Bioorganic & Medicinal Chemistry Letters xxx (2016) xxx–xxx

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

Novel antibody drug conjugates containing exatecan derivative-based cytotoxic payloads

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ARTICLE INFO

Article history:
Received 9 November 2015
Revised 4 February 2016
Accepted 8 February 2016
Available online xxxx

Keywords:
Antibody drug conjugate
Camptothecin
Exatecan
Cytotoxic payload
Linker
Anti-HER2
Trastuzumab
Oncology
Cancer therapy

ABSTRACT

Trastuzumab conjugates consisting of exatecan derivatives were prepared and their biological activities and physicochemical properties were evaluated. The ADCs showed strong efficacy and a low aggregation rate. The exatecan derivatives were covalently connected via a peptidyl spacer (Gly-Gly-Phe-Gly), which is assumed to be stable in circulation, and were cleaved by lysosomal enzymes following ADC internalization into tumor tissue. These anti-HER2 ADCs exhibited a high potency, specifically against HER2-positive cancer cell lines in vitro. The ADCs, bearing exatecan derivatives which have more than two methylene chains, exhibited superior cytotoxicity. It was speculated that steric hindrance of the cleavable amide moiety could be involved in the drug release. The adequate alkyl lengths of exatecan derivatives (**13**, **14**, **15**) were from two to four in terms of aggregation rate. The ADC having a hydrophilic moiety showed good efficacy in a HER2-positive and Trastuzumab-resistant breast carcinoma cell model in mice.

Antibody drug conjugates (ADCs) have been established over the past several years and are now one of the most successful and important strategies for treating patients with hematological malignancies and solid tumors. At present, only three ADCs have been launched; the first ADC (Mylotarg®, gemtuzumab ozogamicin, Pfizer) launched in 2001 for the treatment of patients with acute myelogenous leukemia (AML). However, Mylotarg® was withdrawn in 2010 due to both a lack of evidence confirming its clinical benefits and safety concerns. Since then, two ADCs, Adcetris® (brentuximab vedotin, Seattle Genetics) and Kadcyla® (trastuzumab emtansine, Genetech and Roche), have been launched. And currently, over 30 programs in clinical development have been carried out in pursuit of ADC drug candidates.[1]

Camptothecin (CPT) has been demonstrated to be effective against a broad spectrum of tumors. CPT's target is human DNA topoisomerase I (Topo I). CPT binds to complexes with Topo I and DNA, and thereby is stabilized. This stabilized complex causes apoptosis. Irinotecan hydrochloride (CPT-11, yakult) is a prodrug of a potent CPT analog (SN-38) used for the treatment of patients with various tumors. Exatecan methansulfonate (DX-8951f) is a water soluble CPT which exhibits a stronger Topo I inhibitory activity and antitumor activity than the other CPT analogs. Furthermore, exatecan is effective against P-glycoprotein (P-gp) mediated multi-drug resistant cells.[2]

ADCs are composed of a carrier monoclonal antibody (mAb) and cytotoxic drug payload. The ADCs discussed herein are ADCs composed of a mAb with exatecan derivatives via a peptidyl linker, as shown in Figure 1. It has been previously recognized that glycyl glycyl phenylalanyl glycyl (GGFG) is selectively cleaved by lysosomal enzymes (presumably cathepsins).[3] GGFG is known to release drugs into tumor tissue without releasing them into peripheral circulation.

Human epidermal growth factor receptor 2 (HER2) is a very effective therapeutic target for breast cancer patients. Trastuzumab (Herceptin®, Genetech and Roche) is an anti-HER2 antibody and is used for breast cancer HER2-positive patients in combination with the chemotherapy drugs, anthracycline and taxane. Trastuzumab is the carrier mAb of trastuzumab emtansine. Therefore, the tumor specificity of this mAb, trastuzumab, is suitable for being the ADC's carrier.

