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## **Supporting Information**

## A Fully Synthetic 6-Aza-artemisinin Bearing an Amphiphilic Chain

## **Generates Aggregates and Exhibits Anti-Cancer Activities**

Hikari Koi,<sup>a</sup> Norihito Takahashi,<sup>a</sup> Yasufumi Fuchi,<sup>b</sup> Tomohiro Umeno<sup>b</sup>, Yukiko Muramatsu,<sup>c</sup>

Hiroyuki Seimiya,<sup>c</sup> Satoru Karasawa,<sup>b,\*</sup>and Hiroki Oguri<sup>d,\*</sup>

<sup>a</sup>Division of Applied Chemistry, Graduate School of Engineering, Tokyo University of Agriculture and Technology, 2-24-16 Nakacho, Koganei, Tokyo 184-8588, Japan.

<sup>b</sup>Faculty of Pharmaceutical Sciences, Showa Pharmaceutical University, 3-3165 Higashi-Tamagawagakuen, Machida, Tokyo 194-8543, Japan.

<sup>c</sup>Division of Molecular Biotherapy, Cancer Chemotherapy Center, Japanese Foundation for Cancer Research, Tokyo, 135-8550, Japan

<sup>d</sup>Department of Chemistry, School of Science, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan.

\*Corresponding authors, karasawa@ac.shoyaku.ac.jp, oguri@chem.s.u-tokyo.ac.jp

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#### **General Methods**

All reactions were performed under nitrogen atmosphere unless otherwise specified. NMR spectra were recorded on Bruker VSP 500, JEOL ECA 500 (<sup>1</sup>H/500 MHz, <sup>13</sup>C/125 MHz) spectrometers. Chemical shifts are reported in  $\delta$  (ppm) using chloroform as an internal standard of  $\delta$  7.26 and 77.16 for <sup>1</sup>H and <sup>13</sup>C-NMR, respectively. Data for <sup>1</sup>H NMR are reported as follows: chemical shift (number of hydrogens, multiplicity, coupling constant). Multiplicity is abbreviated as follows: s (singlet), d (doublet), t (triplet), q (quartet), quin (quintet), m (multiplet), br (broad). ESI-Mass spectra were recorded on JEOL AccuTOF LC-Plus JMS-T100 and Bruker micrOTOF control 3.0 systems. Dynamic light scattering (DLS) measurements were performed by a Malvern Zetasizer Nano ZS. The medium pressure liquid chromatography (MPLC) purifications were performed on YAMAZEN YFLC-AI-580 and Biotage Isolera. Where necessary, solvents were distilled from appropriate drying agents prior to use. Reactions were monitored by thin layer chromatography using Merck Millipore TLC Silica gel F<sub>254</sub> plates (0.25 mm) which were visualized using UV light, *p*-anisaldehyde stain, ninhydrin stain and PMS stain. Flash column chromatography was performed using Kanto Silica Gel 60N.

#### Materials

Commercial solvents and reagents were used as received with the following exceptions.

# Description for previously reported synthetic protocols for 6-aza-artemisinins and amphiphilic segments

Synthetic protocols and analytical data for compounds  $(4-5^1 \text{ and } 10-13^2)$  were provided in the supporting information of the previous papers.<sup>1,2</sup> All synthetic procedures and analytical data for new compounds (6-8) were described in this supporting information as follows.

<sup>1.</sup> K. R. Bonepally, T. Hiruma, H. Mizoguchi, K. Ochiai, S. Suzuki, H. Oikawa, A. Ishiyama, R. Hokari, M. Iwatsuki, K. Otoguro, S. Omura and H. Oguri, *Org. Lett.*, 2018, **20**, 4667–4671.

<sup>2.</sup> K. Morishita, Y. Okamoto, S. Murayama, K. Usui, E. Ohashi, G. Hirai, I. Aoki and S. Karasawa, *Langmuir*, 2017, **33**, 7810–7817.

