Development of a High-Affinity Antibody-Binding Peptide for Site-Specific Modification

Kyohei Muguruma,[a, b] Rento Osawa,[a] Akane Fukuda,[a] Naoto Ishikawa,[c] Konomi Fujita,[a] Akihiro Taguchi,[a] Kentaro Takayama,[a, d] Atsuhiko Taniguchi,[a] Yuji Ito,[c] and Yoshio Hayashi*[a]

Immunoglobulin G (IgG)-binding peptides such as 15-IgBP are convenient tools for the site-specific modification of antibodies and the preparation of homogeneous antibody–drug conjugates. A peptide such as 15-IgBP can be selectively crosslinked to the fragment crystallizable region of human IgG in an affinity-dependent manner via the ε-amino group of Lys8. Previously, we found that the peptide 15-Lys8Leu has a high affinity (K_d = 8.19 nM) due to the presence of the γ-dimethyl group in Leu8. The primary amino group required for the crosslinking to the antibodies has, however, been lost. Here, we report the design and synthesis of a novel unnatural amino acid, 4-(2-aminoethylcarbamoyl)leucine (Aecl), which possesses both the γ-dimethyl fragment and a primary amino group. A peptide containing Aecl8 (15-Lys8Aecl) was synthesized and showed a binding affinity ten times higher (K_d = 24.3 nM) than that of 15-IgBP (K_d = 267 nM). Fluorescein isothiocyanate (FITC)-labeled 15-Lys8Aecl with an *N*-hydroxy succinimide ester at the side chain of Aecl8 (FITC-15-Lys8Aecl(OSu)) successfully labeled an antibody (trastuzumab, Herceptin®) with the fluorophore. This peptide scaffold has both strong binding affinity and crosslinking capability, and could be a useful tool for the selective chemical modification of antibodies with molecules of interest such as drugs.

Introduction

Antibodies modified with a molecule of interest such as a fluorophore or a cytotoxic drug (payload) for example, monomethyl auristatin E or DM-1 are frequently utilized in research and drug discovery. Antibody-drug conjugates (ADC) are used clinically to treat cancer patients. An ADC can achieve the target-specific delivery of a cytotoxic drug. This results in the improvement of the therapeutic index by suppressing the adverse effect caused by the payload.[1,2] To date, nine ADCs have been approved by Food and Drug Administration (FDA) for cancer treatment.

In the general preparation of ADCs, including Kadcyla,[3] payloads are randomly conjugated by using their active esters to bind to lysine residues that are abundant on the surface of antibodies. Alternatively, inherent disulfides in the antibody are partially reduced, and resultant thiols are used for the selective conjugation with a payload. However, it is difficult to completely control the partial reduction.[2] Consequently, most of the approved ADCs are heterogeneous as a result of their random modification by payloads.[2] Recent studies have revealed that the heterogeneity of modification sites affects *in vivo* pharmacokinetic, pharmacological efficacy and safety profiles,[2,4] and homogeneity is required to maintain an adequate quality of the ADC. Therefore, development of a method for the site-specific chemical modification of antibody would be valuable for the preparation of homogeneous ADC.

Known site specific antibody modification methods can be broadly classified into two categories;[5] a method using the genetic engineering technique and a method using the site-specific antibody-binding peptide as a directing unit. In the former case, unnatural amino acids or sequences, such as *p*-acetylphenyl-alanine,[6] N^6-((2-azidoethoxy)carbonyl)lysine[7] or an enzyme recognition sequence,[8] is introduced genetically into the antibody, then the payload is crosslinked to the genetically introduced structure. In the second case, an antibody-binding peptide bearing the small molecule with a chemical reactivity or catalytic activity induces a crosslinking reaction near the peptide binding region. In this way, a molecule of interest can be introduced selectively into a certain site.[9] This site-specific crosslinking depends upon the affinity of the peptide, and therefore this strategy is classified as ligand-directed[11] or affinity-guided chemistry.[12] Modification by the

[a] Dr. K. Muguruma, R. Osawa, A. Fukuda, K. Fujita, Dr. A. Taguchi, Dr. K. Takayama, Dr. A. Taniguchi, Prof. Y. Hayashi
Department of Medicinal Chemistry
Tokyo University of Pharmacy and Life Sciences
Hachioji, Tokyo, 192-0392 (Japan)
E-mail: yhayashi@toyaku.ac.jp
[b] Dr. K. Muguruma
Present address:
Department of Chemical Science and Engineering
School of Materials and Chemical Technology
Tokyo Institute of Technology, Tokyo 152-8552 (Japan)
[c] N. Ishikawa, Prof. Y. Ito
Department of Chemistry and Bioscience
Graduate School of Science and Engineering
Kagoshima University
Korimoto, Kagoshima, 890-0065 (Japan)
[d] Dr. K. Takayama
Present address:
Department of Environmental Biochemistry
Kyoto Pharmaceutical University
Kyoto 607-8414 (Japan)

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second method is applicable to the native antibody, which is a big advantage over the first method.

Based on the ligand-directed chemistry in the second concept, Kishimoto et al., developed a method using Fc binding peptide, for an antibody modification with homogeneity (Figure 1a).[10] This is referred to as the chemical conjugation by affinity peptide (CCAP) method. In this method, IgG binding peptide with the payload attached to the N terminus is prepared, the side chain of Lys is reacted with the bivalent crosslinking agent disuccinimidylglutarate (DSG), and then the reactive succinimidyl ester of the DSG on the peptide forms an amide bond with Lys248 which is located near the Fc region of the antibody. As a result, covalent crosslinking between the payload and the antibody is selectively accomplished via the peptide. In this strategy, the binding affinity of IgG-binding peptide is important to achieve the efficient site-specific reaction.