The synthesis of ADC (**1**) was carried out via the route shown in Scheme 1. Commercially available *tert*-butoxycarbonyl glycyl glycyl phenylalanyl glycine (BOC-GGFG-OH) was dissolved in dichloromethane. *N*-Hydroxysuccinimide (HO-Su) and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (WSCI) was

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http://dx.doi.org/10.1016/j.bmcl.2016.02.020
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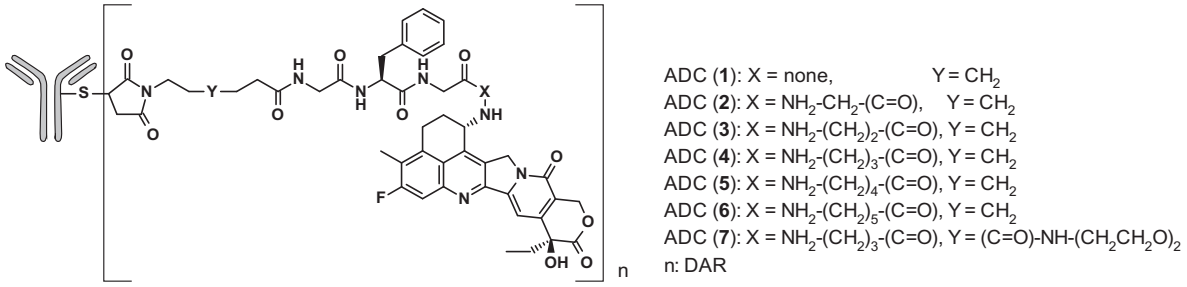
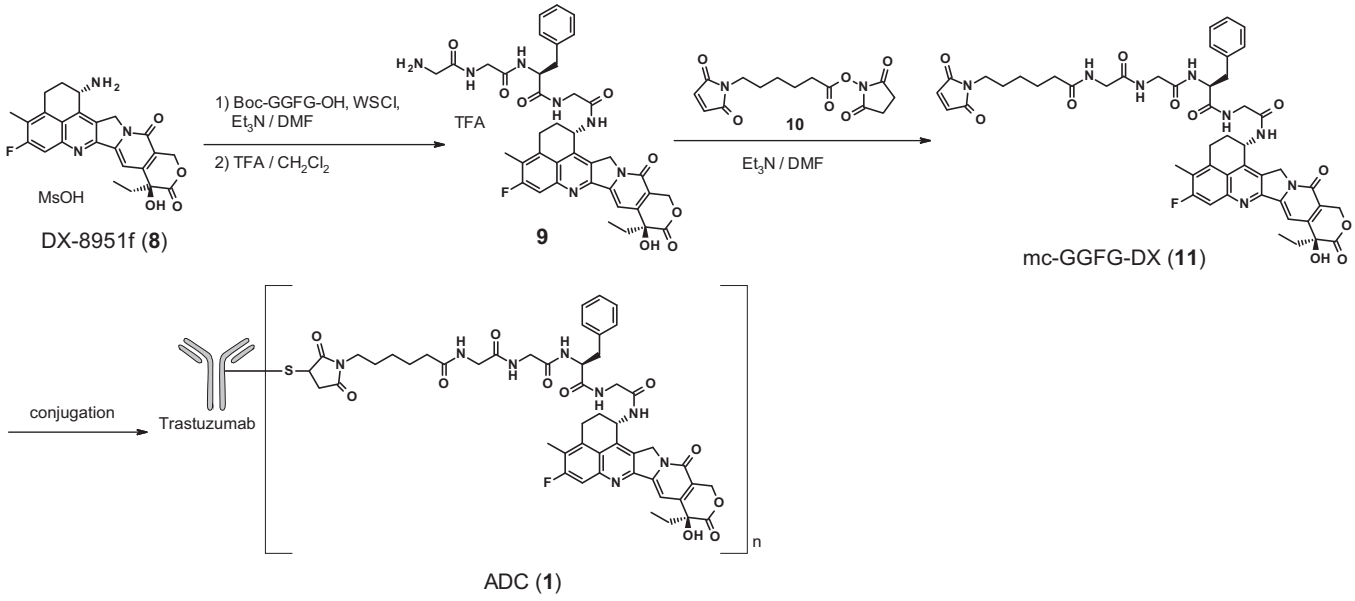


Figure 1. The structure of ADCs.



