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Synthesis of Artemisinin−Estrogen Hybrids Highly Active against HCMV, *P. falciparum*, and Cervical and Breast Cancer

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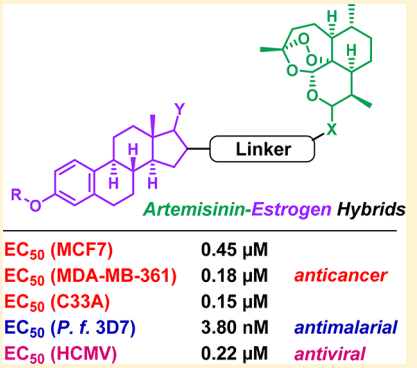
Supporting Information

ABSTRACT: Artemisinin−estrogen hybrids were for the first time both synthesized and investigated for their *in vitro* biological activity against malaria parasites (*Plasmodium falciparum* 3D7), human cytomegalovirus (HCMV), and a panel of human malignant cells of gynecological origin containing breast (MCF7, MDA-MB-231, MDA-MB-361, T47D) and cervical tumor cell lines (HeLa, SiHa, C33A). In terms of antimalarial efficacy, hybrid **8** (EC_{50} = 3.8 nM) was about two times more active than its parent compound artesunic acid (**7**, EC_{50} = 8.9 nM) as well as the standard drug chloroquine (EC_{50} = 9.8 nM) and was, therefore, comparable to the clinically used dihydroartemisinin (**6**) (EC_{50} = 2.4 nM). Furthermore, hybrids **9**−**12** showed a strong antiviral effect with EC_{50} values in the submicromolar range (0.22−0.38 μM) and thus possess profoundly stronger anti-HCMV activity (approximately factor 25) than the parent compound artesunic acid (**7**, EC_{50} = 5.41 μM). These compounds also exerted a higher *in vitro* anti-HCMV efficacy than ganciclovir used as the standard of current antiviral treatment. In addition, hybrids **8**−**12** elicited substantially more pronounced growth inhibiting action on all cancer cell lines than their parent compounds and the reference drug cisplatin. The most potent agent, hybrid **12**, exhibited submicromolar EC_{50} values (0.15−0.93 μM) against breast cancer and C33A cell lines.



KEYWORDS: *Artemisinin, estrogen, antimalarial activity, anticancer activity, antiviral activity*

Over the last three decades, steroids have become a prime focus of research in the field of medicinal chemistry due to their remarkable and diverse pharmacological properties, such as anticancer,[1,2] anti-inflammatory,[3,4] antiparasitic,[5] and antiviral activities.[6,7] In particular, the two steroid hormones estrone (**1**) and 17β-estradiol (**2**) (Figure 1) attracted a lot of attention, as these two estrogens are known to be involved in the development of various cancer types such as breast, colorectal, prostate, and ovarian cancer.[8] This led to the discovery of many different estradiol derivatives, which revealed to possess promising anticancer activity. In 2003, fulvestrant (**3**), an estrogen receptor antagonist, was approved in the USA for the treatment of hormone-related breast cancer, and since then it has been used in clinics.[9] 2-Methoxyestradiol (**4**), an endogenous metabolite of 17β-estradiol (**2**), turned out to effectively inhibit cancer cell proliferation both *in vitro* and *in vivo* and is currently investigated in advanced phases of clinical trials.[10−15] One of the main advantages of 2-methoxyestradiol (**4**) over other biologically active estrogens is that it does not act as an estrogen receptor agonist and consequently is free of the typical hormone-related side effects.[16,17] Furthermore, no serious toxicity was observed in clinical trials when 2-methoxyestradiol (**4**) was applied in pharmacological effective doses, and therefore, it can be regarded as a promising anticancer agent.[16,18]

As of now, no artemisinin-estrogen hybrids were reported in the literature, and our working group already could obtain remarkable results applying the hybridization concept;[19−22] where two different biologically active substances are linked via a covalent bond,[23,24] we planned to use estrogen derivatives as

