Bioconjugate Chemistry

Article

pubs.acs.org/bc

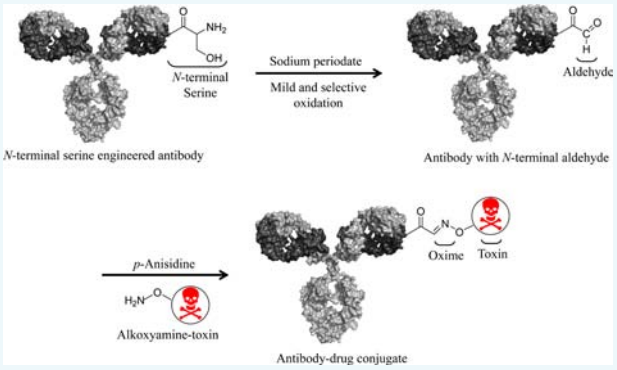
Hydrolytically Stable Site-Specific Conjugation at the *N*-Terminus of an Engineered Antibody

Pamela Thompson,[†] Binyam Bezabeh,[†] Ryan Fleming,[†] Monica Pruitt,[†] Shenlan Mao,[‡] Patrick Strout,[‡] Cui Chen,[‡] Song Cho,[‡] Haihong Zhong,[‡] Herren Wu,[†] Changshou Gao,*[,†] and Nazzareno Dimasi*[,†]

[†]Antibody Discovery and Protein Engineering, [‡]Oncology Research, MedImmune, Gaithersburg, Maryland 20878, United States

Supporting Information

ABSTRACT: Antibody–drug conjugates (ADCs) have emerged as an important class of therapeutics for cancer treatment that combine the target specificity of antibodies with the killing activity of anticancer chemotherapeutics. Early conjugation technologies relied upon random conjugation to either lysine or cysteine residues, resulting in heterogeneous ADCs. Recent technology advancements have resulted in the preparation of homogeneous ADCs through the site-specific conjugation at engineered cysteines, glycosylated amino acids, and bioorthogonal unnatural amino acids. Here we describe for the first time the conjugation of an anti-mitotic drug to an antibody following the mild and selective oxidation of a serine residue engineered at the *N*-terminus of the light chain. Using an alkoxyamine-derivatized monomethyl auristatine E payload, we have prepared a hydrolytically stable ADC that retains binding to its antigen and displays potent in vitro cytotoxicity and in vivo tumor growth inhibition.



INTRODUCTION

Antibody–drug conjugates (ADCs) represent a growing class of therapeutics that combine the target specificity of a monoclonal antibody with the killing ability of a cytotoxic drug conjugated to the antibody. To date, the primary application of ADCs has been in oncology, with two approved drugs, Kadcyla and Adcetris. Kadcyla is composed of the anti-Her2 antibody conjugated with mertansine at lysine residues, while Adcetris is an anti-CD30 antibody conjugated with monomethyl auristatine E (MMAE) at cysteine residues.[1,2] Several other ADCs armed with a variety of chemotherapeutics with distinct mechanisms of action are currently in clinical development for hematological cancers and solid tumors. The two approved ADCs, along with a majority of the ADCs in clinical development, use bioconjugation methods that produce heterogeneous mixtures of conjugates, which complicate analytical characterization. In addition to product heterogeneity, problems with ADC stability in serum have been identified for ADCs prepared by the reaction of native cysteine thiols with maleimides.[3] The realization of these unfavorable characteristics of ADCs prepared using traditional biorthogonal methods has contributed to the design and development of a new class of ADCs which use site-specific conjugation, wherein the site of conjugation and the drug load can be precisely controlled.[4−6]

With the exception of glycoconjugation,[7−10] in which protein engineering is not required, site-specific preparation of ADCs requires the use of antibody modifications to introduce the preferred conjugation site. The most advanced and validated method for site-specific conjugation has been to take advantage of the unique reactivity of cysteines engineered at exposed surfaces of the antibody.[3,11,12] The cysteine-based conjugation approach allows for a fast and specific conjugation with maleimide-functionalized drugs, minimizes structural perturbation, and results in the preparation of homogeneous ADCs with drug-to-antibody ratios (DAR) of 2. Moreover, stabilization of the conjugate can be achieved using a linker which favors hydrolysis of the succinimide ring.[13,14] One disadvantage of this method is that efficient conjugation at engineered cysteines requires reduction of the antibody and subsequent reoxidation to reform the interchain disulfides. This process can potentially generate structural heterogeneity, leaving free thiols at native cysteines in the antibody which are available for conjugation to the drug, and may represent a development liability.

Another site-specific conjugation modality relies on genetically encoding unnatural amino acids.[15,16] An antibody engineered with an amber stop codon can be expressed in mammalian cells that have been engineered with an orthogonal tRNA/aminoacyl-tRNA synthetase pair, which will install the unnatural amino acid. This technique offers minimal structural perturbation of the antibody, no off-site conjugation, and the

Received: June 26, 2015
Revised: September 3, 2015
Published: September 4, 2015

DOI: 10.1021/acs.bioconjchem.5b00355
Bioconjugate Chem. 2015, 26, 2085−2096

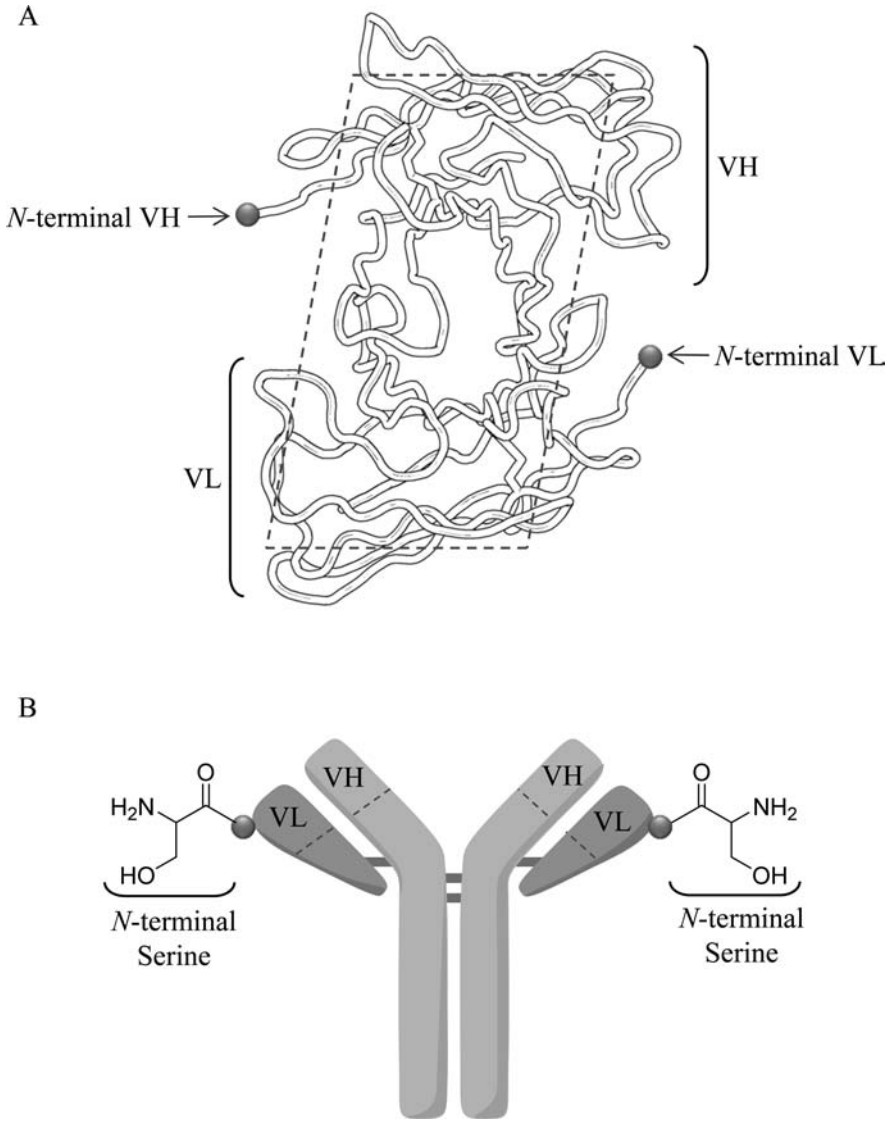


Figure 1. (A) Top-view of a representative Fv domain of a human antibody (PDB: 1F8T; CH_1 and VL structural information not shown). The VL and VH domains and their respective *N*-termini are schematically labeled. The antigen binding domain is shown in the dotted line box. (B) Cartoon representation of an antibody with the serine residue engineered at the *N*-terminus of the light chain.

ability to use a variety of biorthogonal chemistries for conjugation.[17]

From the examples described above, it is evident that site-specific chemistry will help produce ADCs with optimal properties, such as defined drug load and improved in vivo properties. However, site-specific ADCs are still in their nascent stage of development and further improvements such as additional site-specific conjugation modalities may contribute to the development of next wave of ADCs.