Scheme 1. The synthesis of ADC (**1**).

added. To this mixture, was added DX-8951f (**8**) and triethylamine solution in *N*,*N*′-dimethylformamide (DMF). The Boc-GGFG-exatecan was deprotected by trifluoroacetic acid (TFA), followed by condensation with 6-maleimidohexanoic acid *N*-hydroxysuccinimide ester (mc-OSu) in DMF to obtain the maleimide precursor drug linker (mc-GGFG-exatecan, **11**).[4]

The maleimide precursor drug linker (**11**) and mAb conjugation method used a conventional conjugated cysteine (Cys) strategy.[5] A disulfide group with hinges of mAb was reduced with tris(2-carboxyethyl)phosphine hydrochloride (TCEP HCl). And the precursor drug linker (**11**) was added to the reduced antibody. The drug to antibody ratio (DAR) was able to control an amount of the reducing agent, as shown in Table 1. The reaction mixture was purified using size exclusion chromatography (SEC). The DAR[5,6] and aggregation rate of the obtained product were measured.[7]

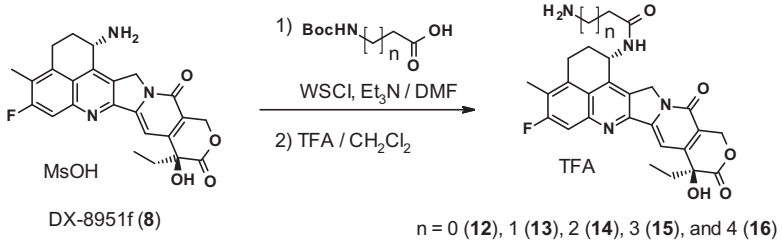
Exatecan derivatives were prepared by condensation and successive deprotection as shown in Scheme 2,[8] and these compounds were used as starting materials to obtain other ADCs (**2**)–(**7**) via similar synthetic routes of ADC (**1**).[4]

These prepared ADCs were shown to express in vitro cytotoxicities in a HER2-positive cell line, KPL-4, as shown in Table 1. GI_{50} was the concentration for 50% of maximal inhibition of cell proliferation. The KPL-4 cells used were a HER2-positive human breast cancer cell line. The KPL-4 was provided by Junichi Kurebayashi from Kawasaki Medical School in Japan. MCF7 cells used were a HER2-negative breast cancer cell line. The cell line was obtained from the European Collection of Cell Cultures (ECACC). None of the ADCs could kill the MCF7 cell line, GI_{50} > 100 nM. These results suggest that the anti-HER2 ADCs showed HER2 selective cytotoxicity. Though all of the ADCs (**1**)–(**7**) showed strong cytotoxicities in vitro, the ADCs (**1**) and (**2**) had a decreased cytotoxicity compared with that of other ADCs. It was speculated that degrading enzymes in tumor cells might not work efficiently because of the steric hindrance of the released drug moiety. The cleavable amide position (C-terminal amide of GGFG tetrapeptide) between the released drug and cleavable peptide linker was important for the affinity of the enzyme. Therefore, the cleavable amide position was changed away from the drug by adding some methylene chains between the drug and cleavable peptide. In particular, the drugs released from the ADCs (**3**)–(**6**) were changed to alkylamine derivatives with methylene alkyl chains from exatecan. These ADCs showed potent in vitro activity. However, the ADC (**6**) indicated more aggregates, possibly due to its hydrophobicity. The ADCs (**3**)–(**5**) had less aggregates than ADC (**6**). The alkyl length of exatecan derivatives influenced the aggregation rate. Having an alkyl length less than hexylene's is preferred from the viewpoint of an ADC's physicochemical properties. Therefore, an alkyl length from two to four seemed the best choice in this case. Moreover, a hydrophilic moiety could be added in the linker portion. ADC (**7a**) which has diethylene glycol showed less of an aggregation rate than ADC (**4**). Even a high drug load ADC (**7b**, DAR 6.2) had an acceptable low aggregation rate, and greater efficacy in vitro.