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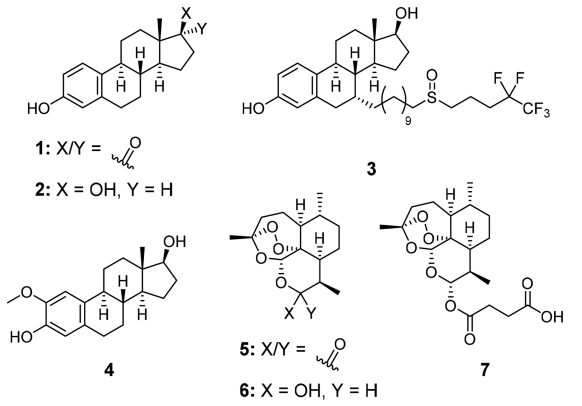

Figure 1. Structures of estrone (**1**), 17β-estradiol (**2**), fulvestrant (**3**), 2-methoxyestradiol (**4**), artemisinin (**5**), dihydroartemisinin (**6**), and artesunic acid (**7**).

precursors for the synthesis of novel artemisinin hybrid molecules. Since its isolation in 1972 from the plant *Artemisia annua* L. by Youyou Tu, for which she received the Nobel Prize in 2015, artemisinin (**5**) was intensively investigated for its pharmacological activities.[25,26] Artemisinin (**5**) exhibits not only antimalarial activity, for what it was mainly used in traditional Chinese medicine for several centuries,[27−29] but it also revealed to possess antiviral[30−33] and anticancer efficacy.[34−38] These promising properties are also reflected in its semisynthetic derivatives dihydroartemisinin (**6**)[39−41] and artesunic acid (**7**),[42−45] bearing an alcohol or a carboxylic acid functionality and for that reason appear to be well-suited for hybridization purposes. Recently, it could be even demonstrated in a phase I clinical trial, which was performed in patients with metastatic breast cancer, that higher cumulative doses of artesunic acid are safe and well tolerated.[46]

Herein, we present the synthesis of five novel artemisinin-estrogen hybrids **8**−**12** (Figure 2) and the evaluation of their *in vitro* biological activity against malaria parasites (*Plasmodium falciparum* 3D7), human cytomegalovirus (HCMV), and a selection of human breast cancer cell lines (MCF7, MDA-MB-231, MDA-MB-361, T47D) and cervical tumor cell lines (HeLa, SiHa, C33A).

Results and Discussion. *Chemistry.* Hybrids **8** and **9** were prepared in moderate to good yields (81%/45%) by standard amide coupling between estradiol amine **13** and either artesunic acid (**7**) or artemisinin-derived carboxylic acid **15** (Scheme 1). The reaction was conducted at room temperature overnight in a 1:1 mixture of CH_3CN and CH_2Cl_2 as solvent , and EDCI was solely used as coupling agent. Surprisingly, under these conditions no ester formation was observed as a side reaction, and the desired amides (**8**, **9**) were the only products. The synthesis of the artemisinin-derived acid **15** was carried out in accordance to an already published protocol starting from dihydroartemisinin (**6**) (Scheme 2).[47] The special feature of this artemisinin derivative is that it is free of the O atom at C-10, and for that reason, it has been referred to a so-called C-10 nonacetal in the previous literature. This derivative has been considered to be more stable compared to the classical C-10-acetals such as artesunic acid (**7**).[48] The other precursor, necessary for the synthesis of hybrids **8** and **9**, 3-methoxy-estradiol-derived amine **13** (Scheme 2), was also prepared in analogy to procedures described in the literature.[49−51] The stereoselectivity of the metal borohydride-mediated reduction of 16α-azido estrone 3-methyl ether (**17**) toward 17α- and 17β-estradiol derivatives **18a**/**b** can be achieved by selecting different alkali metals (Li, Na, or K) as countercation. If bigger countercations like potassium are used, the 17β-isomer is predominantly formed (57% yield), whereas smaller countercations such as lithium lead almost exclusively to the formation of the 17α-isomer (59% yield). 16α-Azido 17β-estradiol 3-methyl ether (**18b**) was then converted to the desired amine **13** by hydrazine monohydrate mediated reduction catalyzed by Raney-Ni (95% yield). The synthesis of hybrid **10** containing a 1,2,3-triazole linkage was realized by a copper-catalyzed 1,3-dipolar cycloaddition between 16α-azido estrone 3-methyl ether (**17**) and artemisinin-derived alkyne **16**, which afforded the desired product in 42% yield. Catalytic amounts of copper(II) sulfate and sodium ascorbate served as a source for copper(I), which was generated *in situ*. Alkyne **16** was prepared according to the literature by etherification of dihydroartemisinin (**6**) with propargyl alcohol.[52] As a final step, 3-benzyloxy-17β-hydroxy-16β-hydroxymethyl-estrone derivative **14** was reacted with either artesunic acid (**7**) or artemisinin-derived acid **15** in a Steglich esterification in order to yield the desired hybrids **11** and **12** in 95/56%. DCC and DMAP were used as coupling agents and CH_2Cl_2 as solvent. The ester formation took place only at the primary alcohol group, which is probably attributed to its higher reactivity and less steric hindrance. The stability of target