With the aim of increasing the binding affinity of peptide **1**, we have studied its structure-activity relationships (SAR), and found several IgG-binding peptides with potent binding properties (Figure 1b).[13] For example, the shortened peptide, 15-IgBP, possesses a binding affinity (15 residues, $K_d = 267$ nM) comparable to that of peptide **1** (17 residues, Ac-DC*AYHKGELVWC*TFH-NH_2, *disulfide bridge, $K_d = 225$ nM). Substitution of Lys by Leu at position 8 improves the binding affinity by a factor of approximately 32 (15-Lys8Leu, $K_d = 8.12$ nM). This peptide shows one of the best antibody binding affinities among the Fc-binding peptides which have been reported.[14] However, 15-Lys8Leu cannot be applied to antibody modification using the CCAP method, because there is no ε-amino group of lysine at position 8 which is required for selective crosslinking to antibodies.

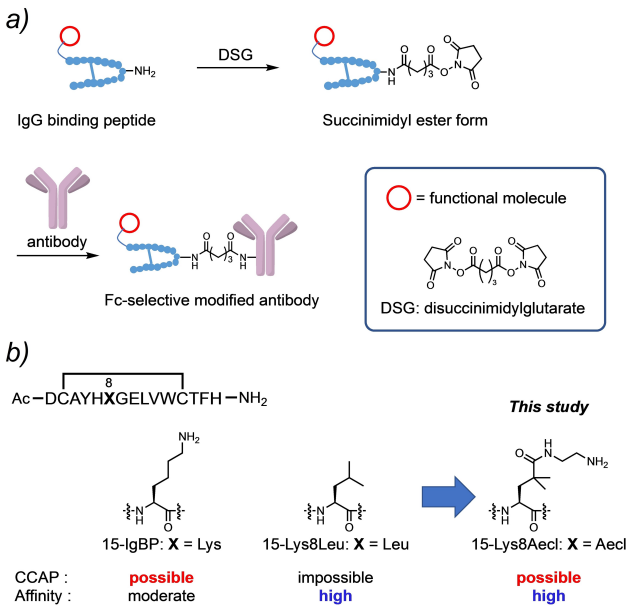


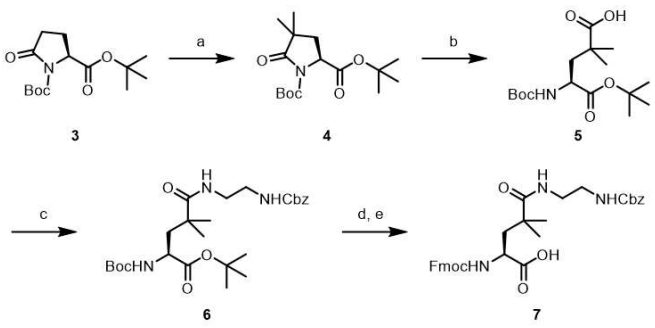
Figure 1. a) Chemical conjugation by affinity peptide (CCAP) method. b) Design concept of high-affinity IgG binding peptide (IgBP) applicable to CCAP method. IgG-binding peptides 15-IgBP,[13a] 15-Lys8Leu[13a] and 15-Lys8Aecl developed by our group.

The previous study also suggests that the high affinity of 15-Lys8Leu can be attributed to the γ-dimethyl structure of leucine, which interacts with the hydrophobic region of the antibody.[13a] In this study, we proposed new unnatural amino acid, 4-(2-aminoethylcarbamoyl)leucine (Aecl), containing both a primary amino group for the crosslinking and γ-dimethyl structure necessary for the high binding affinity (Figure 1b). The novel peptide derivative, 15-Lys8Aecl, was expected to show a high binding affinity due to the presence of the γ-dimethyl structure and to be applicable to site-specific crosslinking with an antibody via the amino group of Aecl by the CCAP method.

Results and Discussion

Fmoc-Aecl(Cbz)-OH, a building block for the Fmoc-based solid-phase peptide synthesis (SPPS), was synthesized as depicted in Scheme 1. The γ-position of *tert*-butyl *N*-Boc-pyroglutamate (**3**) was dimethylated with a moderate yield (40%) by lithium bis (trimethylsilyl)amide (LiHMDS) and methyl iodide. The γ-lactam ring was then opened by hydrolyzation to give the carboxylic acid (**5**), and a subsequent condensation reaction with benzyl (2-aminoethyl)carbamate afforded compound **6** in a yield of 59%. After deprotection of the Boc and *tert*-butyl groups under acidic conditions, the α-amino group was protected with Fmoc-Cl to give the target amino acid Fmoc-Aecl(Cbz)-OH (**7**), in a yield of 20% (5 steps).

An IgG binding peptide, 15-Lys8Aecl, containing an Aecl residue at the position 8 was synthesized by the general Fmoc-based SPPS method. Fmoc-Aecl(Cbz)-OH (**7**) was coupled to the peptide-resin using the HATU/HOAt/DIPEA method. For the final deprotection and cleavage, the resin-bound peptide was treated with a solution of trifluoroacetic acid (TFA)/*m*-cresol/thioanisole: 1,3-dimethoxybenzene (1,3-DMB) (40:1:2:1). As the scavenger in this step, 1,3-DMB was used to suppress the side reaction, *p*-hydroxybenzylation of the C-terminal amide,[13b] and soft-nucleophilic scavengers were used to enhance the deprotection of the Cbz group by the push–pull mechanism reported by Kiso et al.[15] Intramolecular disulfide bond formation in the crude peptide was performed by the Npys-OMe reagent that



Scheme 1. Synthesis of the unnatural amino acid, 4-(2-aminoethylcarbamoyl) leucine (Aecl). a) LiHMDS, MeI, THF, −78 °C, 40%; b) LiOH·H_2O, THF/H_2O = 9:1, quant.; c) benzyl (2-aminoethyl)carbamate, EDC·HCl, HOBt·H_2O, Et_3N, DMF, 59%; d) TFA, CH_2Cl_2; e) Fmoc-Cl, $NaHCO_3$, H_2O/THF = 1:1, 85% (2 steps).

was developed by our group.[16] After HPLC purification, the desired 15-Lys8Aecl was obtained in a total yield of 8% and with a purity of 97%. 15-Lys8Aecl derivatives were also obtained by the modified protocols for the synthesis of 15-Lys8Aecl (see the Experimental Section for more detail).