The in vivo efficacy of the ADC (**7b**) was compared with trastuzumab in mice bearing JIMT-1, a HER2-positive and

Table 1
Drug linker structure, conjugation condition, and in vitro efficacy of ADCs

ADC entry	Drug linker structure		Conjugation condition			Characterization		In vitro
	X	Y	TCEP (equiv)	Drug linker (equiv)	Yield (%)	DAR	Aggregation rate (%)	KPL-4 GI50 (nM)
1	None	—$(CH_2)_3$—	4.6	9.2	55	3.4	26	0.33
2	—NH—CH_2—(C=O)—	—$(CH_2)_3$—	2.3	4.6	83	3.2	3	0.39
3	—NH—$(CH_2)_2$—(C=O)—	—$(CH_2)_3$—	4.6	9.2	62	3.8	2	0.07
4	—NH—$(CH_2)_3$—(C=O)—	—$(CH_2)_3$—	4.6	9.2	75	2.6	3	0.05
5	—NH—$(CH_2)_4$—(C=O)—	—$(CH_2)_3$—	4.6	9.2	59	3.4	4	0.07
6	—NH—$(CH_2)_5$—(C=O)—	—$(CH_2)_3$—	4.6	9.2	61	2.5	20	0.11
7a	—NH—$(CH_2)_3$—(C=O)—	—C(=O)—NH—$(CH_2$—CH_2—$O)_2$—	2.3	4.6	85	3.6	1	0.12
7b	—NH—$(CH_2)_3$—(C=O)—	—C(=O)—NH—$(CH_2$—CH_2—$O)_2$—	4.6	9.2	86	6.2	1	0.04



Scheme 2. The synthesis of exatecan derivatives.

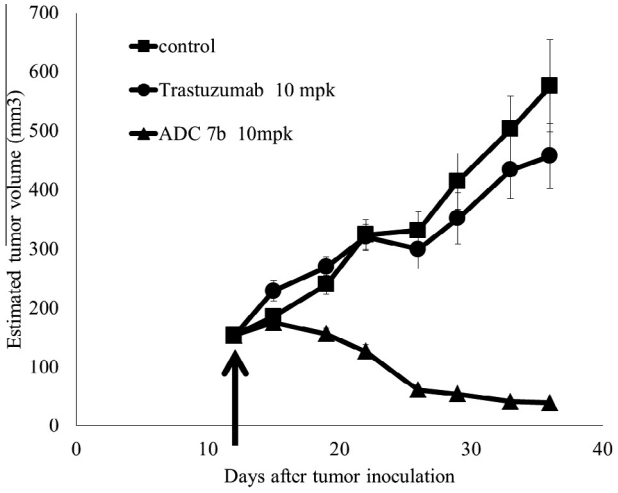


Figure 2. The in vitro efficacy of ADCs in JIMT-1 cells at an average starting tumor volume 150 mm^3 after 12 days.

trastuzumab-resistant breast carcinoma cell line. JIMT-1 was obtained from the Deutshe Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ). Mice were treated with a single i.v. injection of 10 mg/kg ADC (**7b**), or trastuzumab. Trastuzumab had no effect on tumor growth. In contrast, the mice treated with the ADC (**7b**) achieved tumor regressions, as shown in Figure 2.