Inspired by the work of Georghegan and Stroh[18] in which site-directed conjugation of a peptide to a protein was obtained via periodate oxidation of the 2-amino alcohol of a *N*-terminal serine or threonine residue, we envisaged the use of an anti-EphA2 antibody containing an engineered *N*-terminal serine residue for the site-specific attachment of an anticancer drug. The two-step procedure described herein for the preparation of the oxime-linked ADCs is mild and robust. We demonstrate that the chemical modification of engineered serine residue positioned outside the complementarity determining region (CDR) of the antibody does not disrupt antigen binding. Furthermore, we show that these oxime-based ADCs are homogeneous, stable in serum, and have potent in vitro cytotoxicity and in vivo tumor growth inhibition.

RESULTS

Engineering the *N*-Terminal Serine in the Antibody. The site-specific conjugation strategy described herein involves the modification of a 2-amino alcohol engineered at the *N*-terminus of an antibody. To this end, we engineered a serine residue at the *N*-terminus of the light chain domain of an anti-EphA2 antibody (1C1) and a negative control antibody (R347). Figure 1 shows a schematic representation of an antibody showing the structure of the engineered serine at the *N*-terminus of the light chain. The serine engineered antibodies, 1C1-Ser and R347-Ser, were transiently expressed in 293-F cells, with expression levels of 150 mg/L, which are similar to the parental 1C1 and R347 antibodies.

***N*-Terminal Site-Specific Conjugation.** The conjugation process described herein consists of two steps (Figure 2). First, the 2-amino alcohol of the terminal serine residue was oxidized

DOI: 10.1021/acs.bioconjchem.5b00355
Bioconjugate Chem. 2015, 26, 2085−2096

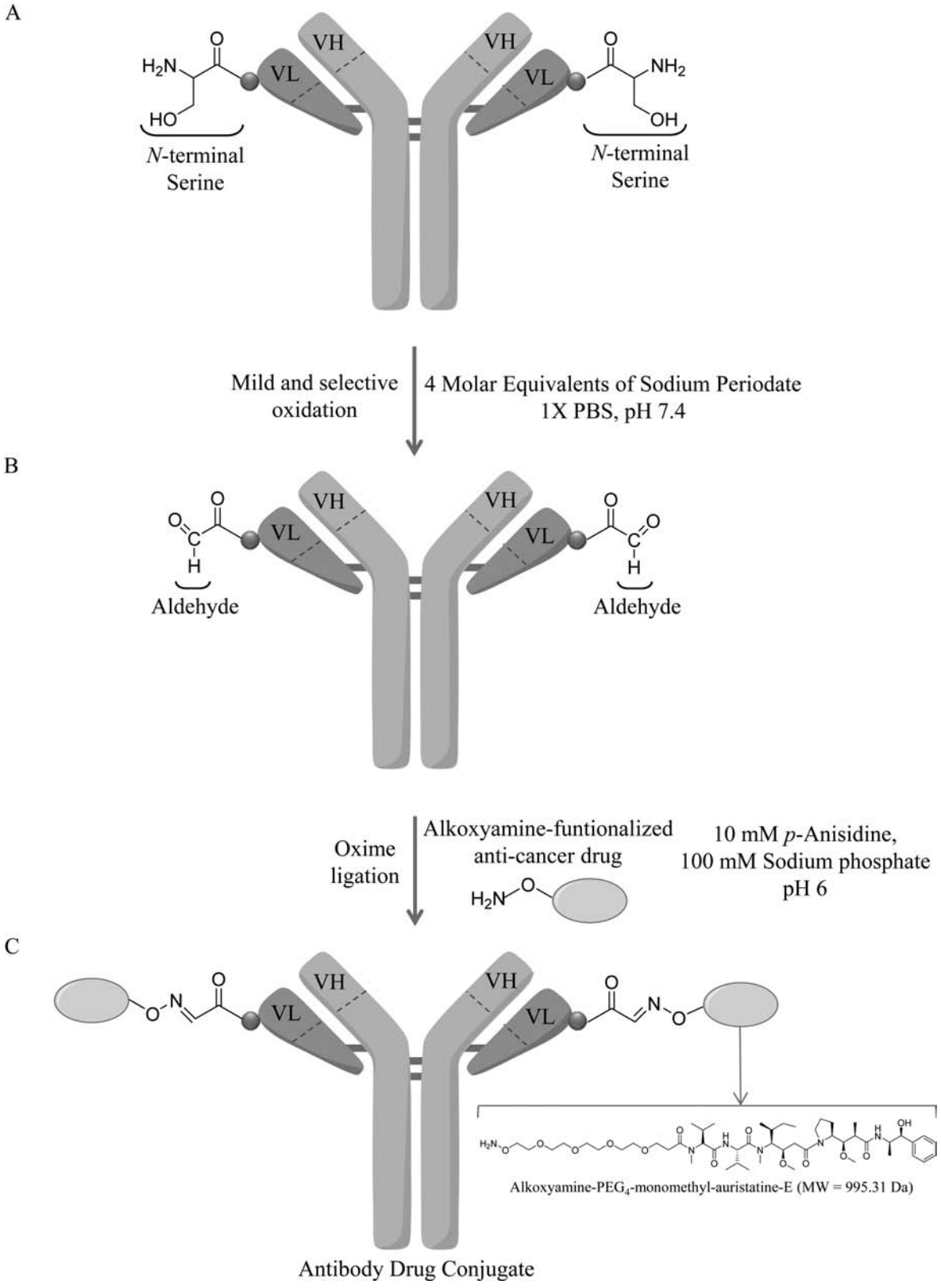


Figure 2. Site-specific oxime-based conjugation process. An antibody with an engineered *N*-terminal serine residue on the light chain (A) is selectively oxidized using $NaIO_4$ to form an aldehyde. The resulting antibody (B), with two glyoxylates, is reacted (C) with alkoxyamine-functionalized monomethyl auristatine E payload in the presence of *p*-anisidine to form a site-specific antibody−drug conjugate with a DAR of 2.

to an aldehyde using 4 mol equiv of $NaIO_4$ in quantitative yields. Second, quantitative formation of the glyoxylic oxime proceeded overnight by the addition of the alkoxyamine-bearing monomethyl auristatine E payload at pH 6 in the presence of *p*-anisidine[19,20] as a catalyst, as detected by reduced