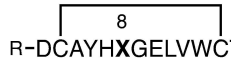
The binding kinetics (k_{on}, k_{off} and K_d) of the peptides were determined by a surface plasmon resonance (SPR) assay using a 1:1 Langmuir fit model (Table 1). As expected, 15-Lys8Aecl showed tenfold higher binding affinity (entry 2, $K_d = 24.3$ nM) than parental 15-IgBP ($K_d = 267$ nM), and both association and dissociation rates were modified ($k_{on} = 1.26\ s^{-1}\cdot\mu M^{-1}$, $k_{off} = 0.0306\ s^{-1}$). The introduced γ-dimethyl structure contributed to improvement of the binding affinity. The affinity was three times weaker than that of 15-Lys8Leu with no amino group (entry 1, $K_d = 8.19$ nM). This result is consistent with a previous result that the tertiary carbon structure at γ-position slightly decreases the affinity in both association and dissociation steps compared to the secondary carbon structure (15-Lys8Ala (*t*Bu)).[13a] 15-Lys8Aecl exhibited, however, the best affinity among the reported peptides with the side chain amino group at the position 8, and is applicable to the CCAP method.[13]

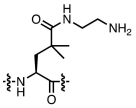
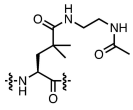
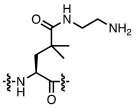
In the CCAP method, this amino group was acylated by DSG to attach the reactive moiety to the peptide. To evaluate the effect of the acyl linker on the binding affinity, 15-Lys8Aecl(Ac) with an acetylated amino group at position 8 was prepared as a mimic of DSG-form. Its binding affinity was found to be slightly decreased (entry 3, $K_d = 89.8$ nM) compared to unacetylated 15-Lys8Aecl (entry 2), mainly due to the slower association step ($k_{on} = 1.26$ vs. $0.478\ s^{-1}\cdot\mu M^{-1}$). The amino group at the position 8 might contribute partially to the high affinity of 15-Lys8Aecl by interacting with Glu380 of the antibody.[13a] However, 15-Lys8Aecl(Ac) still retained a higher binding affinity than 15-Lys8Lys(Ac) ($K_d = 268$ nM), indicating that 15-Lys8Aecl is a promising peptide for the CCAP method.

To estimate the binding of the Aecl-containing peptide with antibody, we performed CD spectral analysis and molecular modeling. The CD spectra of 15-IgBP, 15-Lys8Leu and 15-Lys8Aecl were similar, indicating that these peptides adopt the similar secondary structures (Figure 2a). The positive maximum peak around 230 nm is an important signal relating to the suitable conformation for the antibody binding.[13a] It suggests that the binding mode of 15-Lys8Aecl to the Fc region of antibody may be similar to that of 15-IgBP and other previously examined derivatives.[13] Molecular modeling of 15-Lys8Aecl was performed on the basis of the co-crystal structure of Fc region and Fc-III peptide including Leu residue at the position 8 (PDB ID: 1DN2[14e]), which has a similar sequence and antibody binding mode to peptide **1** (Figure 1b). The dimethyl group of Aecl in 15-Lys8Aecl is located in a similar place to the methyl group of Leu in Fc-III peptide that could interact with hydrophobic region of the antibody. This interaction could induce high binding affinity in 15-Lys8Aecl peptide, and might succeed in directing the primary amino group of Aecl8 to the target residue, Lys248 in the antibody. The distance of 0.63 nm between the both amino groups of Aecl8 and Lys248 could be appropriate for the crosslinking reaction, because the length of DSG linker is 0.77 nm.

To examine the antibody modification using 15-Lys8Aecl, an FITC-labeled peptide (FITC-15-Lys8Aecl) was prepared. The labeled peptide showed a similar binding affinity to that of unlabeled 15-Lys8Aecl (Table 1, entries 4 and 2, $K_d = 25.5$ vs 24.3 nM), suggesting that the Aecl-containing peptide can work as a good affinity-guide for the site-specific conjugation of the payload to the Fc region of the antibody. FITC-15-Lys8Aecl was reacted with DSG in the presence of *N*-methylmorpholine to obtain FITC-15-Lys8Aecl(OSu) bearing the reactive succinimidyl ester (Figure 3a). As extending the reaction time leads to hydrolysis of the unstable succinimidyl ester moiety under basic reaction conditions, excess amounts of reagents were used to complete the reaction before hydrolysis. In HPLC analysis, the peak of starting peptide FITC-15-Lys8Aecl disappeared and the desired product, FITC-15-Lys8Aecl(OSu), was produced after a

Table 1. Antibody binding affinity of peptide derivatives.



	Peptide	R	X	K_d [nM]	k_{on} [$s^{-1}\cdot\mu M^{-1}$]	k_{off} [s^{-1}]
1	15-Lys8Leu	Ac		8.19 ± 2.1[a]	1.47 ± 0.00[a]	0.0116 ± 0.0010[a]
2	15-Lys8Aecl	Ac		24.3 ± 0.4	1.26 ± 0.00	0.0306 ± 0.0005
3	15-Lys8Aecl(Ac)	Ac		89.8 ± 1.7	0.478 ± 0.002	0.0429 ± 0.0008
4	FITC-15-Lys8Aecl	FITC-Acp		25.5 ± 0.7	0.815 ± 0.003	0.0208 ± 0.0006

Binding affinity was measured by using trastuzumab with an immobilized amount of 2000 RU. [a] Values from ref. [13a].