#### Synthetic procedures for self-assembling 6-aza-artemisinins

Synthesis of single-tailed 6-aza-artemisinin (6)



To a mixture of **10** (18.4 mg, 0.0287 mmol) in EtOH (0.96 mL) was added Pd(PPh<sub>3</sub>)<sub>4</sub> (9.18 mg, 7.94  $\mu$ mol), 4-formyl phenylboronic acid (7.43 mg, 0.0496 mmol), and 2 M Na<sub>2</sub>CO<sub>3</sub> (0.718 mL) at room temperature. After being refluxed for 3h at 90°C, the mixture was cooled to room temperature and then treated with water (3.0 mL). After being stirred for 10 min, organic layer was separated, and aqueous layer was extracted with CH<sub>2</sub>Cl<sub>2</sub> (5.0 mL x 3). The combined organic layers were washed with brine, dried over Na<sub>2</sub>SO<sub>4</sub>. After filtration and concentration *in vacuo*, the residue was purified by silica-gel column chromatography (AcOEt / EtOH) to afford **8** (16.7 mg, 0.0270 mmol, 94%) as a colorless oil.

**8**: <sup>1</sup>H-NMR (500 MHz, CDCl<sub>3</sub>): δ 10.02 (1H, s), 7.90 (2H, d, *J* = 8.02 Hz), 7.71-7.67 (3H, m), 7.54 (4H, s), 5.59 (1H, s), 3.66-3.61 (18H, m), 3.58-3.51 (2H, m), 3.53-3.51 (2H, m), 3.48-3.45 (2H, m), 3.34 (3H, s), 3.26 (2H, q, *J* = 6.30 Hz), 1.59-1.53 (4H, m), 1.43-1.40 (4H, m), 1.28-1.23 (2H, m); <sup>13</sup>C-NMR (125 MHz,CDCl<sub>3</sub>): δ 191.99, 156.12, 146.79, 140.74, 134.59, 132.67, 130.33, 127.77, 126.87, 118.89, 71.83, 71.20, 70.62, 70.49, 69.97, 58.97, 39.83, 29.90, 29.32, 26.53, 25.76; HRMS (ESI, *m/z*): calcd. for C<sub>33</sub>H<sub>50</sub>N<sub>2</sub>O<sub>9</sub>Na, [M+Na]<sup>+</sup> 641.3408; found, 641.3438.

To a solution of amine **4** (5.11 mg, 0.0190 mmol) in 1,2-dichloroethane (0.200 mL) was added aldehyde **8** (47.0 mg, 0.0759 mmol), acetic acid (1.30  $\mu$ L, 0.0227 mmol) and Na<sub>2</sub>SO<sub>4</sub> (27.0 mg, 0. 190 mmol) at room temperature. After being stirred for 1 h at 35°C, the reaction mixture was treated with NaBH(OAc)<sub>3</sub> (13.0 mg, 0.0612 mmol). After being stirred for 18 h at the same temperature, the resulting mixture was diluted with CH<sub>2</sub>Cl<sub>2</sub> (3 mL) and quenched with saturated aqueous solution of NaHCO<sub>3</sub> (3 mL) at 0°C. After separation of organic layer, aqueous layer was extracted with CH<sub>2</sub>Cl<sub>2</sub> (10 mL x 3). The combined organic extracts were dried over Na<sub>2</sub>SO<sub>4</sub>. After filtration and concentration *in vacuo*, the residue was purified by HPLC (water / MeCN) to afford **6** (12.0 mg, 0.0138 mmol, 73%) as a colorless oil.

**6**: <sup>1</sup>H-NMR (500 MHz, CDCl<sub>3</sub>):  $\delta$  7.51-7.47 (6H, m), 7.39 (1H, s), 7.29 (2H, d, *J* = 8.02 Hz), 6.32 (1H, s), 5.43 (1H, s), 4.18 (1H, d, *J* = 13.2 Hz), 3.68-3.61 (18H, m), 3.59-3.57 (2H, m), 3.53-3.51 (2H, m), 3.48 (2H, t, *J* = 6.3 Hz), 3.39-3.35 (4H, m), 3.29-3.25 (2H, m), 3.12 (1H, d, *J* = 13.8 Hz), 2.91 (1H, dt, *J* = 11.5, 3.2 Hz), 2.52-2.46 (1H, m), 2.39 (1H, dd, *J* = 10.0, 7.1 Hz), 2.27-2.22 (1H, m), 2.15-2.08 (1H, m), 1.99-1.93 (1H, m), 1.91-1.82 (1H, m), 1.81-1.76 (1H, m), 1.72-1.69 (1H, m), 1.61-1.53 (4H, m), 1.48 (3H, s), 1.42-1.41 (4H, m), 1.30-1.25 (3H, m), 1.23-1.19 (3H, m); <sup>13</sup>C-NMR (125 MHz, CDCl<sub>3</sub>):  $\delta$  171.89, 156.17, 139.95, 139.56, 137.07, 134.36, 129.03, 127.47, 126.72, 119.29, 105.09, 94.03, 71.88, 71.18, 70.72, 70.54, 70.00, 65.18, 59.10, 56.61, 51.38, 44.14, 39.85, 34.55, 32.73, 29.81, 29.20, 26.33, 25.78, 25.15, 23.53, 12.45; HRMS (ESI, *m*/*z*): calcd. for C<sub>46</sub>H<sub>69</sub>N<sub>3</sub>O<sub>13</sub>Na, [M+Na]<sup>+</sup> 894.4722; found, 894.4714.

