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Synthesis of 10β-Substituted Triazolyl Artemisinins and Their Growth Inhibitory Activity against Various Cancer Cells

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The naturally occurring endoperoxide sesquiterpene lactone, artemisinin (**1**), isolated from *Artemisia annua* L. has been used as an important lead compound for antimalarial drug development.[1] Semi-synthetic antimalarial agents, including artemether, arteether, artesuic acid, and artelinic acid, synthesized from dihydroartemisinin (**2**) are now being used in clinical treatments because of their therapeutic efficacy and nontoxicity.[2-5] Recently, research projects have been carried out to develop novel drugs based on the anticancer, antiviral, immunosuppressive, and antifungal properties of various artemisinin-related derivatives.[6] Among the diverse trials to synthesize novel structures derived from artemisinin, the addition of heteroatoms such as sulfur and nitrogen to the C-10 position of **1** deserves special mention because it yielded a different biological property and an improved antimalarial activity which was not reported previously. For example, sulfide and sulfonyl derivatives of artemisinins inhibit the proliferation of human umbilical vein endothelial cells (HUVEC) in response to various growth factors, and they selectively control tumor-related angiogenesis.[7,8] Ziffer synthesized 11-azaartemisinin and its alkyl derivatives exhibiting enhanced antimalarial activity,[9,10] whereas Haynes recently synthesized artemisone (10-alkylaminoartemisinin), *N*-sulfonyl-11-azaartemisinin, and *N*-carbonyl-11-azaartemisinin.[11,12] In particular, the substitution of nitrogen at the C-10 position of artemisinin has been found to improve its bioavailability.

In my previous study, a novel structure possessing nitrogen at the C-10 position had been developed for the primary purpose of introducing the substituted triazole groups *via* the Cu (I)-catalyzed Huisgen 1,3-cylcoaddition between azides and alkynes. The Huisgen 1,3-cylcoaddition is the most widely used synthetic method for realizing a triazole functionality.[13-15] Initially, separatable diastereomeric 10-azido artemisinins (**3**) were obtained by reacting **2** with trimethylsilyl bromide (2.2 eq) and sodium azide (3 eq) at room temperature for 12 h according to a modified Haynes' method.[12] Rapid equilibration of the polonium ions under acidic conditions led to epimerization at the C-9 and C-10 position of **2** to afford three diastereomers, 9α, 10β-azidoartemisinin (**5**), 10β-azidoartemisinin (**6**), and 10α-azidoartemisinin (**7**).[16] Azide **7** reacted with various alkynes to yield a series of type **10** molecules as explained in my previous paper, whereas **5** and **6** did not react with any other alkyne besides phenylacetylene.[16] Because of the relative nonreactivity of diastereomers **5** and **6** under the direct Huisgen cycloaddition conditions and the expectation that the addition of substituted triazole groups at the C-10 position of artemisinin may give rise to unusual biological properties (high anticancer activity in this case), it was necessary to design new synthetic methods in order to complete the stereoisomeric triazole library.[16]

Therefore, rather than using the Huisgen 1,3-cycloaddition of **3** with alkynes to yield a series of structure **4**, substituted 1,2,3-triazoles were first synthesized and then added into the C-10 anomeric position of **2** under acidic conditions. The synthetic strategy involved the synthesis of several 4-substituted-1-*H*-triazoles *via* Huisgen 1,3-cycloaddition of various alkynes and azidotrimethylsilane ($TMSN_3$) (1.5 eq) with 5 mol % of CuI in a mixture of DMF and methanol (5:1)[17] followed by acid (BF_3Et_2O, 0.8 eq)-catalyzed reactions of **2** with the 4-substituted-1-*H*-triazoles in methylene chloride. The reaction resulted in a complex mixture of diastereomers and regioisomers, which included 9-*epi*-10β-(4-substituted triazolyl) artemisinin (**8**), 10α-(4-substituted triazolyl) artemisinin (**10**), and 10α-(5-substituted triazolyl) artemisinin (**11**).[18] A consideration of the