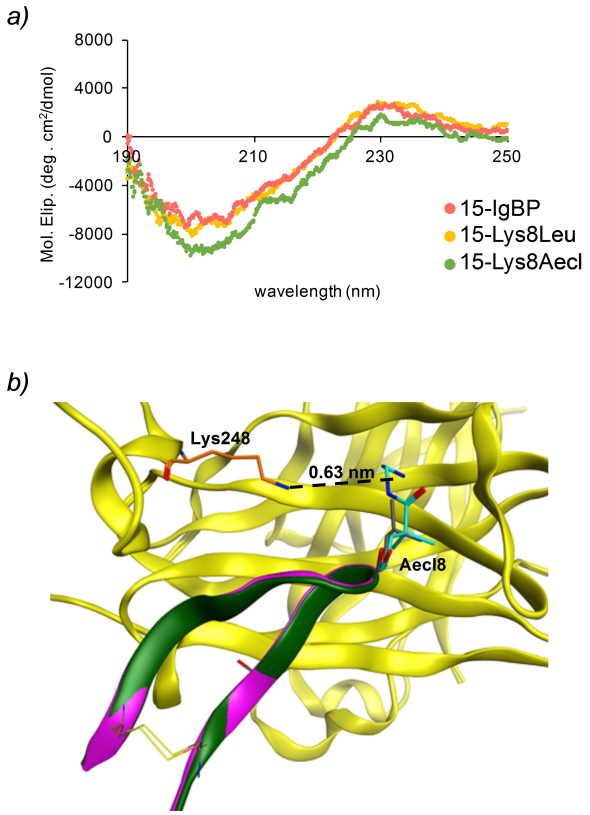


Figure 2. a) CD spectra of 15-IgBP, 15-Lys8Leu and 15-Lys8Aecl. b) Structural model of 15-Lys8Aecl binding with antibody on the basis of the X-ray co-crystal structure of human IgG1 and Fc-III peptide (PDB ID: 1DN2). 15-Lys8Aecl and antibody are shown as pink and yellow ribbons, respectively. The Fc-III peptide in the original PDB file (1DN2) is presented as a green ribbon. Aecl8 of 15-Lys8Aecl, Lys248 of antibody and Leu6 of Fc-III (corresponding to the position 8 in 15-Lys8Aecl) are shown as cyan, orange and gray, respectively.

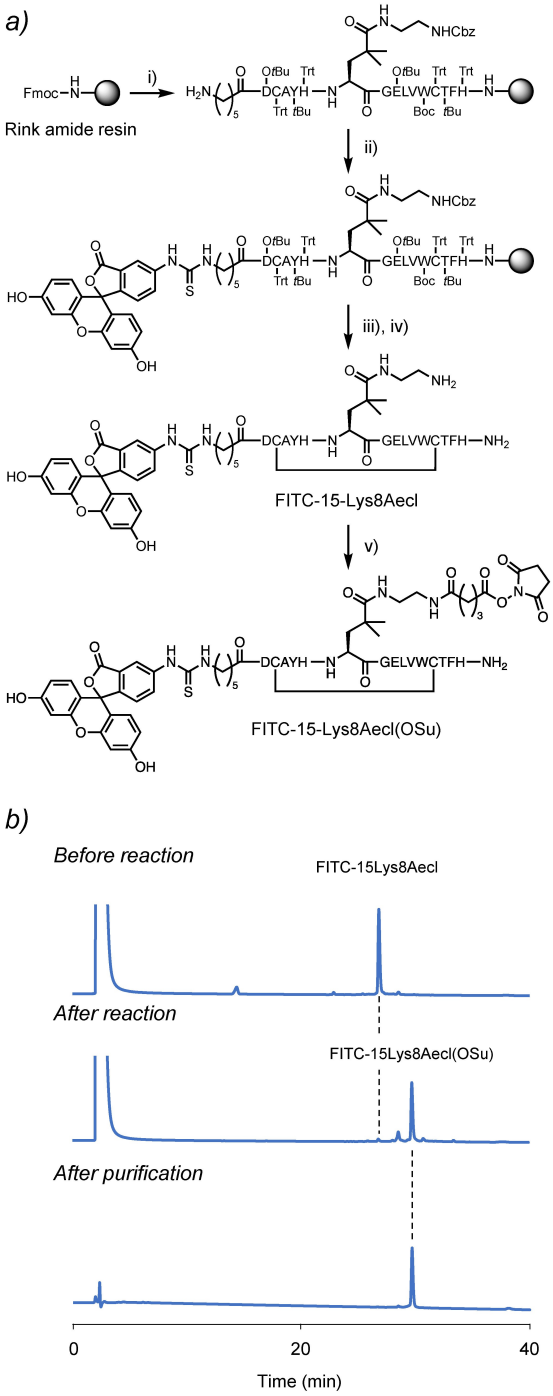


Figure 3. a) Synthesis of FITC-15-Lys8Aecl(OSu). i) Solid-phase peptide synthesis (coupling: Fmoc-amino acid, HATU, HOAt and DIPEA; deprotection: 20% piperidine/DMF), ii) FITC, DMF; iii) TFA/*m*-cresol/thioanisole/1,3-DMB (40:1:2:1); iv) Npys-OMe, 83% aq. CH_3CN, 9% from resin (HPLC purification); v) DSG, *N*-methylmorpholine, DMF, 14% (HPLC purification). b) HPLC chromatograms of the peptide solution before reaction, after reaction of 10 min and after purification. Analysis conditions; Gradient: 0.1% TFA aq./CH_3CN = 90:10 to 50:50 over 40 min, Flow rate: 0.9 mL/min, λ = 230 nm, Column: COSMOSIL 5C18-AR-II.

10 min reaction (Figure 3b). FITC-15-Lys8Aecl(OSu) was obtained after HPLC purification with a purity of 98%.

Finally, we examined the crosslinking reaction of FITC-15-Lys8Aecl(OSu) to trastuzumab (Herceptin®; Figure 4a), monitored by hydrophobic interaction chromatography (HIC), gel filtration chromatography (GFC) and MALDI-TOF MS analysis. In the HIC analysis, the peak for trastuzumab at a retention time of 8.0 min disappeared after the crosslinking reaction with 3 equivalents of FITC-15-Lys8Aecl(OSu) for 1 h (Figure 4b and c, blue lines). A new peak with a retention time of 22.4 min, detected by both UV absorption and fluorescence, was observed after the reaction (Figure 4c, blue and orange lines). These results indicate that the modification of trastuzumab with FITC succeeded completely. In addition, MALDI-TOF MS showed that molecular weight of antibody increased by 4.4 kDa after the reaction, indicating that two peptide molecules are attached to the antibody because the increase of molecular mass by conjugation of single peptide molecule would be 2477 Da (Figures 4d, e and S1). This is reasonable because the antibody possesses two binding sites for the peptide in the symmetric structure of the Fc region. In the GFC analysis, the fluorescence intensity was increased with an equivalent of the peptide reagent, and reached a plateau at 3 equivalents (Figure S2). This result indicates that no overreaction occurs even if a large excess amount of peptide reagent was added.

