68, 21.89, 20.27, 14.87 ppm; GC/MSD (m/z) retention time 15.6 (min) 300 (M^+), 268, 250, 236, 211, 178 (100); Anal. Calcd for $C_{15}H_{24}O_4S$: C, 59.97; H, 8.05; S, 10.67. Found: C, 60.37; H, 7.87; S, 9.84.

3.10. Assay method for HUVEC proliferation inhibition assay

HUVEC in EGM-2 medium containing the growth factor (Cambrex, Walkersville, MD) was plated in a 48-well plate (10,000 cells/well) for 4 h to allow cells to adhere to the plate. The test compounds, diluted in the medium, were added to the appropriate wells and incubated for 72 h at 37 in a 5% CO_2 humidified atmosphere. After incubation, the viability of the HUVEC was assayed using a 3-(4,5-dimethylthiazol-2-y1)-2,5-diphenyltetrazolium bromide (MTT) colorimetric proliferation assay.

3.11. Assay method for HUVEC tube formation inhibition assay

Formation of HUVEC tube on Matrigel (BD Biosciences, Bedford, MA) was assayed in a 96-well plate. HUVEC was plated (5000 cells/well) on 50 μL of Matrigel solidified, and incubated for 16 h at 37 in a 5% CO_2 humidified atmosphere. After incubation, the cells were stained with 0.1% toluidin blue in 4% paraformaldehyde, and photographed with microscope (Olympus, Tokyo, Japan). The total tube length in three randomly chosen microscopic field per well was determined with a image analysis software, Image-Pro plus version 3.0 (Media Cybernetics, MD, USA).

3.12. Assay method for CAM assay

Fertilized chicken eggs were incubated horizontally at 37 °C in a humidified incubator. After 3 days incubation, about 2 mL of albumin was aspirated from the eggs with 18-gauge needle. On day 6, the shell covering the air sac was punched out and membrane of air sac was removed by forceps. Methanol solution of the compounds were applied on polycarbonate membrane (Nuclepore, CA, USA) coated with 1% methycellulose. Then, the disks were placed on the CAM, and the eggs were sealed with adhesive tape. After reincubation for 2 days, an appropriate volume of 20% fat emulsion was injected into the chorioallantois to improve visualization of the vascular network. Inhibition of angiogenesis was determined by measuring the avascular zone in CAM. A positive anti-angiogenic response was assessed as an avascular zone of 3 mm in diameter and results were expressed as frequency (%) of avascular zone.

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