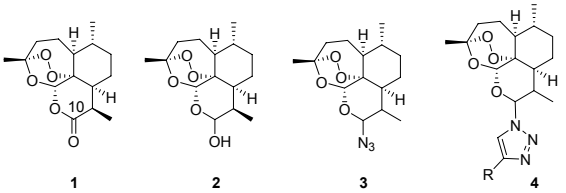


Figure 1

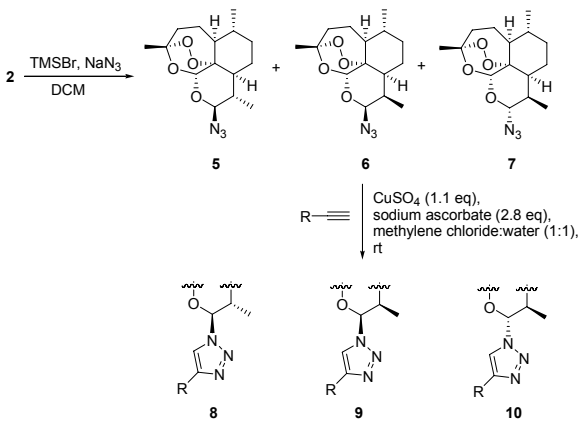


Figure 2. Huisgen 1,3-cycloaddition of 10-azido artemisinin (**5**, **6**, and **7**) with alkynes.

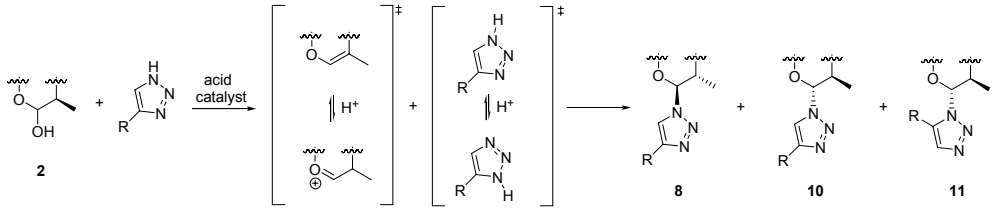
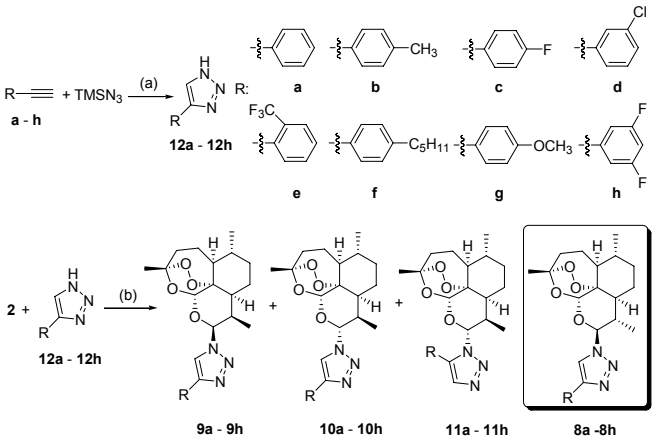


Figure 3. Addition of a 1,2,3-triazolyl substituent to the anomeric position of dihydroartemisinin (**2**).

results of two the synthetic methods shown in Figures 2 and 3, reveals a clear difference in the kinds of products obtained. Equilibration of the oxonium intermediate with 1, 2, 3-triazoles under acidic conditions resulted in epimerization at the C-9 and C-10 positions of **2** such that it can form two possible diastereomers (**8** and **10**) and one regioisomer (**11**).[19,20] Thus, diastereomeric and regioisomeric derivatives of **2** have the substituted triazole moieties at the C-10 position. Although the new synthetic trial met with a relative amount of success, in that the two series of structures **8** and **10** were obtained using the acidic reaction between **2** and various triazoles and additionally the new regioisomeric structure of **11** was obtained, nonetheless the library of type **9** shown in Figure 2 was not achieved by this method. The new synthetic triazoyl artemisinin libraries of type **8** and type **11** molecules were, however, confirmed to inhibit the growth of cancer cells and the type **10** analogs were shown to have a strong anticancer activity.[18]