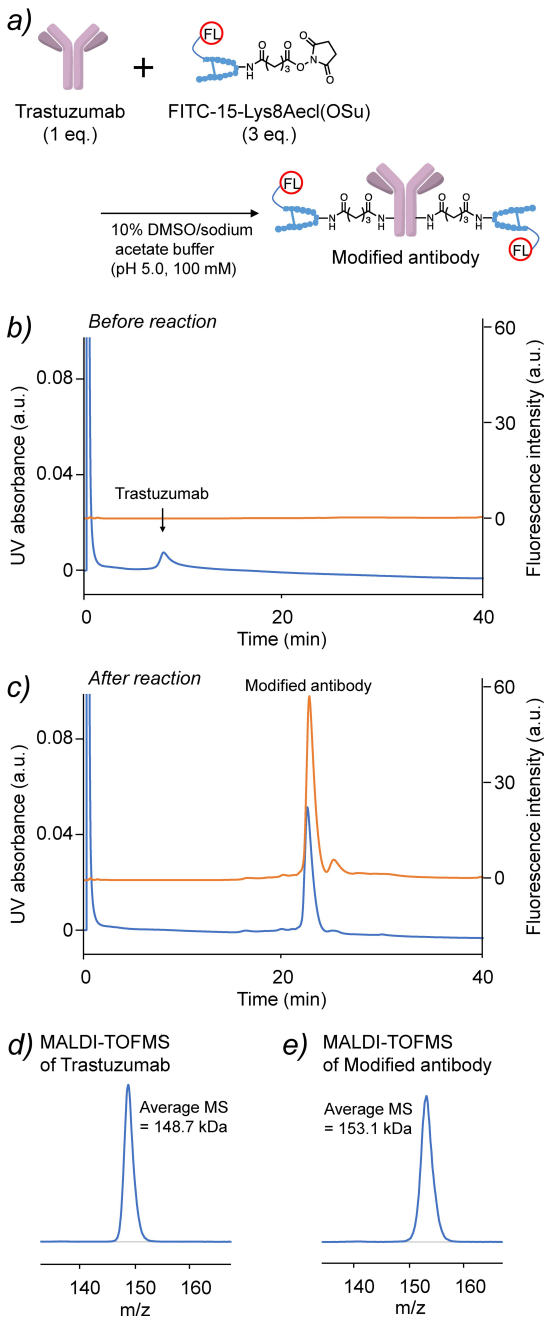


Figure 4. a) Antibody modification using FITC-labeled 15-Lys8Aecl peptide. HIC chromatograms of the reaction solution b) before and c) after adding the peptide reagent, detected by absorbance (230 nm, blue lines) and fluorescence (λ_{ex} = 490 nm, λ_{em} = 520 nm, orange lines). MALDI-TOF mass-spectra of the reaction solution d) before and e) after adding the peptide reagent.

Additionally, the peptide–antibody conjugate was purified by dialysis with a yield of 60% (calculated on the basis of protein amount, which is determined by bicinchoninic acid (BCA) assay; Figure S4). Taken together, the peptide reagent based on 15-Lys8Aecl sequence could rapidly provide the homogeneously modified antibody which would be applicable to preparing homogeneous ADCs.

Conclusion

We have designed and synthesized a novel IgG binding peptide containing an unnatural amino acid, Aecl, with both the γ-dimethyl and primary amino groups at the position 8. The building block for SPPS, Fmoc-Aecl(Cbz)-OH, was synthesized in five steps from the commercially available amino acid (**3**) with a total yield of 20%. 15-Lys8Aecl shows the best binding affinity (K_d = 24.3 nM) among the reported peptides[13] and can be applied to antibody modification based on the CCAP method. Moreover, the FITC-labelled peptide, FITC-15Lys8Aecl, retained a high binding affinity (K_d = 25.5 nM), and was successfully applied to the efficient FITC labelling of the antibody. Therefore, the peptide with Aecl can be a useful tool for the site-specific antibody modification on the basis of ligand-directed or affinity-guided chemistry.

Experimental Section

General procedures

Reagents and solvents were purchased from FujiFilm Wako Pure Chemical Industries (Osaka, Japan), Kanto Chemical Co., Inc. (Tokyo, Japan), Sigma–Aldrich (St. Louis, MO), Watanabe Chemical Industries (Hiroshima, Japan), Tokyo Chemical Industries (Tokyo, Japan) and Nacalai tesque (Kyoto, Japan). All were used as received. Trastuzumab was purchased from Chugai Pharmaceutical Co., Ltd. Column chromatography was performed on silica gel 60 N (spherical, neutral; 40–50 μm) and thin-layer chromatography (TLC) was performed on precoated plates (0.25 mm, silica gel Merk Kieselgel 60F245). ^{1}H NMR spectra were measured in $CDCl_3$ or CD_3OD solution and referenced to TMS (0.00 ppm) using a Bruker DPX-400 (400 MHz) NMR spectrometer. ^{13}C NMR spectra were measured in $CDCl_3$ or CD_3OD solution and referenced to a residual solvent peak of $CDCl_3$ (77.05 ppm) or CD_3OD (49.00 ppm) using a Bruker DPX-400 (100 MHz) NMR spectrometer. When peak multiplicities are reported, the following abbreviations are used: s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet. IR spectra were recorded on JASCO FT/IR 4100 spectrometer. Mass spectra were obtained on a Waters MICRO MASS LCT-premier (ESI) or Shimadzu Biotech AXIMA Assurance (MALDI). Melting points were measured with a Yanaco MP-500D melting point apparatus. Optical rotations were measured with a JASCO Polarimeter P-1030 at the sodium-D line (589 nm) at the concentrations (*c*, in g/dL). The measurements were carried out at 25 °C in a cell with path length of 1 dm.