Synthesis of double-tailed 6-aza-artemisinin (7)



To a mixture of **12** (50.2 mg, 0.0466 mmol) in EtOH (1.6 mL) was added Pd(PPh<sub>3</sub>)<sub>4</sub> (14.2 mg, 0.0123 mmol), 4-formyl phenylboronic acid (10.2 mg, 0.0680 mmol) and 2 M Na<sub>2</sub>CO<sub>3</sub> aq. (1.17 mL) at room temperature. After being refluxed for 18h at 90°C, the mixture was cooled to room temperature and then treated with water (3 mL). After being stirred for 10 min, organic layer was separated, aqueous layer was extracted with AcOEt (20 mL x 3). The combined organic layers were washed with brine, dried over Na<sub>2</sub>SO<sub>4</sub>. After filtration and concentration *in vacuo*, the residue was purified by HPLC (water / MeCN) to afford crude **9** (47.2 mg including inseparable impurities) as a colorless oil. Formation of **9** was confirmed by HRMS (ESI, *m/z*): calcd. for C<sub>53</sub>H<sub>90</sub>N<sub>4</sub>O<sub>17</sub>Na, [M+Na]<sup>+</sup> 1077.6193; found, 1077.6165. The crude **9** was subjected to the next step without further purification.

To a solution of amine **4** (6.77 mg, 0.0251 mmol) in 1,2-dichloroethane (0.212 mL) was added the crude aldehyde **9** (49.6 mg, approx. 0.0470 mmol), acetic acid (2.00  $\mu$ L, 0.0349 mmol) and Na<sub>2</sub>SO<sub>4</sub> (45.2 mg, 0.0318 mmol). After being stirred for 30 min at 35°C, the mixture was treated with NaBH(OAc)<sub>3</sub> (13.1mg, 0.0618 mmol). After being stirred for 5 h at the same temperature, the reaction mixture was then treated with additional amounts of NaBH(OAc)<sub>3</sub> (14.2 mg, 0.0670 mmol). After

being stirred for 4 h at 35 °C, the resulting mixture was diluted with  $CH_2Cl_2$  (2 mL), quenched with saturated aqueous solution of NaHCO<sub>3</sub> (1 mL) at 0°C, and then treated with water (2 mL). After separation of organic layer, aqueous layer was extracted with  $CH_2Cl_2$  (20 mL x 3). The combined organic extracts were washed with brine, dried over Na<sub>2</sub>SO<sub>4</sub>. After filtration and concentration *in vacuo*, the residue was purified by HPLC (water/ MeCN) to afford 7 (14.3 mg, 0.0109 mmol, 44%) as a colorless oil.

7: <sup>1</sup>H-NMR (500 MHz, CDCl<sub>3</sub>):  $\delta$  7.65 (2H, s), 7.54-7.50 (4H, m), 7.21 (2H, d, *J* = 8.0 Hz), 6.99 (1H, s), 6.29 (1H, s), 5.70 (2H, s), 4.13 (1H, d, *J* = 13.2 Hz), 3.63-3.60 (39H, m), 3.56-3.51 (8H, m), 3.43-3.40 (4H, m), 3.37-3.33 (7H, m), 3.18 (4H, q, *J* = 6.3 Hz), 3.08 (1H, d, *J* = 13.2 Hz), 2.85 (1H, d, *J* = 11.5 Hz), 2.50-2.43 (1H, m), 2.35 (1H, dd, *J* = 10.0, 7.0 Hz), 2.24-2.04 (5H, m), 1.94-1.89 (1H, m), 1.86-1.74 (2H, m), 1.70-1.65 (1H, m), 1.55-1.52 (3H, m), 1.46-1.44 (6H, m), 1.32-1.31 (8H, m), 1.24-1.23 (1H, m), 1.22-1.17 (3H, m); <sup>13</sup>C-NMR (125 MHz, CDCl<sub>3</sub>):  $\delta$  171.92, 156.27, 142.15, 140.77, 140.21, 137.56, 128.85, 127.28, 111.80, 108.32, 105.07, 94.03, 77.36, 71.93, 71.31, 70.74, 70.58, 70.04, 65.16, 59.09, 56.66, 51.34, 44.10, 39.88, 34.52, 32.72, 29.99, 29.82, 29.37, 26.57, 25.83, 25.72, 25.14, 23.49, 12.45; HRMS (ESI, *m*/*z*): calcd. for C<sub>66</sub>H<sub>111</sub>N<sub>5</sub>O<sub>21</sub>, [M+2H]<sup>2+</sup> 654.8880; found, 654.8860.