The final objective of the project, under the overarching goal to make the 10-substituted triazolyl artemisinin library, was to synthesize all possible libraries having the 10-βstereochemistry of the substituentsincluding type **9** in Figure **2**. As was mentioned, the Huisgen 1,3-cycloaddition reaction of **6** with various alkynes and acid-catalyzed condensation reaction between **2** and triazole groups failed to generate the type **9** library. The number of alternative methods to obtain the type **9** artemisinin library was somewhat limited. Many previous attempts to synthesize the type **9** using the Huisgen 1,3-cycloaddition reaction and acid-catalyzed addition reaction, involving the variation of reaction conditions such as the solvent system, catalyst, *etc*., had already been attempted without success.[16,18] However, the clue leading to the breakthrough started from the idea that unexpected unexpected regioisomeric change in the 1,2,3-triazole system originated from the migration of the *N*-1 proton under acidic conditions. Without this proton migration, the 5-substituted-1-*H*-triazoles could not be obtained. The proton migration appears to be thermodynamically controlled process in which the thermodynamically stable isomer is preferred. Because the addition reaction does not occur without an acid catalyst, the only other possible variation to the reaction conditions was to increase the quantity of triazole compound so that it would be present in excess. It was anticipated that the presence of an excess of 4-substituted-1-*H*-triazoles in the reaction mixture, the possibility of triazole at the C-10 of artemisinin would occur before the equilibration of the 4-substituted-1-*H*-triazoles to 5-substituted-1-*H*-triazoles would be increased. Fortunately, the postulate that presence of excess triazoles would exert kinetic control of the reaction environment and that compound **9** would form under this condition is confirmed by the ratio of the reaction products. With this change in the reaction conditions, a series of type **9** structures were obtained as shown in Scheme 1 and the ratio of each of the stereoisomers produced is summarized in Table 1. A consideration of the ratio of products based on the molar equivalent of triazoles used in the reaction, shows that the type **11** compound is the main product when 1 equivalent of triazole is used,[18] whereas type **9** is the main product of the reaction with 3 equivalents of triazole. This shows that the excess of triazole precluded the isomerization of dihydroartemisinin and the migration of the hydrogen of *N*-1 to the *N*-3 position of the triazole, as shown in Figure 3.

The growth inhibitory effect of the members of the synthe-



Scheme 1. *Reagents and conditions*: (a) Azidotrimethylsilane (1.5 eq), CuI (5 mole %), DMF:MeOH = 5:1, reflux, 24 h. Isolated yields: **5a**, 85%; **5b**, 66%; **5c**, 89%; **5d**, 75%; **5e**, 61%; **5f**, 91%; **5g**, 70%; **5h**, 79%; (b) Triazole (**5a-5h**, 1 eq), BF_3Et_2O (0.8 eq) methylene chloride, rt, 24 h

Table 1. Yields and ratios of 10-substituted triazolylartemisinins synthesized *via* an acid-catalyzed reaction

	Yield[a] (%)	Ratio[b]		
		9	**10**	**11**
a	75	2	1	1.3
b	60	3.3	1	1.7
c	55	1	1.3	2
d	79	1.5	1.1	1
e	70	2.8	1	2.8
f	85	5.8	1.7	1
g	65	5.5	2	1
h	75	2.9	1	3.4

[a]Isolated yield. [b]Ratios of each product were calculated by integrating the H-9 peak in the ^{1}H NMR of non-purified product mixtures.

Table 2. Growth inhibitory concentration of the derivatives against cancer cells

Growth inhibitory concentration of the derivatives against cancer cells (GI_{50},[a] μM)													
		DLD-1	U87	Hela	SiHa	A172	B16			DLD-1	U87	Hela	SiHa	A172	B16
a	**8**	0.28	015	0.03	0.14	0.11	0.20	**e**	**8**	1.07	0.53	0.35	0.62	0.40	0.48
	9	**0.60**	**0.20**	**0.04**	**0.14**	**0.15**	**0.22**		**9**	**0.10**	**0.29**	**0.19**	**0.32**	**0.29**	**0.34**
	10	0.93	0.50	0.58	0.73	0.57	0.78		**10**	2.38	0.29	0.19	0.32	0.29	0.34
	11	0.60	0.20	0.04	0.14	0.15	0.22		**11**	0.17	0.08	0.07	0.08	0.08	0.08
b	**8**	0.16	1.34	0.73	1.58	1.74	0.70	**f**	**8**	0.84	0.27	0.24	0.44	0.26	0.34
	9	**0.26**	**0.27**	**0.11**	**0.12**	**0.09**	**0.12**		**9**	**0.14**	**0.07**	**0.09**	**0.08**	**0.07**	**0.07**
	10	0.40	0.28	0.11	0.12	0.09	0.12		**10**	0.14	0.07	0.09	0.08	0.07	0.07
	11	0.29	0.11	0.04	0.19	0.10	0.11		**11**	0.22	0.09	0.04	0.10	010	0.08
c	**8**	2.15	0.63	0.41	0.96	0.56	0.84	**g**	**8**	0.22	0.07	0.10	0.07	0.07	0.10
	9	**0.26**	**0.16**	**0.05**	**0.14**	**0.13**	**0.11**		**9**	**0.34**	**0.39**	**0.13**	**0.36**	**0.30**	**0.13**
	10	0.48	0.16	0.05	0.14	0.13	0.11		**10**	0.49	0.39	0.13	0.36	0.30	0.13
	11	0.31	0.09	0.06	0.08	0.08	0.77		**11**	0.88	0.23	0.09	0.24	0.21	0.29
d	**8**	1.17	0.35	0.21	0.41	0.33	0.28	**h**	**8**	1.28	0.45	0.24	0.46	0.54	0.70
	9	**0.15**	**0.25**	**0.09**	**0.24**	**0.31**	**0.21**		**9**	**0.13**	**0.13**	**0.05**	**0.12**	**0.14**	**0.13**
	10	0.29	0.25	0.09	0.24	0.31	0.21		**10**	0.30	0.13	0.05	0.12	0.14	0.13
	11	0.04	0.10	0.06	0.16	0.13	0.10		**11**	0.17	0.17	0.08	0.07	0.10	0.10
Taxol	0.01	0.02	0.02	0.03	0.01	0.01									