Benzyl (2-aminoethyl)carbamate

Benzyl chloroformate (1.3 mL, 9 mmol) in CH_2Cl_2 (25 mL) was added dropwise at 0 °C to a solution of ethylene diamine (0.6 mL, 9 mmol) in CH_2Cl_2 (90 mL). After stirring for 1 h, the reaction solution was washed with brine, dried over Na_2SO_4, filtered and concentrated. The product was used in next step without further purification. White solid, yield 95% (1.57 g). Characterization data matched with previously reported data of this known compound;[17] m.p. 74.3–75.1 °C; ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.32-7.27 (m, 5H), 5.82 (s, 1H), 5.07 (s, 2H), 3.17 (q, J = 5.6 Hz, 2H), 2.73 (t, J = 5.6 Hz, 2H), 1.23 (s, 1H); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 156.7, 136.6, 128.5, 128.1, 66.7, 43.8, 41.7; IR (KBr) cm^{-1}: 3320, 1692, 1541, 1454, 1266; HRMS (ESI): *m*/z calcd. for $C_{10}H_{14}N_2O_2$ $[M+H]^+$ 195.1134, found 195.1128.

di-*tert*-Butyl (S)-4,4-dimethyl-5-oxopyrrolidine-1,2-dicarboxylate (4)

This reaction was performed in oven-dried glassware under an argon atmosphere. *tert*-Butyl *N*-(*tert*-butoxycarbonyl)-L-pyroglutamate **3** (5 g, 17.5 mmoL) in THF was added dropwise at −78 °C to a solution of lithium bis(trimethylsilyl)amide (LiHMDS) in THF (36.8 mL, 36.8 mmol). After stirring for 15 min at −78 °C, methyl iodide (2.3 mL, 36.8 mmol) in THF (25 mL) was added dropwise. Then, after stirring for 2 h at room temperature (RT), the reaction solution was neutralized with sat. NH_4Cl aq., and then extracted with EtOAc. The extract was washed with brine, dried over Na_2SO_4, filtered, and concentrated. The residual oil was purified by silica gel chromatography (hexane/EtOAc 5:1), resulting in a white solid (yield 40%, 2.2 g). Characterization data matched with those in a previous report of this known compound (**4**);[18] $[\alpha]_D^{25} = -22.30°$ (*c* 1.02, MeOH) (lit. $[\alpha]_D^{20} = -26.24°$ (*c* 1.0, $CHCl_3$); m.p. 104.6–105.8 °C (lit. 100–102 °C); ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 4.41$ (dd, $J = 4.3$ and 9.7 Hz, 1H), 2.19 (dd, $J = 9.8$ and 13.3, 1H), 1.89 (dd, $J = 4.3$ and 13.3 Hz, 1H), 1.52 (s, 9H), 1.48 (s, 9H), 1.21 (s, 3H), 1.21 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): $\delta = 178.3$, 170.7, 149.8, 83.2, 82.2, 58.5, 41.6, 36.7, 27.9, 25.8, 25.3; IR (KBr) cm^{-1}: 2980, 1784, 1731, 1308, 1164, 1255; HRMS (ESI): *m*/z calcd for $C_{16}H_{27}NO_5$ $[M+H]^+$ 336.1787, found 336.1777.

(*S*)-5-(*tert*-Butoxy)-4-((*tert*-butoxycarbonyl) amino)-2,2-dimethyl-5-oxopentanoic acid (5)

$LiOH{\cdot}H_2O$ (370 mg, 8.8 mmol) was added at 0 °C to a solution of compound **4** in THF/H_2O (9:1, 40 mL) After stirring at RT for 4 h, the reaction solution was acidified to pH 3 with 1 M HCl aq. and extracted with EtOAc. The extract was washed with brine, dried over Na_2SO_4, filtered, and concentrated, resulting in a white solid (2.7 g). This compound was used without further purification. m.p. 105.2–106.5 °C; $[\alpha]_D^{25} = -8.29°$ (*c* 0.40, MeOH); ^{1}H NMR (400 MHz, MeOD): $\delta = 4.11$–4.07 (m, 1H), 1.96–1.94 (m, 2H), 1.45 (s, 9H), 1.43 (s, 9H), 1.23 (s, 3H), 1.20 (s, 3H); ^{13}C NMR (100 MHz, MeOD): $\delta = 181.1$, 173.8, 157.7, 82.6, 80.5, 53.3, 42.0, 41.9, 28.7, 28.2, 26.8, 24.8; IR (KBr) cm-1: 3254, 2978, 1737, 1709, 1402, 1369, 1150; HRMS (ESI): *m*/z calcd for $C_{16}H_{29}NO_6$ $[M+H]^+$ 354.1893, found 354.1885.

***tert*-Butyl (*S*)-5-((2-(((benzyloxy)carbonyl)amino)ethyl) amino)-2-((*tert*-butoxycarbonyl) amino)-4,4-dimethyl-5-oxopentanoate (6)**

EDC·HCl (260 mg, 1.35 mmol), $HOBt{\cdot}H_2O$ (210 mg, 1.35 mmol) and benzyl (2-aminoethyl)carbamate (262 mg 1.35 mmol) in DMF were added at 0 °C to a solution in DMF of compound **5** (300 mg, 0.9 mmol). After stirring for 2 h at RT, the solvent was removed *in vacuo*, and the residue was extracted with EtOAc. The extract was washed with brine, filtered and concentrated. Then, the residue was purified by flash column chromatography (hexane/EtOAc 1:1) to obtain compound **6** as a clear oil, yield 60% (270 mg); $[\alpha]_D^{25} = -5.47°$ (*c* 0.35, MeOH); ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 7.36$–7.31 (m, 5H), 6.42 (s,1H), 5.56 (s, 1H), 5.09 (s, 2H), 5.04 (d, $J = 9.0$ Hz, 1H), 4.13–4.07 (m, 1H), 3.51–3.25 (m, 4H), 2.11–2.05 (m, 1H), 1.82-1.79 (m, 1H), 1.44 (s, 9H), 1.38 (s, 9H), 1.21 (s, 3H), 1.18 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): $\delta = 178.3$, 172.0, 157.2, 155.6, 136.4, 128.3, 128.0, 128.0, 81.5, 79.7, 66.6, 52.1, 41.4, 40.8, 40.7, 40.4, 28.2, 27.8, 23.3; IR (KBr) cm^{-1}: 3361, 2977, 1715, 1530, 1367, 1256, 1154; HRMS (ESI): *m*/z calcd for $C_{26}H_{41}N_3O$ $[M+H]^+$ 530.2842, found 530.2838.