These novel ADCs using exatecan derivatives having alkyl amine release the drug efficiently. Furthermore, adding a hydrophilic group to the C-terminus of the peptide moiety was effective in reducing aggregation rates. The ADC (**7b**), which has a maximum 8 DAR, showed an acceptable aggregation rate and high efficacy in vitro and in vivo. No change in mice body weight was observed by the ADC treatment indicating that the ADC was well tolerated.

Camptothecins are an especially compelling drug class for ADCs because they can be produced synthetically and modified to give analogs. However, aqueous insolubility of the camptothecins presents a significant challenge for ADCs. Design of camptothecin derivatives and implementation of a high polarity moiety is an ideal solution to overcome this. ADCs bearing exatecan derivatives of camptothecin analogs show a high potency for cancer therapy.

References and notes

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3. Inoue, K.; Kumazawa, E.; Kuga, H.; Susaki, H.; Masubuchi, N. *Adv. Exp. Med. Biol.* **2003**, *519*, 145.
4. Nuclear magnetic resonance (NMR) and liquid chromatography–mass (LC–MS) analysis. LC–MS ionization mode was electrospray ionization (ESI) or atmospheric pressure chemical ionization (APCI). For precursor drug linker (**11**) of ADC (**1**); ^{1}H NMR (400 MHz, DMSO-d_6) δ/ppm: 0.87 (3H, t, J = 7.4 Hz), 1.15–1.21 (2H, m), 1.41–1.50 (4H, m), 1.80–1.90 (2H, m), 2.07–2.12 (4H, m), 2.17–2.23 (1H, m), 2.35–2.40 (1H, m), 2.41 (3H, s), 2.73–2.81 (1H, m), 2.98 (1H, dd, J = 13.7, 4.6 Hz), 3.15–3.20 (2H, m),3.53 (1H, dd, J = 16.6, 5.7 Hz), 3.62–3.77 (5H, m), 4.39–4.45 (1H, m), 5.22 (1H, d, J = 18.9 Hz),5.27 (1H, d, J = 18.9 Hz),5.39 (1H, d, J = 16.0 Hz), 5.44 (1H, d, J = 16.0 Hz), 5.55–5.60 (1H, m), 6.53 (1H, s), 6.98 (2H, s), 7.13–7.24 (5H, m), 7.32 (1H, s), 7.81 (1H, d, J = 10.3 Hz), 7.95–8.00 (1H, m), 8.05–8.09 (2H, m), 8.28–8.31 (1H, m), 8.41 (1H, d, J = 8.6 Hz). MS (APCI) m/z: 947 (M+H)$^+$. For precursor drug linker of ADC (**2**); ^{1}H NMR (400 MHz, DMSO-d_6) δ/ppm: 0.86 (3H, t, J = 7.2 Hz), 1.18–1.19 (2H, m), 1.45–1.48 (4H, m), 1.83–1.85 (2H, m), 2.12–2.17 (4H, m), 2.39 (3H, s), 2.68 (1H, dd, J = 24.4, 14.7 Hz), 2.83–2.87 (1H, m), 3.17–3.78 (12H, m), 4.42–4.45 (1H, m), 5.23 (2H, s), 5.41 (2H, s), 5.58–5.60 (1H, m), 6.53 (1H, s), 6.99 (2H, s), 7.15–7.29 (6H, m), 7.76 (1H, d, J = 10.9 Hz), 7.97–8.00 (1H, m), 8.09–8.12 (3H, m), 8.25–8.28 (1H, m), 8.44 (1H, d, J = 8.2 Hz). MS (APCI) m/z: 1004 (M+H)$^+$. For precursor drug linker of ADC (**3**); ^{1}H NMR (400 MHz, DMSO-d_6) δ/ppm: 0.86 (3H, t, J = 7.2 Hz), 1.12–1.22 (2H, m), 1.39–1.51 (4H, m), 1.79–1.91 (2H, m), 2.02–2.20 (2H, m), 2.07 (2H, t, J = 7.4 Hz), 2.30–2.42 (4H, m), 2.40 (3H, s), 2.78 (1H, dd, J = 14.1, 9.4 Hz), 3.02 (1H, dd, J = 14.7, 4.9 Hz), 3.12–3.21 (2H, m), 3.26–3.42 (2H, m), 3.50–3.80 (6H, m), 4.40–4.51 (1H, m), 5.19 (1H, d, J = 19.6 Hz), 5.26 (1H, d, J = 19.2 Hz), 5.42 (2H, brs), 5.51–5.62 (1H, m), 6.53 (1H, s), 6.99 (2H, s), 7.13–7.28 (5H, m), 7.31 (1H, s), 7.74–7.84 (2H, m), 8.01 (1H, t, J = 5.3 Hz), 8.06 (1H, t, J = 5.7 Hz), 8.14 (1H, d, J = 8.2 Hz), 8.25 (1H, t, J = 5.7 Hz), 8.53 (1H, d, J = 8.6 Hz). MS (ESI) m/z: 1018 (M+H)$^+$. For precursor drug linker of ADC (**4**); ^{1}H NMR (400 MHz, DMSO-d_6) δ/ppm:0.87 (3H, t, J = 7.3 Hz), 1.12–1.22 (2H, m), 1.40–1.51 (4H, m), 1.66–1.76 (2H, m), 1.80–1.91 (2H, m), 2.05–2.21 (6H, m), 2.39 (3H, s), 2.79 (1H, dd, J = 14.0, 9.8 Hz), 2.98–3.21 (5H, m), 3.55–3.77 (8H, m), 4.41–4.48 (1H, m), 5.15 (1H, d, J = 18.9 Hz), 5.24 (1H, d, J = 18.9 Hz), 5.40 (1H, d, J = 17.1 Hz), 5.44 (1H, d, J = 17.1 Hz), 5.54–5.60 (1H, m), 6.53 (1H, s), 6.99 (2H, s), 7.20–7.27 (5H, m), 7.30 (1H, s), 7.70 (1H, t, J = 5.5 Hz), 7.80 (1H, d, J = 11.0 Hz), 8.03 (1H, t, J = 5.8 Hz), 8.08 (1H, t, J = 5.5 Hz), 8.14 (1H, d, J = 7.9 Hz), 8.25 (1H, t, J = 6.1 Hz), 8.46 (1H, d, J = 8.5 Hz). MS (APCI) m/z: 1032 (M+H)$^+$. For precursor drug linker of ADC (**5**); ^{1}H NMR (400 MHz, DMSO-d_6) δ/ppm: 0.87 (3H, t, J = 7.4 Hz), 1.13–1.21 (2H, m),