DOI: 10.1021/acs.bioconjchem.5b00355
Bioconjugate Chem. 2015, 26, 2085−2096

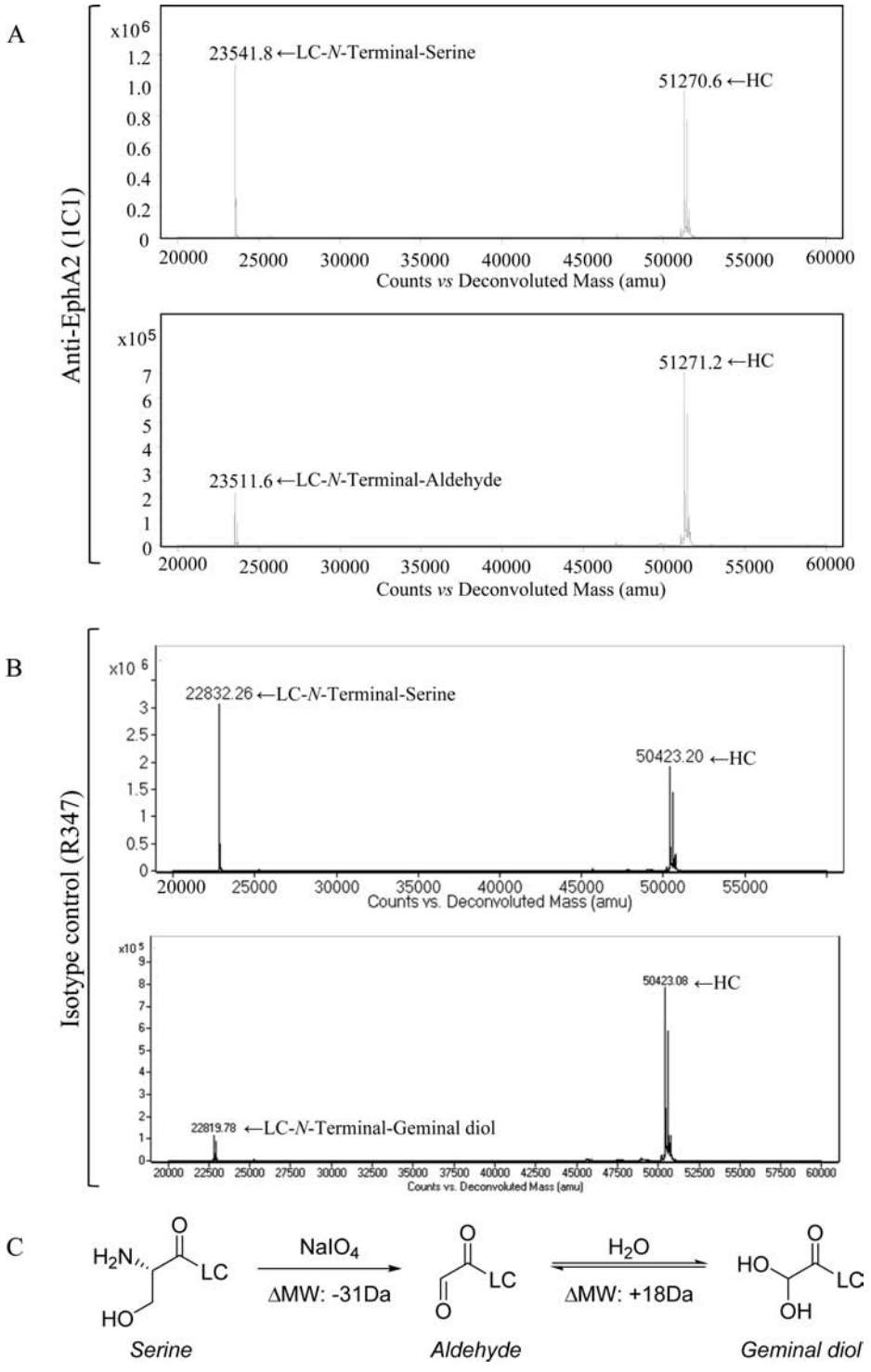


Figure 3. Quantitative oxidation of (A) 1C1 and (B) R347 antibodies by $NaIO_4$ was determined by rLCMS. (C) Equilibrium of an aldehyde and geminal diol under aqueous conditions. Expected masses (Da): 1C1-Ser, 23541; 1C1-Aldehyde, 23511; 1C1-geminal diol, 23539; R347-Ser, 22832; R347-Aldehyde, 22801; R347-geminal diol, 22819.

reverse phase liquid chromatography mass spectrometry (rLCMS).

Analytical characterization of the ADCs. Quantitative oxidation of the *N*-terminal serine residue in both 1C1-Ser and R347-Ser was confirmed using rLCMS (Figure 3A,B). The periodate oxidation of an *N*-terminal serine residue results in the loss of 31 Da; under aqueous conditions, the resulting aldehyde is present in equilibrium as a geminal diol (Figure 3C). Ions corresponding to both the aldehyde and the geminal diol were identified in the reduced mass spectra for oxidized 1C1-Ser and R347-Ser. Oxidation and subsequent conjugation of the *N*-terminus did not generate in process aggregate, as shown by analytical size-exclusion chromatography (SEC-HPLC, Figure 4A). The oxime formation was highly efficient, which was demonstrated by analytical hydrophobic interaction chromatography (HIC-HPLC, Figure 4B), with 95% of ADC

DOI: 10.1021/acs.bioconjchem.5b00355
Bioconjugate Chem. 2015, 26, 2085−2096

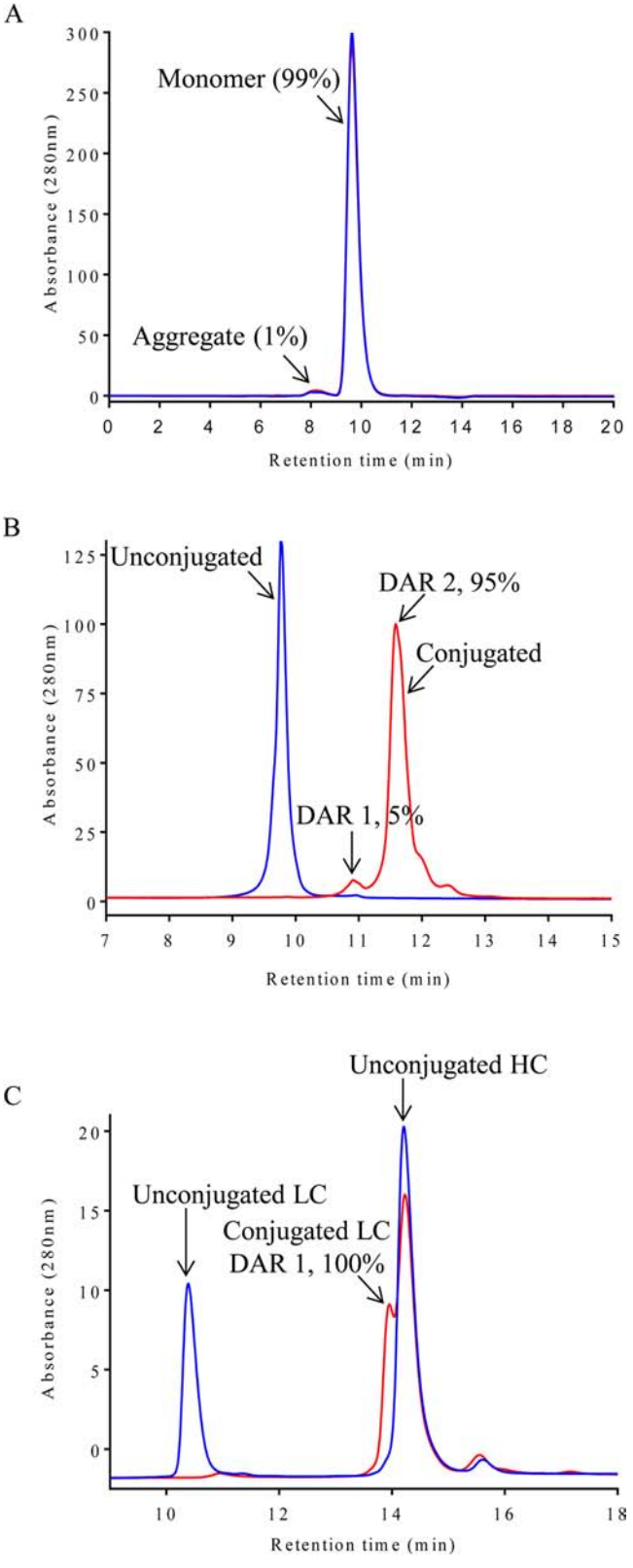


Figure 4. Analytical characterization of the antibody–drug conjugate: (A) size-exclusion chromatography, (B) hydrophobic interaction chromatography, and (C) reduced reverse phase chromatography of the oxidized (blue) and conjugated (red) 1C1 antibody.

having a drug-to-antibody ratio (DAR) of 2. Moreover, reduced reverse phase chromatography (rRP-HPLC) confirmed that the conjugation was specific to the light chain (Figure 4C). Additionally, a DAR of 2 was determined by rLCMS (Figure 5).