(S)-2-((((9*H*-Fluoren-9-yl)methoxy)carbonyl) amino)-5-((2-(((benzyloxy)carbonyl)amino)ethyl) amino)-4,4-dimethyl-5-oxopentanoic acid (7)

TFA (4.8 mL) was added at 0 °C to a solution of compound **6** (220 mg, 0.43 mmol) in CH_2Cl_2 (2.4 mL). After stirring at RT for 1 h, the solvent was removed *in vacuo* to obtain the compound with deprotected amino and carboxyl groups. The compound was dissolved in THF/H_2O (1:1, 14.4 mL), and NaOH (180 mg, 2.15 mmol) and Fmoc-Cl (120 mg, 0.47 mmol) were added to the solution. After stirring at RT for 2 h, the reaction solution was acidified to pH 3 by adding 1 M HCl aq. at 0 °C. The mixture was extracted with EtOAc, washed with brine, dried over Na_2SO_4, filtered and concentrated. The residue was purified by flash column chromatography (hexane/EtOAc 5:1 to 4:1) to obtain compound **7** as a white solid, yield 85% (140 mg). $[\alpha]_D^{25} = -2.09°$ (*c* 0.25, CH_3CN); m.p. 69.5–70.4 °C; ^{1}H NMR (400 MHz, MeOD): $\delta = 7.75$ (d, $J = 7.5$ Hz, 2H), 7.63-7.57 (m, 2H), 7.35 (t, $J = 7.5$ Hz, 2H), 7.29–7.18 (m, 7H), 5.00 (s, 2H), 4.26 (d, $J = 7.0$ Hz, 2H), 4.13 (t, 6.8 Hz, 1H), 4.06 (d, $J = 8.2$ Hz, 1H), 3.28-3.13 (m, 4H), 2.17 (d, $J = 14.2$ Hz, 1H), 1.89–1.78 (m, 1H), 1.22–1.08 (m, 6H); ^{13}C NMR (100 MHz, MeOD): $\delta = 180.3$, 175.9, 159.2, 158.3, 145.3, 145.2, 142.6, 142.5, 138.3, 129.4, 129.0, 129.0, 128.9, 128.8, 128.2, 128.1, 126.4, 126.2, 120.9, 68.1, 67.5, 52.9, 42.6, 42.4, 41.5, 41.3, 41.2, 27.1, 25.0; IR (KBr) cm^{-1}: 3403, 1715, 1525, 1236, 739; HRMS (ESI): *m*/z calcd for $C_{32}H_{35}N_3O_7$ $[M+H]^+$574.2553, found 574.2555.

Solid-phase peptide synthesis (SPPS)

IgG binding peptides were synthesized by the Fmoc-based solid-phase peptide synthetic method[19] using an automatic peptide synthesizer (Prelude). Using the Fmoc-NH-SAL resin (40 μmol), the peptide chain was elongated with the Fmoc-amino acid (5 equiv), 1-[bis(dimethylamino)-methylene]-1*H*-1,2,3-triazolo[4,5-b] pyridinium 3-oxid hexafluorophosphate (HATU, 5 equiv), 1-hydroxy-7-azabenzotriazole (HOAt, 5 equiv) and *N*,*N*-diisopropylethylamine (DIPEA, 10 equiv) for 30 min. The following amino acid derivatives were used as the building blocks; Fmoc-Asp(O*t*Bu)-OH, Fmoc-Cys (Trt)-OH, Fmoc-Ala-OH, Fmoc-Tyr(*t*Bu)-OH, Fmoc-His(Trt)-OH, Fmoc-Gly-OH, Fmoc-Glu(O*t*Bu)-OH, Fmoc-Leu-OH, Fmoc-Val-OH, Fmoc-Trp (Boc)-OH, Fmoc-Thr(*t*Bu)-OH and Fmoc-Phe-OH. The Fmoc group was deprotected with 20% piperidine in DMF for 10 min twice. These reactions were repeated to lengthen the desired peptide. The N-terminal amino group was acetylated by acetic anhydride (3 equiv) and DIPEA (3 equiv) in DMF for 15 min. For FITC-labelled peptide, the N-terminal amino group of the peptide-resin was coupled with Fmoc-aminocaproic acid, the Fmoc group was deprotected, and then mixed with FITC (2.5 equiv) in DMF. Cleavage from resin and final deprotection were performed by treating with TFA/*m*-cresol/thioanisole[15]/1,3-DMB[13b] (40:1:2:1) for 7 h at RT. The crude peptide was precipitated with Et_2O and washed twice. After drying, the residual solid was dissolved in 83% aq. CH_3CN, and then methyl 3-nitro-2-pyridinesulfenate (Npys-OMe, 5 equiv)[16] was added to form the intramolecular disulfide bond. After stirring for 7–9 h at RT, the solution was lyophilized and washed with Et_2O to remove the excess reagent. After drying, the residual solid was purified by RP-HPLC [SunFire PrepC18 OBD 19 x 150 mm (5 μm)] to give the desired peptide. The purity of synthesized peptides was analyzed by RP-HPLC (Cosmosil 5 C18 AR-II, 4.6 i.d. ×150 mm) using a binary solvent system with a linear gradient starting from 10% CH_3CN in 0.1% aqueous TFA to 50% CH_3CN in 0.1% aqueous TFA at a flow rate of 0.9 mL/min, and detected at UV 230 nm. The yield and analytical data of peptides are shown in Supporting Information.