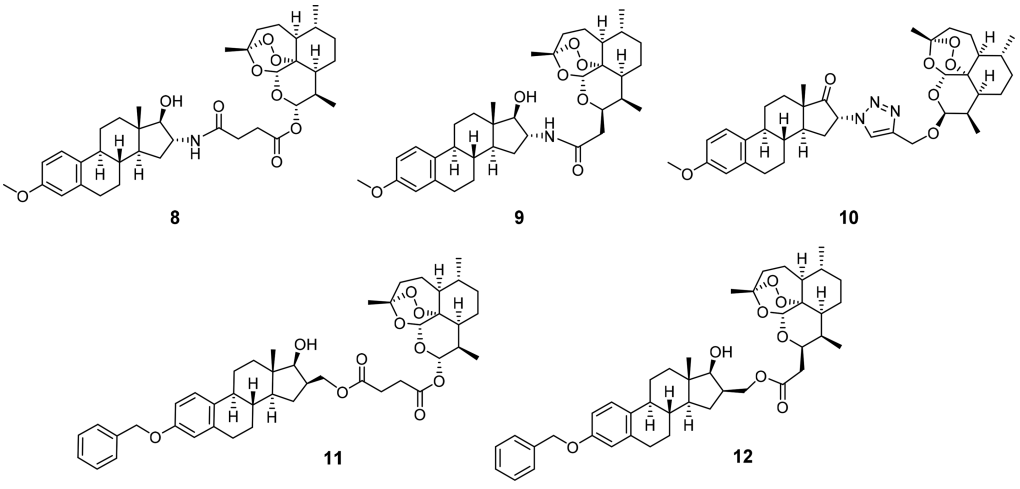

Figure 2. Novel hybrids **8**−**12** applied for biological tests against *P. falciparum* 3D7, HCMV, and breast and cervical cancer.