Binding of Serine Engineered Antibodies and ADCs to Human EphA2. Binding of 1C1, 1C1-Ser, and 1C1-Ser-MMAE to human EphA2 expressed on PC-3 cells and recombinant EphA2 was demonstrated by flow cytometry and ELISA, respectively (Figure 6A,B). PC-3 cells, which express EphA2 on cell surface,[21] were incubated with antibodies and ADCs and the binding was detected using a fluorescently labeled secondary antibody. As shown in Figure 6A, the parental anti-EphA2 antibody 1C1, 1C1-Ser, and 1C1-Ser-MMAE bind to PC-3 with equivalent maximal binding, with EC_{50} values ranging from 0.22 to 0.47 μg/mL. The controls antibodies have no measurable binding to PC-3 cells. In addition, specific binding to EphA2 was further confirmed using antigen negative SKBR3 cells (data not shown). Binding of 1C1, 1C1-Ser, and 1C1-Ser-MMAE to recombinant human EphA2 was verified using ELISA, as shown in Figure 6B. All 1C1 antibodies were found to have similar EC_{50} values ranging from 5.8 to 6.4 μg/mL for human EphA2, while all negative controls R347 did not bind to EphA2 (Figure 6B). Additionally, no binding was observed by all constructs when human serum albumin was used as a negative control in the ELISA assay (data not shown).

Hydrolytic Stability of the Oxime-Linked ADC. To assess the hydrolytic stability of the oxime-linked ADC, we incubated 1C1-Ser-MMAE formulated at pH 7.2, for 6 days at 4 and 40 °C and at pH 4.5 for 4 days at 37 °C. As shown in Figure 7, the antibody–drug conjugate was found to be stable with negligible drug loss in all tested conditions.

In Serum Stability of the ADC. One important attribute of an ADC is its stability in serum. 1C1-Ser-MMAE was incubated at 200 μg/mL in rat serum at 37 °C for 4 days. After 4 days, the ADC was affinity purified from the rat serum using an anti-human Fc specific antibody and analyzed using rLCMS. Over the course of 4 days, we observed no detectable loss of the oxime-linked MMAE (Figure 8).

In Vitro Cytotoxicity of the ADC. To test the cytotoxicity of 1C1-Ser-MMAE toward cells expressing EphA2, we incubated the 1C1, 1C1-Ser, 1C1-Ser-MMAE, R347, R347-Ser, and R347-Ser-MMAE with PC-3 cells for 6 days at 37 °C and measured cell viability. As shown in Figure 9, 1C1-Ser-MMAE was able to specifically kill PC-3 cells in a dose dependent manner with an IC_{50} of 12.98 ng/mL, whereas the unconjugated 1C1 and negative control antibodies were inactive.

In Vivo Antitumor Activity. Mouse xenograft studies were conducted with PC-3 cells using nude mice. Tumor-bearing mice were treated once weekly with an intravenous injection of 3 mg/kg of 1C1-Ser, 1C1-Ser-MMAE, R347-Ser, and R347-Ser-MMAE for a total of 3 weeks. Significant tumor growth inhibition was observed for the 1C1-Ser-MMAE (Figure 10), while 1C1-Ser, R347-Ser, and R347-Ser-MMAE had no antitumor activity (Figure 10). Moreover, mouse body weights were unaffected in all treatment groups (data not shown).

DISCUSSION

In recent years intense effort has been devoted to improving the therapeutic index (TI, defined as maximum tolerated dose/minimum efficacious dose) of ADCs.[22] Some advancements in TI improvement have come from the development of site-specific ADCs which offer precise drug load and optimal in serum stability.[23]

DOI: 10.1021/acs.bioconjchem.5b00355
Bioconjugate Chem. 2015, 26, 2085–2096

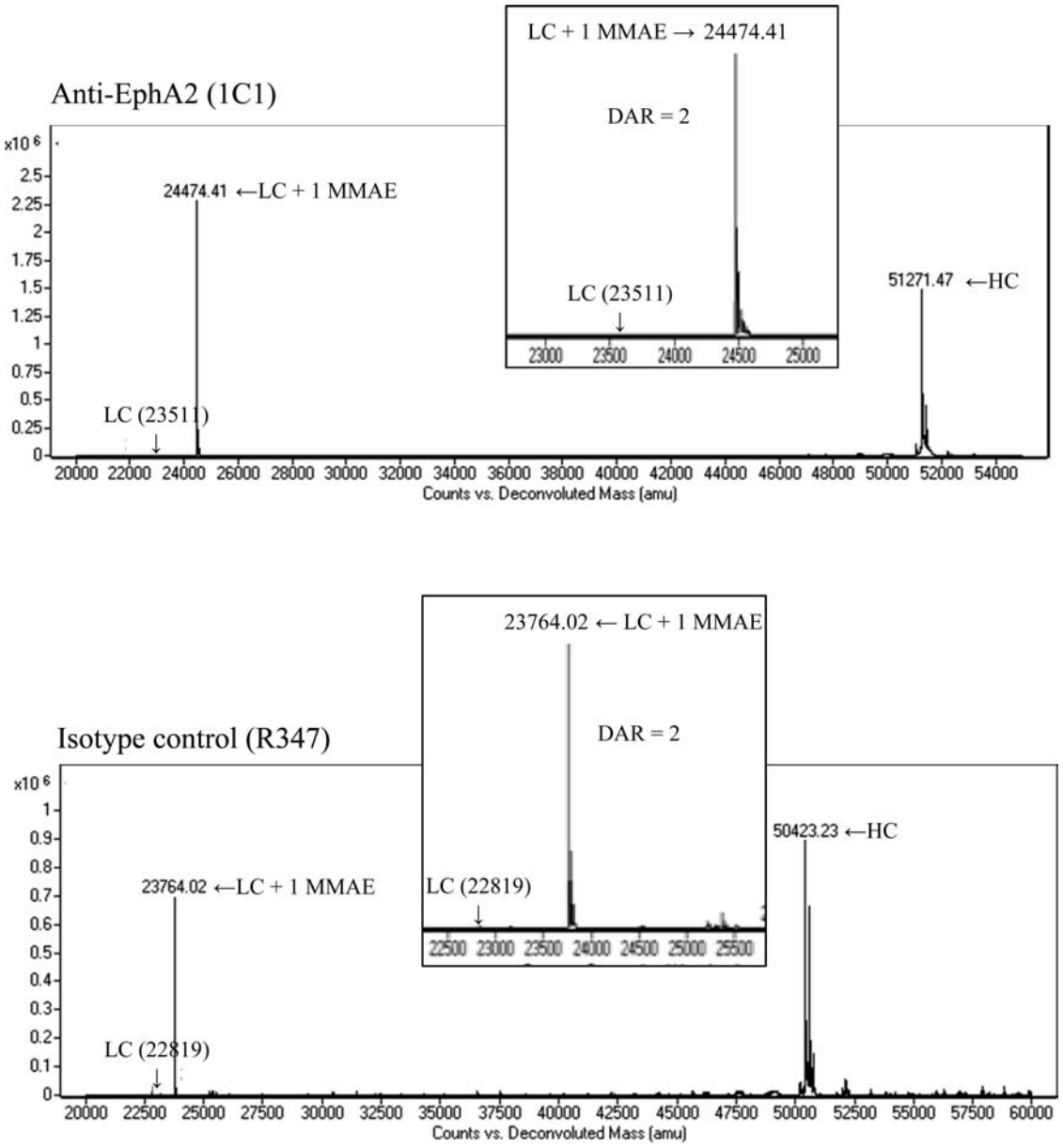


Figure 5. Conjugation of the alkoxyamine-PEG$_4$-MMAE to the *N*-terminal aldehyde determined by rLCMS. Expected masses (Da): 1C1-Ser-MMAE, 22474; R347-Ser-MMAE, 23764.