Acylation of peptide side chains

Acylation of side chains of 15-Lys8Aecl and FITC-15-Lys8Aecl was performed in solution with the previously described conditions.[13a] After disulfide bridging, the purified peptide was dissolved in DMSO at a concentration of 2.5 μM. Then, the peptide was treated with *N*-succinimidyl acetate (10 equiv) or DSG (25 equiv) in the presence of *N*-methylmorpholine (10–20 equiv) at RT. After 10–30 min, the desired peptide was directly purified by RP-HPLC [SunFire PrepC18 OBD 19×150 mm (5 μm)].

Circular dichroism (CD) spectra

The circular dichroism spectra of the peptides were measured on the basis of the previous conditions[20] using a Jasco J-1500CD spectrometer (Jasco, Japan) in a quartz cell with a 0.5 cm path length. Spectra were collected between 190–250 nm with a scan speed of 100 nm/min, a response time of 1 s, and a bandwidth of 1 nm at 25 °C. The peptides were dissolved in a 10 mM phosphate buffer (pH 7.4) including 10% 2,2,2-trifluoroethanol at a concentration of 2.5 μM.

Surface plasmon resonance (SPR) assay

The binding kinetics were determined by a Biacore T-200 system on the basis of the previous conditions.[20] Trastuzumab (human IgG1), human serum albumin or mouse IgG_1 was dissolved in 10 mM acetate buffer (pH 5.5) and immobilized by premixed *N*-hydroxysuccinimide and EDC·HCl (1-(3-dimethylaminopropyl)-3-ethyl-carbodiimide hydrochloride) onto a CM5 censor chip. The analytes (peptide derivatives) were adjusted to the desired concentration (ranging from 62.5 to 100 nM) by a serial dilution in a running buffer (HBS-EP; 0.01 M HEPES, 0.15 M NaCl, 3 mM EDTA, 0.005% tween 20, pH 7.4). The sensorgrams were obtained with an association time of 180 s, dissociation time of 600 s, and flow rate of 50 μL/min. To determine the binding kinetics (k_{on}, k_{off} and K_d), the obtained sensorgrams were analyzed by the Biacore T200 Evaluation software Ver.1.0, using 1:1 binding model.

Molecular modelling

The crystal structure of the Fc region of human IgG1 in complex with Fc-III peptide (PDB ID: 1DN2) was obtained from the Protein Data Bank. The 15-Lys8Aecl peptide was constructed on the basis of the secondary structure of the Fc-III peptide by using molecular operating environment (MOE) software. The plausible conformation of the side chain of the Aecl residue was obtained by scanning a separately created side chain library. Energy minimization process was performed using the Amber10:EHT force field.

Antibody modification reaction

Several equivalents of FITC-15-Lys8Aecl(OSu) in DMSO were added at RT to a solution of trastuzumab in 100 mM sodium acetate buffer (pH 5.0). The final concentration of trastuzumab was adjusted to 4 μM in 10% DMSO/buffer solution. After 1 h, the reaction was analyzed by hydrophobic interaction chromatography (HIC) or gel filtration chromatography (GFC).

Hydrophobic interaction chromatography (HIC)

For HIC, TSKgel Butyl-NPR column (4.6 mm i.d. ×3.5 cm) was used in the elution conditions with [25 mM phosphate buffer (pH 7.0) + 1.5 M $(NH_4)SO_4$]/[25 mM phosphate buffer (pH 7.0) + 5% IPA] = 20:80 to 100:0 over 40 min at a flow rate of 0.7 mL/min. The signals were detected by UV absorbance at 230 nm and fluorescence with excitation of 490 nm and emission of 520 nm.

Gel filtration chromatography (GFC)

For GFC, TSKgel G3000SWXL column (7.8 mm i.d. ×30 cm) was used in the elution conditions with 0.1 M sodium phosphate buffer (pH 7.0) at a flow rate of 0.7 mL/min. The signals were detected by UV absorbance at 230 nm and fluorescence with excitation of 490 nm and emission of 520 nm.

Mass spectrometry analysis

Samples were desalted with ZipTip Pipette Tips C4 (Millipore Co.) and analyzed by MALDI-TOF MS in linear mode using sinapinic acid as a matrix. The mass value is calibrated with mass of Trastuzumab as 148.0 kDa

Dialysis

The reaction mixture (100 μL) was diluted with 25 mM phosphate buffer (pH 6.0, 100 μL), and dialyzed with 25 mM phosphate buffer (pH 6.0) at RT for 1 day twice using a Pur-A-Lyzer Maxi 3500 Dialysis Kit (Sigma-Aldrich) according to the manufacturer's instruction. The protein concentration after dialysis (25 μL) was measured with Pierce BCA Protein Assay Kit (Thermo) according to the manufacturer's instruction.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords: IgG-binding peptide · bioconjugation · antibody-drug conjugate · structure activity relationship · unnatural amino acid

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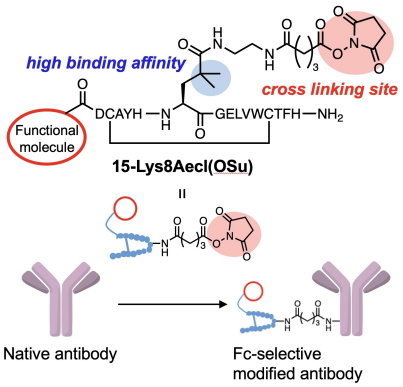
FULL PAPERS

Two in one: A novel unnatural amino acid 4-(2-aminoethylcarbamoyl) leucine (Aecl) was synthesized and used to develop a high-affinity IgG-binding peptide. Synthesized Aecl-containing peptides not only showed their strong binding affinity but also efficiently labeled trastuzumab. This suggests that 15-Lys8Aecl is a useful tool for the site-specific modification of native antibodies, leading to the preparation of homogeneous antibody–drug conjugates.



*Dr. K. Muguruma, R. Osawa, A. Fukuda, N. Ishikawa, K. Fujita, Dr. A. Taguchi, Dr. K. Takayama, Dr. A. Taniguchi, Prof. Y. Ito, Prof. Y. Hayashi**