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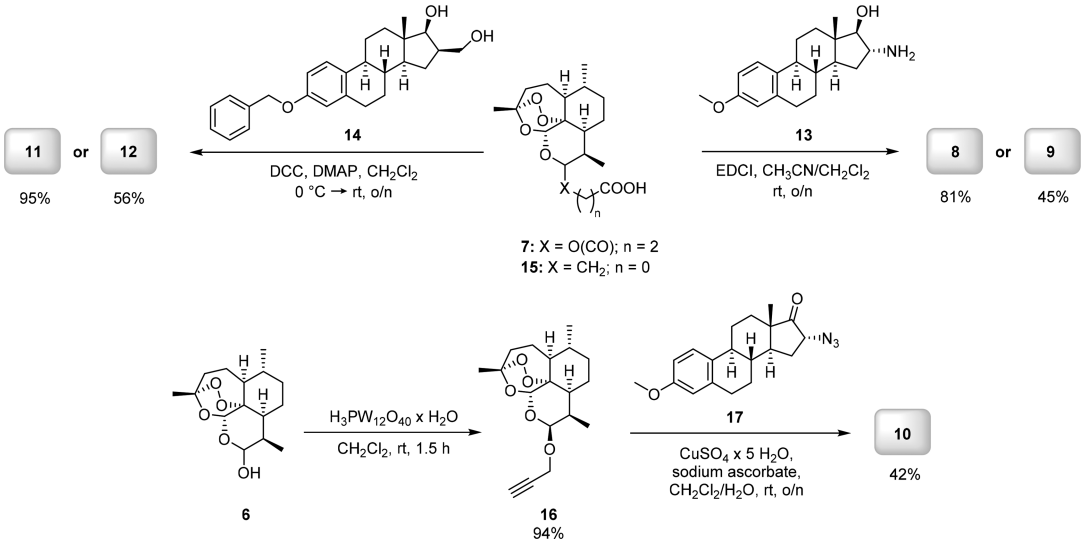
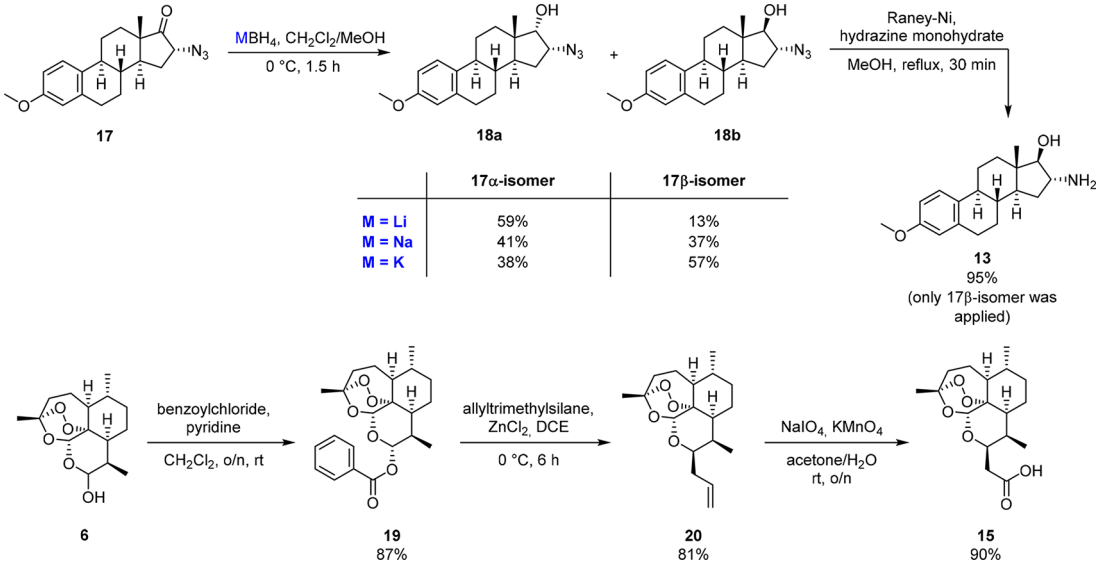
Scheme 1. Synthesis Route for Hybrids 8−12



Scheme 2. Synthesis of Estrogen Precursor 13 and Artemisinin-Derived Acid 15



	17α-isomer	17β-isomer
M = Li	59%	13%
M = Na	41%	37%
M = K	38%	57%

compounds **8−12** was examined by heat exposure at 65 °C for 24 h or 40 °C for 48 h, respectively. After applying these conditions, ^{1}H NMR spectroscopy revealed that all synthesized hybrids remained sufficiently stable, i.e., less than 5% decomposition was detected in the recorded spectra.

The hydroxy group at C-3 of all artemisinin-estrogen hybrids **8−12** was protected via an ether group (benzyloxy or methoxy) to decrease the binding affinities of these novel compounds to the estrogen receptors and consequently reduce eventual hormone-related side effects.

Biological Activity of the Hybrids. Antimalarial Activity. All synthesized hybrids **8−12** as well as their precursors, dihydroartemisinin (**6**), artesunic acid (**7**), estrone diol **14**, and estrone azide **17** were investigated for their antimalarial activity against chloroquine-sensitive *Plasmodium falciparum* 3D7 parasites (Table 1). Hybrids **8−12** exhibited excellent to moderate antimalarial efficacy with EC_{50} values ranging from 3.8 to 128.8 nM, while their estrogen precursors **14** and **17** showed no activity (EC_{50} > 16,000 nM). The best performing hybrid **8** was roughly two times more active than its parent compound artesunic acid (**7**) (EC_{50} = 8.9 nM) as well as the standard drug chloroquine (9.8 nM) and was therefore in terms of antimalarial efficacy comparable to the clinically used dihydroartemisinin (**6**) (EC_{50} = 2.4 nM). Hybrids **9** and **12** containing a C-10 nonacetal artemisinin moiety were found to be two and four times, correspondingly, less active (EC_{50} values of 7.7 and 128.8 nM) than their C-10 acetal counterparts (EC_{50}(**8**) = 3.8 nM; EC_{50}(**11**) = 34.2 nM). The same behavior was also observed in connection with artemisinin-quinazoline hybrids,[53] which is in contrast to that of artemisinin-derived dimers.[54] This indicates that different mechanisms might be involved for artemisinin-derived hybrids than for its dimeric structures. In addition, these EC_{50} values also demonstrate that a benzyloxy subunit at C-3 of the estrogen moiety (hybrids **11** and **12**) seems to be unbeneficial for antimalarial activity of artemisinin-estrogen hybrids, as compounds **8** and **9** with a methoxy group were much more active. This result might be explained by the fact that hybrids **11** and **12** are more lipophilic than compounds **8** and **9**, and as a result, their cellular uptake into the malaria parasites is probably more limited.