Herein we report a bioorthogonal site-specific conjugation strategy for preparing ADCs based on the engineering of a *N*-terminal serine residue into an antibody. This approach is broadly applicable to any IgG since it involves introducing the serine residue at the *N*-terminus of the light chain, which is positioned outside the antigen binding site and far from the antibody domains that are critical for binding to FcγRs and FcRn (Figure 1). Similarly, engineering a serine residue at the *N*-terminus of the heavy chain and its subsequent use for conjugation is also possible. Moreover, introducing serine residues at both *N*-termini of the variable domains is possible if a DAR of 4 is desired.

It has been reported that the use of excess $NaIO_4$ can oxidize the vicinal diols of both fucose and sialic acid on an antibody.[7,24] To ensure oxidation of only the engineered serine residue, the light chain of the antibody was selected for the introduction of the *N*-terminal serine, which allows for simultaneous monitoring of both light and heavy chains during the treatment with $NaIO_4$. The stoichiometry of the $NaIO_4$ was optimized to control the reaction, leaving the reactive glycans and amino acids, such as lysine, histidine, and cysteine[25] intact, as determined by rLCMS in Figure 3. Subsequent conjugation of the aldehyde with an alkoxyamine-bearing monomethyl auristatine E in the presence of *p*-anisidine as a catalyst allowed for a complete reaction after 16 h at pH 6. Biophysical analysis of the *N*-terminally conjugated ADCs showed the conjugate to be homogeneous (Figure 4B,C), with negligible in process aggregate formation (Figure 4A), which are both indications of manufacturability.

Having demonstrated the robust formation of the ADCs at near-neutral pH, we next confirmed the hydrolytic stability of the oxime-linked conjugates under both neutral and acidic pH. While the instability of aldoxime bioconjugates has been reported,[26] we observed no significant hydrolysis of the oxime-linked drug at both pH 7.2 and 4.5 over the course of 6 days (Figure 7).

DOI: 10.1021/acs.bioconjchem.5b00355
Bioconjugate Chem. 2015, 26, 2085−2096

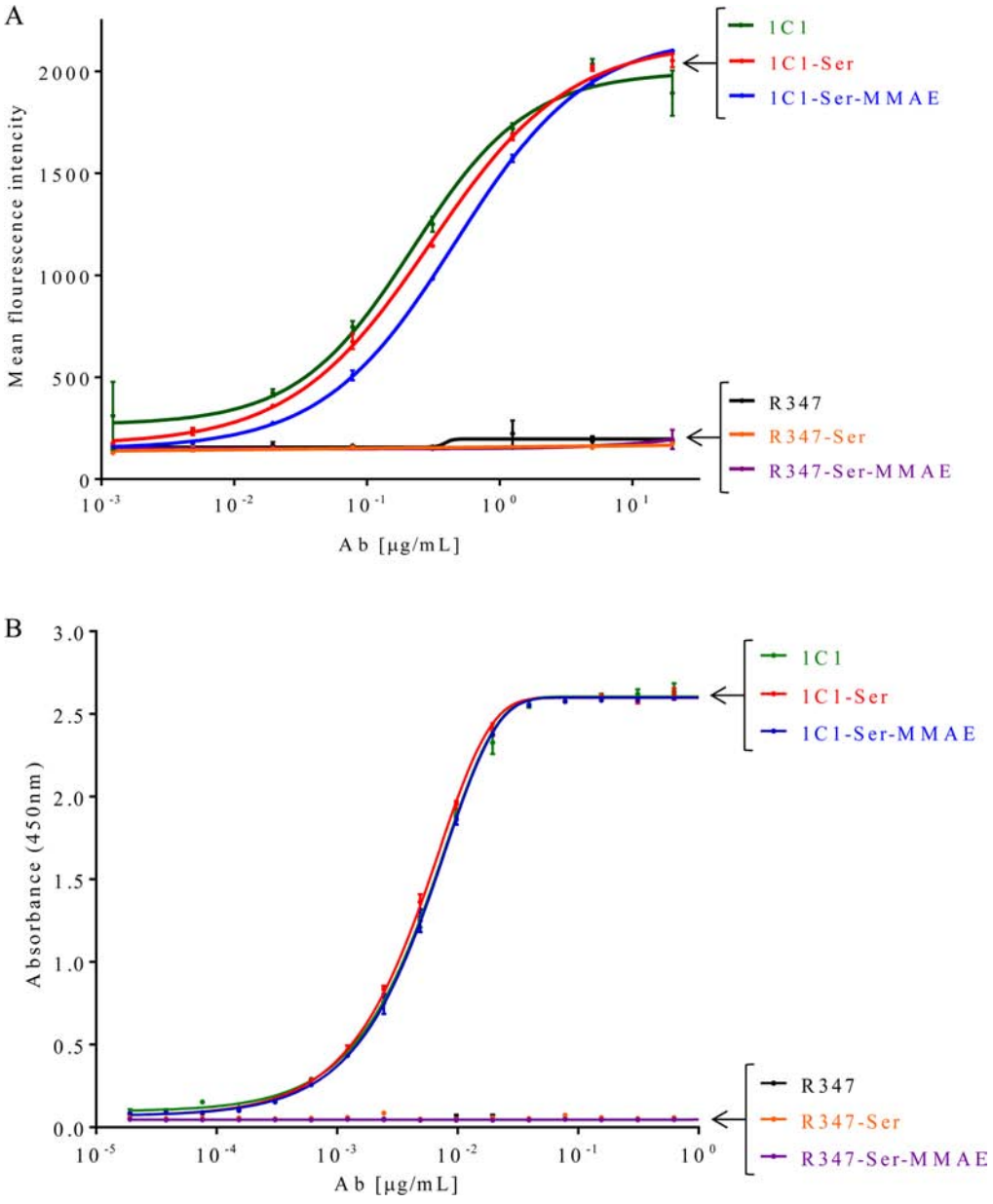


Figure 6. Binding (n = 3) of wild-type antibodies, *N*-terminal serine engineered antibodies, and ADCs to (A) cell surface and (B) recombinant human EphA2 by flow cytometry and ELISA, respectively.

To address the in serum stability of the novel oxime-linked ADC, the 1C1 conjugate was incubated in rat serum for 4 days. An aldehyde-tagged protein conjugated via oxime chemistry with Alexa Fluor 488 showed evidence of degradation in plasma after 1 day.[27] We, however, have found that the conjugation of an alkoxyamine-bearing drug to the *N*-terminus of the light chain of an antibody results in a stable oxime-linked conjugate, with no appreciable drug loss after a four day incubation in serum (Figure 8). This enhanced stability may be attributed to the extended π system of the oxime.

Analysis by flow cytometry and ELISA did not detect any significant difference in either 1C1-Ser or 1C1-Ser-MMAE's binding to the EphA2 antigen when compared to the parental antibody (Figure 6). In addition, when conjugated to the antimitotic MMAE, specific and potent cytotoxic activity is observed in antigen positive PC-3 cells (Figure 9).

The 1C1-Ser-MMAE demonstrated specific and potent antitumor activity in a xenograft prostate cancer model expressing EphA2 (Figure 10). The in vitro cytotoxicity and in vivo tumor growth inhibition indicate that the 1C1-Ser-MMAE is efficiently internalized into target positive cells.