#### Determinations of critical aggregation concentration (CAC) <sup>3,4</sup>

Various concentrations (1 - 200 M) of **6** and **7** were prepared using an aqueous solution with 5% DMSO or a pH 7.4 citrate-phosphate buffer with 5% DMSO. To the solutions, 1 M pyrene solutions were added and stirred by a vortex mixer for 10 s. Fluorescence measurements (ex. 330 nm) of the resulting solutions were performed using a JASCO FP-8500 spectrofluorimeter. The signal intensity ratio of I<sub>3</sub> related to I<sub>1</sub> for a pyrene were plotted as a function of sample concentration (logC). The given plots were fitted by a least square using a liner model and the crossing points between the lines were regarded as the CAC values. In an aqueous solution with 5% DMSO, the CAC values of **6** and **7** were 10.3 and 15.8  $\mu$ M, respectively. Measurements under a pseudo-physiological condition (pH 7.4 citrate-phosphate buffer with 5% DMSO) resulted in decrease of CAC values, **6** (5.3  $\mu$ M) and **7** (15.0  $\mu$ M).



**Figure S1.** Critical aggregation concentration (CAC) of amphiphilic 6-aza-artemisinins measured with pyrene as the fluorescent dye: (a) compound **6**, (b) compound **7**.

#### Dynamic light scattering (DLS)<sup>5</sup>

DLS measurements were performed with a Malvern Zetasizer Nano ZS instrument, with a heliumneon laser (633 nm, 3 mW) at a scattering angle of 173°. The 100  $\mu$ L solution sample in a micro cuvette (Aldrich; BRAND<sup>®</sup> UV cuvette micro center H 8.5 mm, volume 70-850  $\mu$ L) was used and measured three times per one temperature. The hydrodynamic diameter (*D*<sub>H</sub>) values were determined by the average of three scans. The temperature-dependence measurements were performed after maintaining the temperature for 120 s.



**Figure S2.** DLS profiles of 6-aza-artemisinins (**6** and **7**) under a pseudo-physiological condition (pH 7.4 citrate-phosphate buffer with 5% DMSO),  $D_{\rm H}$  values: **6** (1  $\mu$ m), **7** (7.5 and 51 nm).

- 3. E. Tanimoto, S. Karasawa, S. Ueki, N. Nitta, I. Aoki and N. Koga, *RSC Adv.*, 2013, **3**, 3531–3534.
- M. Anaya, M. Kwak, A. J. Musser, K. Müllen and A. Herrmann, *Chem.-Eur. J.*, 2010, 16, 12852– 12859.
- 5. T. Araki, S. Murayama, K. Usui, T. Shimada, I. Aoki and S. Karasawa, *Nano Lett.*, 2017, **17**, 2397–2403.

#### Lower critical solution temperature (LCST) behavior

The LCST behavior of the solution was tracked using a JASCO V570 spectrometer attached to the temperature controller JASCO 420. The LCST value was determined by the inflection point of the transmittance at 800 nm, as a function of temperature. The temperature of the sample in a quartz cuvette was set at 10°C, and increased by interval of one °C up to a final temperature. Each measurement was performed after maintaining the temperature for 120 s.



**Figure S3.** Plot of  $D_{\rm H}$  as a function of temperature of aqueous solutions (0.5 mM, containing 5% DMSO) of **6** and **7**.