[a]GI_{50} values were calculated by nonlinear regression analysis using the GraphPad Prism software ($R^2 > 0.95$).

tic 10-substituted triazolylartemisinin library against cancer cell lines such as DLD-1, U-87, Hela, SiHa, A172, and B16[21] was examined using the MTT colorimetric method.[22] Although the growth inhibition activities of **8**, **10**, and **11** were previously published, [16,18] all inhibition activity results including newly synthesized type **9** compounds are summarized in Table 2 in order to provide a understanding of the biological properties according to the structural diversity. As shown in Table 2, all the stereoisomers have shown a strong inhibition activity against the tested cancer cells in the sub-micromolar range. Based on this preliminary investigation of the structure-activity relationship, no appreciable differences in the growth inhibitory effects based on the stereo- and regio-chemistry were observed. However, the strength of the growth inhibitory effect shows a dependence on the functional group attached to the triazole ring. Among the eight functionalities that were attached to the ring, the substituted triazolyl artemisinin compounds with a pentylbenzene group (**8f**, **9f**, **10f**, and **11f**) showed the highest anticancer activity.

In conclusion, we have established a library of 10-substituted triazolyl artemisinins (**8a-8h**, **9a-9h**, **10a-10h**, and **11a-11h**) comprising compounds that strongly inhibit the growth of cancer cell lines, such as DLD-1, U-87, Hela, SiHa, A172, and B16. These compounds were synthesized by an acid-catalyzed reaction of **2** with various substituted triazoles (**12a-12h**) in methylene chloride at room temperature. In particular, when 3 equivalents of triazole are reacted with dihydroartemisinin (**2**), the 10β-(4-substituted triazolyl) artemisinins (**9a-9h**) are obtained, which means that the type of product obtained differs according to the quantity of the triazole reactants employed in the synthesis. From the viewpoint of antiproliferation activity against the various cancer cells, **8f**, **9f**, **10f**, and **11f**, which have a pentylphenyltriazole moiety, exhibit potent activity; therefore, these compounds will be analyzed in preclinical trials in order to evaluate their *in vivo* anticancer activity and toxicity.

Experimental Section

The Following Procedure is Typically Adopted for the Synthesis of 10-(Substituted triazolyl) artemisinin (9, 10, and 11). BF_3Et_2O (0.40 g, 2.8 mmole) was added to a solution of dihydroartemisinin (**2**, 1.0 g, 3.5 mmole) and 4-phenyl-1*H*-1,2,3-triazole (**a**, 0.51 g, 10.5 mmole) in dichloromethane (85 mL); and the reaction mixture was stirred for about 12 hours at room temperature. The reaction progress was monitored using TLC. When the starting materials were no longer observed on the TLC plate, the reaction mixture was diluted with water (100 mL), and extracted with dichloromethane (3 × 50 mL). The organic layer was dried over $MgSO_4$ and then evaporated under reduced pressure. The residue was purified by silica-gel chromatography using an ethyl acetate/hexane (5:2) eluent, to afford **9a**, **10a**, and **11a**. The spectral data of type **10** and type **11** structures were previously reported in ref 18.