In conclusion, this is the first report of a stable ADC conjugated at the *N*-terminus of an antibody using oxime chemistry. We have demonstrated that for antibody conjugations, the oxime ligation at the *N*-terminus is a robust reaction that occurs at near-neutral pH. Additionally, the resulting oxime-linked ADC demonstrates excellent hydrolytic stability in not only neutral and acidic environments, but also in rat serum. The two-step procedure described above promises to be a valuable method for the scalable preparation of site-specific antibody−drug conjugates. Furthermore, when used in combination with bioorthogonal conjugation modalities, like the engineered cysteine approach, this *N*-terminal conjugation

DOI: 10.1021/acs.bioconjchem.5b00355
Bioconjugate Chem. 2015, 26, 2085−2096

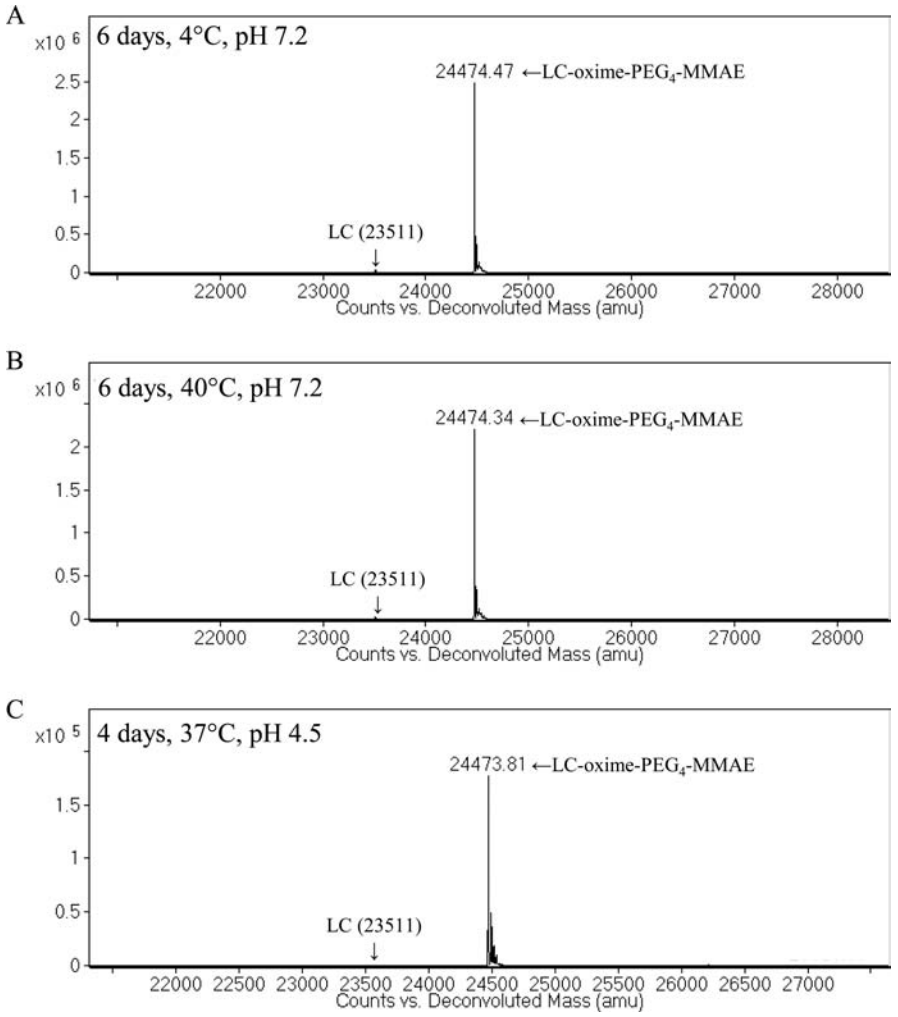


Figure 7. Hydrolytic stability of 1C1-Ser-MMAE. 1C1-Ser-MMAE was incubated in (A) 1× PBS, pH 7.2 at 4 °C and (B) 40 °C for 6 days and (C) 5 mM sodium acetate, pH 4.5 at 37 °C for 4 days and then analyzed by rLCMS.

strategy offers an opportunity to combine conjugation partners, including fluorophores and other anticancer therapeutics, like the DNA alkylating pyrrolobenzodiazepine dimers.[11,12] Current efforts in our laboratory are devoted to studying (i) the in vivo pharmacology in multiple murine models, (ii) the biodistribution, pharmacokinetics, and tolerability in animal models, (iii) preparation of ADCs in which the serine residue is engineered at the *N*-terminus of the heavy chain, and (iv) preparation of ADCs with a DAR of 4 where serine residues are introduced at both *N*-termini of the light and heavy chains.

EXPERIMENTAL SECTION

General Remarks. All reagents were purchased from Thermo Scientific or Sigma-Aldrich and were used without further purification, unless otherwise stated. The payload used in this study, alkoxyamine-PEG_4-MMAE, was purchased from Concortis (Sorrento Therapeutics) and was 95% pure as judged by analytical HPLC. An Agilent 1100 series HPLC system equipped with an autosampler, diode-array detector (DAD), and ChemStation software was used to carry out analytical characterization of the ADCs. All antibodies and ADCs were detected using a wavelength of 280 nm.

Monoclonal Antibodies. An anti-EphA2 IgG1 antibody[21] (1C1, with a kappa light chain) and negative-control antibody (R347, with a lambda light chain) were used for the *N*-terminal serine engineering on the light chain (1C1-Ser and R347-Ser). Standard molecular biology methods were used to generate the *N*-terminal serine antibody mutants. The antibodies were produced and purified at MedImmune as described by Dimasi et al.[28] Expression and secretion of the recombinant antibodies were under the control of the cytomegalovirus promoter and the native immunoglobulin light chain signal peptide (Supplementary Figure 1), respectively. Antibodies were formulated into 1× PBS, pH 7.2. The 3D-structural information on the Fv domain was retrieved from the Protein Data Bank using entry code 1F8T. The structure illustration was prepared using the program Pymol.

Oxidation of the *N*-Terminal Serine Engineered Antibodies. 1C1-Ser and R347-Ser antibodies were treated with 4 mol equiv of sodium periodate ($NaIO_4$) for 20 min at room temperature. The excess $NaIO_4$ was quenched by the addition of 70 mol equiv of serine.[29] The antibodies were dialyzed using 10 kDa molecular weight cut off (MWCO) cassettes into 1× PBS, pH 7.2.

Conjugation of Alkoxyamine-PEG_4-MMAE to Aldehyde-Functionalized Monoclonal Antibodies. To a solution of glyoxylate-functionalized antibodies was added 1 M sodium phosphate, pH 6, and 500 mM *p*-anisidine (in acetonitrile) to a final concentration of 100 mM and 10 mM, respectively. Fifteen molar equivalents of alkoxyamine-PEG_4-