1.36–1.52 (6H, m), 1.53–1.65 (2H, m), 1.79–1.92 (2H, m), 2.05–2.15 (4H, m), 2.19 (2H, s), 2.40 (3H, s), 2.79 (1H, dd, J = 13.7, 10.2 Hz), 2.98–3.10 (3H, m), 3.12–3.21 (2H, m), 3.29–3.37 (2H, m), 3.53–3.79 (6H, m), 4.41–4.50 (1H, m), 5.16 (1H, d, J = 18.8 Hz), 5.23 (1H, d, J = 18.8 Hz), 5.43 (2H, s), 5.52–5.60 (1H, m), 6.53 (1H, s), 6.99 (2H, s), 7.12–7.28 (5H, m), 7.31 (1H, s), 7.63 (1H, t, J = 5.7 Hz), 7.80 (1H, d, J = 10.6 Hz), 8.02 (1H, t, J = 5.9 Hz), 8.08 (1H, t, J = 5.7 Hz), 8.12 (1H, d, J = 7.8 Hz), 8.24(1H, t, J = 5.7 Hz), 8.45 (1H, d, J = 8.6 Hz). MS (ESI) m/z: 1046 $(M+H)^+$. For precursor drug linker of ADC (**6**); ^{1}H NMR (400 MHz, CD_3OD) δ/ppm: 0.99 (3H, t, J = 7.4 Hz), 1.27 (2H, td, J = 11.6, 6.1 Hz), 1.38–1.44 (2H, m), 1.50–1.63 (6H, m), 1.65–1.80 (2H, m), 1.89–1.98 (2H, m), 2.17–2.25 (3H, m), 2.26–2.36 (3H, m), 2.40 (3H, s), 2.95 (1H, dd, J = 14.3, 9.2 Hz), 3.12 (1H, dd, J = 13.7, 5.7 Hz), 3.15–3.25 (4H, m), 3.44 (2H, t, J = 7.2 Hz), 3.65 (1H, d, J = 17.2 Hz), 3.76 (1H, d, J = 17.2 Hz), 3.79––3.86 (4H, m), 4.43 (1H, dd, J = 8.9, 6.0 Hz), 5.10 (1H, d, J = 18.9 Hz), 5.25 (1H, d, J = 18.9 Hz), 5.35 (1H, d, J = 16.6 Hz), 5.56 (1H, d, J = 16.0 Hz), 5.60–5.64 (1H, m), 6.76 (2H, s), 7.12–7.24 (6H, m), 7.58 (1H, s), 7.60 (1H, d, J = 10.9 Hz), 7.68 (1H, t, J = 5.7 Hz). MS (ESI) m/z: 1060 $(M+H)^+$. For precursor drug linker of ADC (**7**); ^{1}H NMR (400 MHz, DMSO-d_6) δ: 0.85 (3H, t, J = 7.4 Hz), 1.65–1.74 (2H, m), 1.77–1.90 (2H, m) 2.07–2.19 (4H, m), 2.30 (2H, t, J = 7.2 Hz), 2.33–2.36 (2H, m), 2.38 (3H, s), 2.76 (1H, dd, J = 13.7, 9.8 Hz), 2.96–3.18 (9H, m), 3.42–3.44 (4H, m), 3.53–3.76 (10H, m), 4.43 (1H, td, J = 8.6, 4.7 Hz), 5.14 (1H, d, J = 18.8 Hz), 5.23 (1H, d, J = 18.8 Hz), 5.38 (1H, d, J = 17.2 Hz), 5.42 (1H, d, J = 17.2 Hz), 5.52–5.58 (1H, m), 6.52 (1H, s), 6.98 (2H, s), 7.12–7.17 (1H, m), 7.18–7.25 (4H, m), 7.29 (1H, s), 7.69 (1H, t, J = 5.5 Hz), 7.78 (1H, d, J = 11.3 Hz), 7.98–8.03 (2H, m), 8.11 (1H, d, J = 7.8 Hz), 8.16 (1H, t, J = 5.7 Hz), 8.23 (1H, t, J = 5.9 Hz), 8.44(1H, d, J = 9.0 Hz). MS (APCI) m/z: 1149 $(M+H)^+$.