9a: ^{1}H-NMR (300 MHz, $CDCl_3$) δ 7.84 (1H, s, triazol), 7.78 (2H, d, J = 7.0 Hz, phenyl), 7.44 (2H, t, J = 7.1 Hz, phenyl), 7.36 (H, t, J = 7.3 Hz, phenyl), 6.48 (1H, s, H-12), 6.29 (1H, d, J = 5.7 Hz, H-10), 3.20 (1H, m), 2.43 (1H, td, J = 14.5, 4.0 Hz), 2.10 (1H, m), 1.48 (3H, s, 3-CH_3), 0.99 (3H, d, J = 6.2 Hz, 9-CH_3), 0.92 (3H, d, J = 7.5 Hz, 6-CH_3); ^{13}C-NMR (75M Hz, $CDCl_3$) δ 147.1, 130.3, 128.6, 128.4, 125.9, 104.3, 91.3, 90.5, 80.8, 52.6, 43.7, 37.2, 36.2, 34.4, 30.8, 25.9, 24.6, 22.4, 20.3, 13.2.

9b: ^{1}H-NMR (300 MHz, $CDCl_3$) δ 7.80 (1H, s, triazol), 7.66 (2H, d, J = 8.1 Hz, phenyl), 7.25 (2H, d, J = 8.1 Hz, phenyl), 6.46 (1H, s, H-12), 6.27 (1H, d, J = 5.7 Hz, H-10), 3.21 (1H, m), 2.42 (1H, td, J = 14.5, 4.0 Hz), 2.38 (s, 3H, Toluyl), 2.10 (1H, m), 1.47 (3H, s, 3-CH_3), 0.98 (3H, d, J = 6.4 Hz, 9-CH_3), 0.92 (3H, d, J = 7.4 Hz, 6-CH_3); ^{13}C-NMR (75 MHz, $CDCl_3$) δ 147.2, 138.3, 130.1, 129.5, 127.6, 125.9, 104.3, 91.2, 90.5, 80.8, 52.7, 43.8, 37.2, 36.3, 34.5, 30.1, 25.9, 24.6, 22.4, 21.3, 20.3, 13.2.

9c: ^{1}H-NMR (300MHz, $CDCl_3$) δ 7.79 (1H, s, triazol), 7.74 (2H, q, J = 5.1 Hz, phenyl), 7.12 (2H, t, J = 8.8 Hz, phenyl),

6.44 (1H, s, H-12), 6.28 (1H, d, J = 5.7 Hz, H-10), 3.20 (1H, m), 2.43 (1H, td, J = 14.5, 4.0 Hz), 2.09 (1H, m), 1.47 (3H, s, 3-CH_3), 0.99 (3H, d, J = 6.2 Hz, 9-CH_3), 0.92 (3H, d, J = 7.5 Hz, 6-CH_3); ^{13}C-NMR (75 MHz, $CDCl_3$) δ 146.3, 130.1, 127.7, 126.6, 116.0, 115.7, 104.3, 91.3, 90.5, 80.8, 52.6, 43.8, 37.3, 36.3, 34.4, 30.8, 25.9, 24.6, 22.4, 20.3, 13.2.

9d: ^{1}H-NMR (300MHz, $CDCl_3$) δ 7.84 (1H, s, triazol), 7.76 (1H, s, phenyl), 7.65 (1H, d, J = 7.0 Hz, phenyl), 7.37 (1H, t, J = 7.9 Hz, phenyl), 7.30 (1H, t, J = 1.8 Hz, phenyl), 6.44 (1H, s, H-12), 6.29 (1H, d, J = 5.7 Hz, H-10), 3.21 (1H, m), 2.43 (1H, td, J = 14.6, 4.0 Hz), 2.09 (1H, m), 1.47 (3H, s, 3-CH_3), 1.00 (3H, d, J = 6.2 Hz, 9-CH_3), 0.93 (3H, d, J = 7.5 Hz, 6-CH_3); ^{13}C-NMR (75 MHz, $CDCl_3$) δ 145.9, 134.8, 132.1, 130.5, 130.1, 128.4, 126.0, 124.0, 104.3, 91.5, 90.5, 80.7, 52.6, 43.7, 37.2, 36.2, 34.4, 30.7, 25.9, 24.6, 22.4, 20.3, 13.2.

9e: ^{1}H-NMR (300 MHz, $CDCl_3$) δ 7.76 (1H, t, J = 7.7 Hz, phenyl), 7.74 (1H, s, triazol), 7.61 (2H, d, J = 3.8 Hz, phenyl), 7.53 (1H, t, phenyl), 6.44 (1H, s, H-12), 6.32 (1H, d, J = 5.7 Hz, H-10), 3.21 (1H, m), 2.43 (1H, td, J = 14.3, 3.8 Hz), 2.08 (1H, m), 1.47 (3H, s, 3-CH_3), 0.99 (3H, d, J = 5.3 Hz, 9-CH_3), 0.95 (3H, d, J = 4.2 Hz, 6-CH_3); ^{13}C-NMR (75 MHz, $CDCl_3$) δ 144.6, 133.2, 132.0, 128.7, 126.4, 104.4, 91.5, 90.5, 80.8, 52.6, 43.7, 37.1, 36.3, 34.4, 30.8, 25.9, 24.6, 22.2, 20.3, 13.1.