DOI: 10.1021/acs.bioconjchem.5b00355
Bioconjugate Chem. 2015, 26, 2085−2096

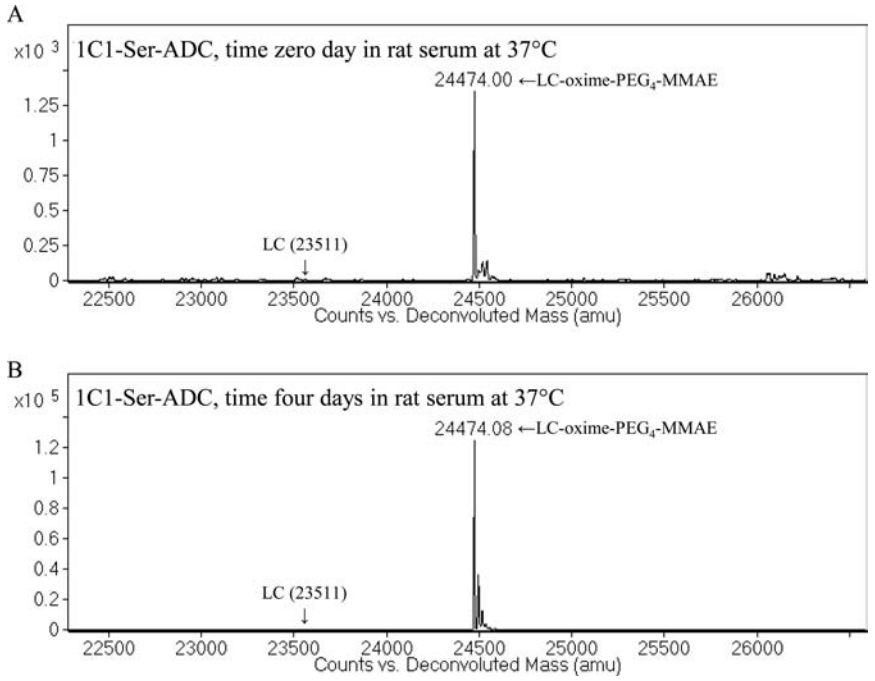


Figure 8. 1C1-Ser-MMAE demonstrates stability in serum. The ADC was incubated in rat serum for 4 days at 37 °C, affinity purified using an anti-human Fc-specific antibody, and analyzed using rLCMS.

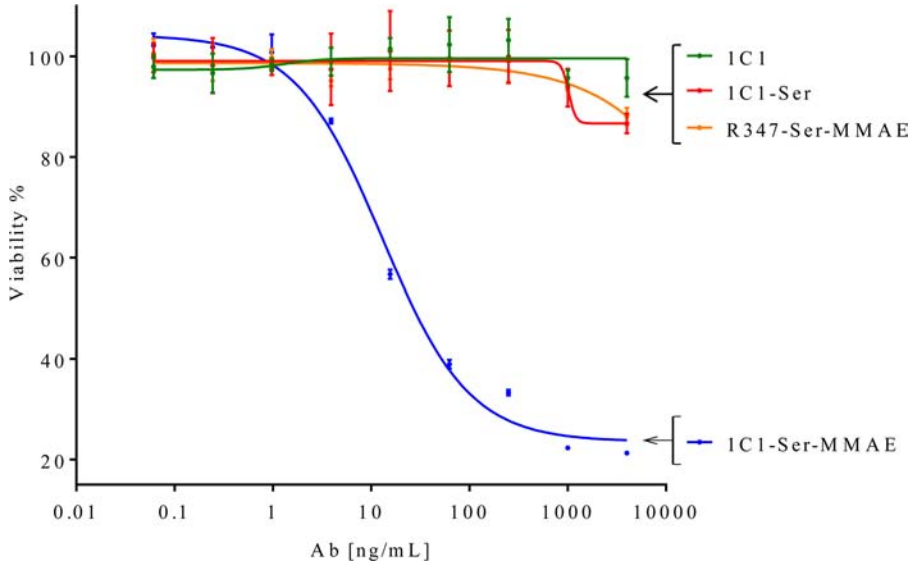


Figure 9. 1C1-Ser-MMAE is highly cytotoxic against EphA2 positive PC-3 cells.

MMAE were then added to the reaction mixture, which was gently rotated in the dark for 16 h.

Purification and Formulation of Antibodies and *N*-Terminally Conjugated ADCs. The crude ADC reaction mixtures were diluted 4-fold with water and purified using type II ceramic hydroxyapatite column chromatography (Biorad). Each ADC was eluted with a linear gradient of 0 to 2 M sodium chloride in 10 mM sodium phosphate pH 7 over 20 min. The purified ADCs and antibodies were concentrated to 2 mg/mL and formulated by buffer exchange into 1× PBS, pH 7.2.

Analytical Characterization of Antibodies and ADCs. The purity of antibodies and ADCs was determined by SEC-HPLC, as described by Dimasi et al.,[28] with the addition of 10% isopropanol to the running buffer. 100 μg (100 μL of volume) of antibodies and ADCs were used for the SEC-HPLC analysis.

Hydrophobic interaction chromatography (HIC-HPLC) was used to determine the conjugation efficiency of the ADCs and drug load distribution. HIC-HPLC was carried out using a Butyl-NPR column (4.6 μm ID × 3.5 cm, 2.5 μm, Tosoh Bioscience) and 25 mM Tris-HCl, 1.5 M $(NH_4)_2SO_4$, pH 7.0 (buffer A), and 25 mM Tris-HCl, 5% isopropanol, pH 7.0 (buffer B). 100 μg (100 μL of volume) of antibody or ADC was loaded and eluted at a flow rate of 0.8 mL/min with a gradient of 5% B to 100% B over 13 min. Efficiency of conjugation and drug-to-antibody ratio (DAR) were determined by integration of the observed peaks at 280 nm.

DOI: 10.1021/acs.bioconjchem.5b00355
Bioconjugate Chem. 2015, 26, 2085−2096

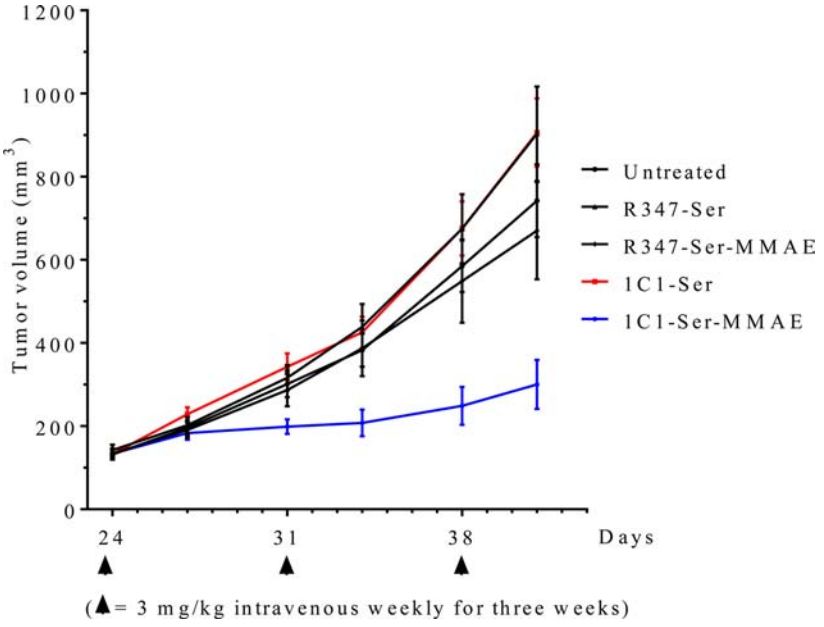


Figure 10. 1C1-Ser-MMAE has potent and specific antitumor activity in a mouse xenograft model of human prostate cancer.

Reduced reverse-phase chromatography (rRP-HPLC) was used to confirm conjugation to the *N*-terminus of the engineered light chain. Antibodies and ADCs were reduced at 37 °C for 20 min using 42 mM dithiothreitol (DTT) in PBS pH 7.2. 10 μg (20 μL of volume) of reduced antibodies or ADCs was loaded onto a PLRP-S, 1000 Å column (2.1 × 50 mm, Agilent) and eluted at 80 °C at a flow rate of 1 mL/min with a gradient of 5% B to 100% B over 25 min (solvent A, 0.1% Trifluoroacetic acid in water; and solvent B, 0.1% Trifluoroacetic acid in acetonitrile).

Conjugation efficiency and DAR were determined using reduced reverse phase mass spectrometry (rLCMS) on an Agilent 1200 series HPLC coupled to an Agilent 6520 Accurate-Mass Q-TOF LC/MS with an electrospray ionization source. 2 μg (4 μL of volume) of reduced glycosylated antibodies or ADCs was loaded onto a Poroshell 300SB-C3 column (2.1 × 75 mm, Agilent) and eluted at a flow rate of 0.4 mL/min using a step gradient of 60% B after 6 min (solvent A, 0.1% Formic acid in water; and solvent B, 0.1% Formic acid in acetonitrile). Agilent MassHunter was used for data acquisition and chromatogram processing.