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Table 1. EC_{50} Values for Hybrids 8−12 and Selected Reference Compounds Tested against *P. falciparum* 3D7 Parasites, HCMV, and Various Human Breast and Cervical Cancer Cell Lines

				EC_{50} (μM)[e]						
compound	MW (g/mol)	EC_{50} (nM) *P.f.* 3D7	EC_{50} (μM) HCMV	MCF7	MDA-MB-231	MDA-MB-361	T47D	HeLa	SiHa	C33A
chloroquine	319.87	9.8 ± 2.8[a]								
ganciclovir	579.98		2.60 ± 0.50[b]							
cisplatin	300.05			5.78	19.13	3.76	9.78	12.43	7.87	3.69
artemisinin (**5**)	282.14		>10[c]	-	-	-	-	-	-	-
DHA (**6**)	284.35	2.4 ± 0.4[a]	>10[c]	8.24	10.69	1.71	4.60	10.46	29.80	1.71
artesunic acid (**7**)	384.42	8.9 ± 1.9	5.41 ± 0.60[d]	4.21	10.04	2.27	2.22	12.03	>30	1.83
estrone amine **13**	301.43	-	-	11.90	15.95	4.58	5.56	13.30	17.35	13.25
estrone diol **14**	392.54	17,250 ± 586	-	12.89	12.75	2.77	8.32	12.80	7.75	12.20
estrone azide **17**	325.41	>50,000	-	>30	>30	>30	>30	>30	>30	>30
8	667.84	**3.8 ± 0.8**	2.44 ± 0.13	4.69	6.89	**0.64**	**0.74**	11.45	26.00	**0.87**
9	609.80	7.7 ± 2.4	**0.23 ± 0.20**	1.02	1.85	0.69	1.17	1.65	6.21	**0.57**
10	647.81	13.1 ± 1.8	**0.24 ± 0.01**	1.77	1.78	**0.17**	**0.16**	15.40	28.90	2.05
11	758.95	34.2 ± 3.2	**0.38 ± 0.10**	**0.76**	2.30	**0.20**	**0.22**	>30	28.43	1.73
12	700.91	128.8 ± 13.0	**0.22 ± 0.00**	**0.45**	**0.86**	**0.18**	**0.93**	14.22	16.12	**0.15**

[a]EC_{50} values have been previously reported.[19] [b]EC_{50} value has been previously reported.[43] [c]EC_{50} values have been previously reported. [d]EC_{50} value has been previously reported.[58] [e]Mean values from two independent determinations with five parallel wells.

Anticytomegaloviral Activity. Furthermore, hybrids **8**−**12** were analyzed for antiviral activity, focusing on human cytomegalovirus (recombinant HCMV AD169-GFP) used for the infection of cultured primary human foreskin fibroblasts (HFFs). Experimental determination of EC_{50} values was carried out in accordance to a previously established protocol,[55−58] and the results thereof are summarized in Table 1. Hybrids **9**−**12** exerted a high antiviral efficacy with EC_{50} values in the submicromolar range (0.22−0.38 μM) and thus possessed a profoundly stronger anti-HCMV activity (approximately factor 25) than the parent compound artesunic acid (**7**). These compounds were also more effective than ganciclovir used as the gold standard of current antiviral treatment. In contrast to the determined antimalarial activities, C-10 nonacetal-linked artemisinin-derived hybrids **9** and **12** were more potent in anti-HCMV activity than their C-10 acetal-linked counterparts (hybrids **8** and **11**). This difference was most pronounced between compounds **8** and **9**. In this case, hybrid **8** (EC_{50} = 2.44 μM) was approximately ten times less active than hybrid **9** (EC_{50} = 0.23 μM). Cell morphology, growth behavior, and signs of cytotoxicity were routinely monitored by microscopic inspection under compound treatment along the period of infection (7 days, referring to a situation of multiround viral replication), and no cytotoxicity was observed within the range of all concentrations tested.