#### Transmission electron microscopy (TEM)<sup>6</sup>

TEM images were obtained using a JEM-1400 Plus electron microscope. The sample (5  $\mu$ L) was mounted on a copper grid with an elastic carbon supporting membrane and, after 30 s, the residual solution was absorbed using a filtrate paper. A diluted EM stain (5  $\mu$ L) was then added to the grid, and, after 30 s, the residual solution was absorbed using filtrate papers and dried over 24 h. The 2.0 wt% EM stain was prepared using phosphomolybdic acid diluted with distilled water and filtered using a 0.45  $\mu$ M filter.

R. Shiraishi, S. Matsumoto, Y. Fuchi, T. Naganuma, D. Yoshihara, K. Usui, K. Yamada and S. Karasawa, *Langmuir*, 2020, 36, 5280–5286.

#### In vitro cell proliferation assay

MTS assay for HeLa cell:<sup>7,8</sup> HeLa cells were seeded in a 96-well plate at the density of 5,000 cells/well and incubated at 37°C overnight. Compounds (2, 5, 6, 7, 11, and 13) in DMSO were diluted with culture medium (100  $\mu$ L/well), consequently, 0–100  $\mu$ M (*n*= 3) in 1.0% DMSO solution were prepared in individual wells. The treated cells were incubated at 37°C for 48 h, following which the solutions were discarded and the cells were washed with PBS twice and the solutions were replaced with fresh DMEM containing FBS. These plates were further incubated with CellTiter 96 (Promega, 20  $\mu$ L/well) containing 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium salt (MTS), for 3 h at 37°C. The absorption of each well was measured at 490 nm using Varioskan Flash plate reader (Thermo Fischer Scientific). The ratio of absorbance of treated and untreated wells was used to estimate cell viability and the respective percentages were plotted as a function of compound concentrations.



Figure S4. *In vitro* activities of 6-aza-artemisinins (5–7), artesunate (2), and the amphiphiles (11 and 13) against HeLa cells.

8. M. Sakuma, Y. Fuchi, K. Usui, and S. Karasawa, Chem. Asian. J. 2019, 14, 3938-3945

Y. Fuchi, M. Sakuma, K. Oyama, M. Kono, K. Hamada, A. Mizutani, and S. Karasawa, *Sci. Rep.*, 2019, 9, 17723.

MTT assay for DMS114 and HCT116 cells: Human lung cancer cell line DMS114 and colorectal cancer cell line HCT116 were maintained in RPMI1640 medium (Nacalai Tesque, Kyoto, Japan) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 100 µg/mL kanamycin as described previously.<sup>9,10</sup> These cell lines were cultured in a humidified atmosphere of 5% CO<sub>2</sub> at 37°C.

To evaluate cell viability, the cells treated with compounds for 2 days were incubated with thiazol blue tetrazolium bromide (MTT) at a final concentration of 1 mg/mL at 37°C for 4 hours. Then the medium containing MTT was removed and dimethyl sulfoxide was added to the cells. Following that optical density at 570 nm and 630 nm for reference was measured using an xMark microplate spectrophotometer (Bio-RAD, Hercules, CA, USA).



**Figure S5.** Drug concentration–cell number curve using MTT, (a) cell proliferation assay with DMS114 and (b) HCT116 cell.

- T. Mashima, Y. Taneda, M.-K. Jang, A. Mizutani, Y. Muramatsu, H. Yoshida, A. Sato, N. Tanaka, Y. Sugimoto and H. Seimiya, *Oncotarget*, 2017, 8, 47902–47915.
- T. Mashima, T. Oh-hara, S. Sato, M. Mochizuki, Y. Sugimoto, K. Yamazaki, et al. J. Natl. Cancer Inst., 2005, 97, 765–777.



Figure S6. <sup>1</sup>H-NMR spectrum (500 MHz) of 8 in CDCl<sub>3</sub>



Figure S7. <sup>13</sup>C-NMR spectrum (125 MHz) of 8 in CDCl<sub>3</sub>



Figure S8. <sup>1</sup>H-NMR spectrum (500 MHz) of 6 CDCl<sub>3</sub>



Figure S9. <sup>13</sup>C-NMR spectrum (125 MHz) of 6 in CDCl<sub>3</sub>



Figure S10. <sup>1</sup>H-NMR spectrum (500 MHz) of 7 CDCl<sub>3</sub>



Figure S11. <sup>13</sup>C-NMR spectrum (125 MHz) of 7 in CDCl<sub>3</sub>