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6. DAR was calculated by UV absorption measurement of 280 nm and 370 nm.
7. Aggregation rate was measured by high performance liquid chromatography (HPLC). Column; Tosoh TSK gel G3000SWXL (300 × 7.8 mm). Mobile phase; 0.5 mL/min 0.03 M phosphate buffer (0.2 M arginine, pH 6.8). Sample; 10 μL (1 mg/mL) injection; ADC (**1**) in phosphate buffered saline (pH 7.5), ADC (**2**), (**7a**), and (**7b**) in acetate buffer red 5% sorbitol (pH 5.5), and ADC (**3**)–(**6**) in phosphate buffered saline (pH 6.0).
8. NMR and LC–MS analysis. Compound (**12**); ^{1}H NMR (400 MHz, CD_3OD) δ/ppm: 0.96 (3H, t, J = 7.0 Hz), 1.89–1.91 (2H, m), 2.14–2.16 (1H, m), 2.30 (3H, s), 2.40–2.42 (1H, m), 3.15–3.21 (2H, m), 3.79–3.86 (2H, m), 4.63–4.67 (1H, m), 5.00–5.05 (1H, m), 5.23 (1H, d, J = 16.0 Hz), 5.48 (1H, d, J = 16.0 Hz), 5.62–5.64 (1H, m), 7.40–7.45 (2H, m). MS (APCI) m/z: 493 $(M+H)^+$. Compound (**13**); ^{1}H NMR (400 MHz, DMSO-d_6) δ/ppm: 0.87 (3H, t, J = 7.2 Hz), 1.86 (2H, dquin, J = 14.6, 7.2 Hz), 2.06–2.27 (1H, m), 2.41 (3H, s), 2.46–2.57 (2H, m), 3.08 (2H, t, J = 6.8 Hz), 3.14–3.24 (2H, m), 5.22 (1H, d, J = 18.8 Hz), 5.29 (1H, d, J = 18.8 Hz), 5.43 (2H, s), 5.58 (1H, dt, J = 8.5, 4.5 Hz), 6.55 (1H, s), 7.32 (1H, s), 7.74 (3H, brs), 7.82 (1H, d, J = 11.0 Hz), 8.67 (1H, d, J = 8.6 Hz). MS (ESI) m/z: 507 $(M+H)^+$. Compound (**14**); ^{1}H NMR (400 MHz, DMSO-d_6) δ/ppm: 0.87 (3H, t, J = 7.2 Hz), 1.79–1.92 (4H, m), 2.10–2.17 (2H, m), 2.27 (2H, t, J = 7.0 Hz), 2.40 (3H, s), 2.80–2.86 (2H, m), 3.15–3.20 (2H, m), 5.15 (1H, d, J = 18.8 Hz), 5.26 (1H, d, J = 18.8 Hz), 5.42 (2H, s), 5.54–5.61 (1H, m), 6.55 (1H, s), 7.32 (1H, s), 7.72 (3H, brs), 7.82 (1H, d, J = 11.0 Hz), 8.54 (1H, d, J = 8.6 Hz). MS (APCI) m/z: 521 $(M+H)^+$. Compound (**15**); ^{1}H NMR (400 MHz, DMSO-d_6) δ/ppm: 0.88 (3H, t, J = 7.4 Hz), 1.52–1.71 (4H, m), 1.87 (2H, tt, J = 14.4, 6.9 Hz), 2.07–2.18 (2H, m), 2.22 (2H, t, J = 7.0 Hz), 2.40 (3H, s), 2.76–2.88 (2H, m), 3.13–3.22 (2H, m), 5.18 (1H, d, J = 18.8 Hz), 5.24 (1H, d, J = 18.8 Hz), 5.43 (2H, s), 5.53–5.61 (1H, m), 6.55 (1H, s),7.33 (1H, s), 7.65 (3H, brs), 7.81 (1H, d, J = 11.3 Hz), 8.49 (1H, d, J = 8.6 Hz). MS (ESI) m/z: 535 $(M+H)^+$. Compound (**16**); ^{1}H NMR (400 MHz, DMSO-d_6) δ/ppm: 0.88 (3H, t, J = 7.2 Hz), 1.31–1.41 (2H, m), 1.52–1.70 (4H, m), 1.80–1.94 (2H, m), 2.05–2.18 (2H, m), 2.21 (2H, t, J = 7.4 Hz), 2.40 (3H, s), 2.81 (2H, t, J = 7.4 Hz), 3.10–3.25 (2H, m), 3.33 (2H, brs), 5.18 (1H, d, J = 19.8 Hz), 5.22 (1H, d, J = 19.8 Hz), 5.41 (2H, d, J = 16.6 Hz), 5.45 (2H, d, J = 16.6 Hz), 5.53–5.60 (1H, m), 6.55 (1H, s), 7.32 (1H, s), 7.80 (1H, d, J = 10.9 Hz), 8.49 (1H, d, J = 9.2 Hz). MS (ESI) m/z: 549 $(M+H)^+$.