Anticancer Activity. In a next step, hybrids **8**−**12** as well as their artemisinin and estrone precursors were investigated for their anticancer potential by means of MTT assay against a panel of human breast (MCF7, MDA-MB-231, MDA-MB-361, T47D) and cervical (HeLa, SiHa, C33A) cancer cell lines (Table 1). Estrone derivatives **13** and **14** exhibited antiproliferative action similar to that of reference agent cisplatin in terms of potency, while estrone azide **17** proved to be ineffective. Both artemisinin-derived compounds **6** and **7** elicited growth inhibitory effects comparable to cisplatin with exception for SiHa cell line, which was not sensitive toward them. All of the synthesized hybrids **8**−**12** exhibited substantially pronounced antiproliferative action on breast cancer cells. The most potent hybrid **12** displayed submicromolar EC_{50} values (0.18−0.93 μM) indicating an outstanding increase in the efficacy when compared with the actions of the building elements of the molecule. In the case of cervical cell lines, the actions of the precursors were modest, and the increase in the anticancer potency were less dynamic though compound **9** was remarkable on all utilized cells, and hybrid **12** exhibited promising action on C33A cell line.

Conclusion. In conclusion, several estradiol/estrone derivatives could be coupled to artemisinin for the first time, thereby forming five novel artemisinin-estrogen hybrids **8**−**12**. These were investigated for their *in vitro* biological activity against malaria parasites (*Plasmodium falciparum* 3D7), human cytomegalovirus (HCMV), and a selection of human breast and cervical cancer cell lines. All synthesized hybrids exhibited a strong antimalarial effect with EC_{50} values in the nanomolar range (3.8−128.8 nM). The most active hybrid in terms of antimalarial efficacy, compound **8**, was about two times more active than its parent compound artesunic acid (**7**) (EC_{50} = 8.9 nM) as well as the standard drug chloroquine (9.8 nM) and was therefore comparable to the clinically used dihydroartemisinin (**6**) (EC_{50} = 2.4 nM). Furthermore, hybrids **9**−**12** exhibited high antiviral activity (EC_{50} = 0.22−0.38 μM) and thus represent a group of very attractive, novel chemical structures exerting a pronounced anti-HCMV activity mostly in the submicromolar range, which appears even superior to the in vitro efficacy of reference drug ganciclovir. Besides the antimicrobial properties of the prepared agents, they exhibited a pronounced growth inhibitory action against a panel of human cancer cells. EC_{50} values of the hybrids were lower by orders of magnitude when compared with those of the building blocks. Based on the results of the presented antiproliferative assays, hybrid molecules designed and synthesized from artemisinin and estrone elements can be regarded as potential lead molecules for development of innovative anticancer agents. All in all, a relatively low level of effort in chemical synthesis was sufficient to generate very promising pharmacological candidate compounds, which once again highlights the attractiveness of the hybridization concept. We like to stress that this concept possesses a broad translational potential and might be useful for a number of future drug and biomedical developments.

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■ ASSOCIATED CONTENT

Supporting Information

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Experimental conditions and procedures as well as spectral data for precursors **13**, **15**, **16**, **18a**/**b**, **19**, and **20** and target compounds **8**−**12**; recorded spectra of target compounds; details of cell lines and reagents as well as cell viability assay for biological evaluation (PDF); SMILES data (XLSX)

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Notes

The authors declare no competing financial interest.

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■ DEDICATION

This paper is dedicated to Professor Youyou Tu.

■ ABBREVIATIONS

DCC, *N*,*N*′-dicyclo-hexylcarbodiimide; DCE, 1,2-dichloroethane; DHA, dihydroartemisinin; DMAP, 4-(dimethylamino)-pyridine; EDCI, *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide; EtOAc, ethyl acetate; equiv, equivalent; GFP, green fluorescent protein; HCMV, human cytomegalovirus; HFFs, human foreskin fibroblasts.

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