9f: ^{1}H-NMR (300 MHz, $CDCl_3$) δ 7.80 (1H, s, triazol), 7.68 (2H, d, J = 7.9 Hz, phenyl), 7.25 (2H, t, J = 8.3 Hz, phenyl), 6.47 (1H, s, H-12), 6.28 (1H, d, J = 5.7 Hz, H-10), 3.20 (1H, m), 2.63 (2H, t, J = 7.5 Hz), 2.41 (1H, m), 1.49 (3H, s, 3-CH_3), 0.99 (3H, d, J = 6.2 Hz, 9-CH_3), 0.91 (3H, d, J = 7.5 Hz, 6-CH_3); ^{13}C-NMR (75 MHz, $CDCl_3$) δ 147.2, 135.0, 130.1, 129.0, 127.8, 125.8, 104.3, 91.2, 80.8, 79.0, 44.4, 37.5, 37.2, 36.3, 36.2, 35.7, 34.5, 34.1, 31.1, 30.0, 25.9, 24.4, 22.5, 20.2, 14.0.

9g: ^{1}H-NMR (300 MHz, $CDCl_3$) δ 7.76 (1H, s, triazol), 7.70 (2H, d, J = 9.0 Hz, phenyl), 6.95 (2H, d, J = 6.8 Hz, phenyl), 6.46 (1H, s, H-12), 6.27 (1H, d, J = 5.7 Hz, H-10), 3.85 (3H, s, -OCH_3), 3.19 (1H, m), 2.43 (1H, td, J = 13.6, 4.0 Hz), 2.09 (1H, m), 1.47 (3H, s, 3-CH_3), 0.99 (3H, d, J = 6.2 Hz, 9-CH_3), 0.92 (3H, d, J = 7.3 Hz, 6-CH_3); ^{13}C-NMR (75 MHz, $CDCl_3$) δ 159.8, 147.0, 129.8, 127.2, 123.1, 114.3, 104.3, 91.2, 90.5, 80.8, 55.4, 52.6, 43.8, 37.2, 36.3, 34.430.8, 25.9, 24.6, 22.4, 20.3, 13.2.

9h: ^{1}H-NMR (300 MHz, $CDCl_3$) δ 7.83 (1H, s, triazol), 7.28 (2H, m, phenyl), 6.80 (1H, m, phenyl), 6.42 (1H, s, H-12), 6.29 (1H, d, J = 5.7 Hz, H-10), 3.21 (1H, m), 2.43 (1H, td, J = 14.5, 3.8 Hz), 2.09 (1H, m), 1.47 (3H, s, 3-CH_3), 0.99 (3H, d, J = 6.2 Hz, 9-CH_3), 0.92 (3H, d, J = 7.3 Hz, 6-CH_3); ^{13}C-NMR (75 MHz, $CDCl_3$) δ 165.1, 161.8, 145.2, 135.0, 130.7, 108.9, 104.0, 100.6, 91.6, 90.5, 80.7, 52.6, 43.7, 37.3, 36.2, 34.4, 30.7, 25.9, 24.6, 22.4, 20.3, 13.2.

Growth Inhibitory Activity of the Artemisinin Derivatives was Evaluated with the MTT Assay.[22] Cancer cells were plated in 96-well culture plates at a density of 5×10^3 cells/well in a final volume of 100 μL of DMEM medium containing 10% FBS, pre-incubated for 4 h, and treated with serial concentrations of artemisinin derivatives for 72 h. After treatment, the cells were incubated for 4 h at 37 °C with a solution of MTT at a concentration of 1 mg/mL. The culture supernatant was aspirated and DMSO (100 μL) was added to dissolve the formed formazan crystals. The plate was then read at 570 nm in a microplate spectrophotometer (SpectraMax 250, Molecular Devices, CA, USA). Each assay was performed in triplicate. GI_{50} was calculated by nonlinear regression analysis from a sigmoid dose-response curve using the GraphPad Prism software ver 3.0 (GraphPad Software, CA, U.S.A.) when $R^2 > 0$.

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