Binding of Antibodies and ADCs to Human EphA2. Binding of antibodies and ADCs to soluble and membrane bound human EphA2 was carried out using ELISA and flow cytometry (FACS), respectively. For the flow cytometry binding, PC-3 (ATCC CRL-1435) and SK-BR-3 (ATCC HTB-30) cells were dissociated using TrypLE (Life Technologies) and suspended at a concentration of 0.5^6 cells/mL in FACS buffer (1× PBS + 2% FBS). 100 μL of this cell suspension (50K cells) was pipetted into each well of a 96 well U-bottomed plate (Thermo Scientific), washed with 150 μL of FACS buffer, and centrifuged at 1500 rpm for 5 min. The resulting cell pellets were suspended in 1:4 serial dilutions of antibodies in FACS buffer in concentrations ranging from 20 μg/mL to 1.22 ng/mL. Cells were then incubated with the antibody for 2 h on wet ice. After 2 h, cells were spun down at 1200 rpm for 5 min and washed twice with 250 μL of FACS buffer. After the second wash step, the resulting cell pellets were suspended with 150 μL of 1:250 dilution of goat anti-human AlexaFluor 647 secondary antibody (Life Technologies). Cells were incubated with the secondary antibody in wet ice for 1 h and washed twice with FACS buffer. After the last centrifugation, the FACS buffer was removed from the plate, and 150 μL of 3 μM concentration of DAPI (Life Technologies) was added to the wells to determine cell viability. Stained cells were acquired using a BD LSRII flow cytometry system (BD Biosciences), and data from the LSRII were analyzed using the software FlowJo.

For the ELISA binding assay 60 ng/well of human EphA2-FLAG (MedImmune) and Bovine Serum Albumin (Thermo Fisher) were coated on 96 well plate (Corning) overnight at 4 °C in 1× PBS. The plates were washed three times with 1× PBS containing 0.1% Tween-20 (PBST) and blocked with 150 μL of 3% nonfat dry milk (Biorad) in PBST at room temperature. After 1 h incubation, the plates were washed with PBST and a 2-fold serial dilution of the antibodies (starting from 10 μg/mL) in blocking buffer were added and incubated for 1 h at room temperature. The antibody solution was removed by washing with PBST followed by 1 h incubation at room temperature with a 1:3000 dilution of an anti-human-Fc-HRP antibody (Thermo Scientific) prepared in PBST. Binding was visualized with the addition of 30 μL of SureBlue Reserve TMB substrate (KPL) for 5 min at room temperature and the reaction was stopped by adding 30 μL of TMB Stop Solution (KPL). The absorbance at 450 nm was measured using a microtiter plate reader. The data were analyzed using Prism 5 software (GraphPad).

Hydrolytic Stability Studies of the ADCs. 1C1-Ser-MMAE, at 2 mg/mL in 1× PBS, pH 7.2, was subjected to incubation at 4 and 40 °C for 6 days. The ADC was reformulated into 5 mM sodium acetate, pH 4.5, by dialysis using 10 kDa MWCO cassettes and subjected to incubation at 37 °C for 4 days. All samples were analyzed by rLCMS to detect changes in DAR.

Rat Serum Stability Analysis of the 1C1-Ser-ADC. Rat serum was purchased from Jackson ImmunoResearch Laboratories. The serum was filtered through a 0.2 μm syringe filter into sterile polypropylene tubes and kept on ice. 100 μL of the

DOI: 10.1021/acs.bioconjchem.5b00355
Bioconjugate Chem. 2015, 26, 2085−2096

ADC solution at 2 mg/mL was added to 900 μL of rat serum to a final concentration of 200 μg/mL. An aliquot of 300 μL was taken from the sample at time zero, placed on dry ice, and stored at −80 °C within 1 min of the ADC addition to serum. Following incubation for 4 days at 37 °C, the sample was stored at −80 °C until analysis. Anti-human Fc-specific agarose-immobilized antibody (Sigma-Aldrich) was used to affinity capture the ADC from serum. For each time point, 25 μg of immobilized anti-human IgG was mixed with 300 μL 1× PBS and 100 μL serum sample for 30 min at room temperature under continuous and gentle rotation. The mixture was washed in triplicate with 1× PBS to remove any unbound serum proteins and the ADC was eluted using 100 μL IgG elution buffer (Thermo Scientific) and neutralized with 20 μL 1 M Tris-HCl pH 8. To prepare the ADC for rLCMS analysis, 55 μL of ADC was treated with 3 μL 0.5 M DTT at 37 °C for 30 min. 25 μL of the reduced ADC sample was analyzed by rLCMS.

In Vitro Cytotoxicity. 1C1, 1C1-Ser, 1C1-Ser-MMAE, and R347-Ser-MMAE were analyzed for in vitro cytotoxicity using the human EphA2-positive PC-3 prostate cancer cell lines. PC-3 cells were obtained from ATCC, maintained in RPMI1640 media (Life Technologies), and supplemented with 10% heat-inactivated fetal bovine serum (HI-FBS) (Life Technologies) at 37 °C in 5% CO_2. The cells in exponential growth phase were seeded in 96-well culture plates at 1500 cells/well and then treated on the following day with the antibodies and ADCs at 4-fold serial dilution at 9 concentrations in duplicate starting from 4000 ng/mL. The treated cells were cultured for 6 days and cell viability was determined using the CellTiter-Glo Luminescent Viability Assay (Promega) following the manufacturer's protocol. Cell viability was calculated as a percentage of control untreated cells. IC_{50} for 1C1-Ser-MMAE was determined using logistic nonlinear regression analysis with Prism software (GraphPad).

In Vivo Efficacy. In vivo efficacy studies were performed using 4- to 6-week-old female athymic (nu/nu) mice (Harlan Sprague−Dawley Inc.). A total of 5×10^6 PC-3 cells were implanted into the right flank of mice to establish a subcutaneous tumor disease model. When the mean tumor volume was 100 to 150 mm^3, the tumor-bearing mice were randomly divided into groups (n = 8, each group), and treated once a week for a total of 3 weeks with intravenous dose of R347-Ser, 1C1-Ser, R347-Ser-MMAE, and 1C1-Ser-MMAE at 3 mg/kg. Untreated mice were included as control. Mice were monitored daily and tumors were measured twice weekly using calipers. The tumor volumes were calculated using the formula $1/2 \times L \times W2$ (L = length; W = width). Body weights were measured daily to assess tolerability of the treatments. The study was terminated 41 days from tumor implantation or when the tumor volumes reached ∼2000 mm^3. The tumor growth inhibition was plotted using Prism software. Tumor volumes are expressed as mean ± SEM. The efficacy studies were approved by the MedImmune Institutional Animal Care and Use Committee and were conducted in accordance with the Guide of the Care and Use of Laboratory Animals.[30]

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.bioconjchem.5b00355.

Supplementary figure (PDF)

■ AUTHOR INFORMATION

Corresponding Authors

*E-mail: gaoc@medimmune.com.

*E-mail: dimasin@medimmune.com.

Notes

The authors declare the following competing financial interest(s): All authors are employees of MedImmune and may be stockholders of AstraZeneca.

■ ACKNOWLEDGMENTS

This work was supported by MedImmune. We would like to thank our colleague Manuel Baca for scientific discussion.

■ REFERENCES

(1) Chari, R. V., Miller, M. L., and Widdison, W. C. (2014) Antibody-drug conjugates: an emerging concept in cancer therapy. *Angew. Chem., Int. Ed. 53*, 3796−3827.

(2) Beck, A., and Reichert, J. M. (2014) Antibody-drug conjugates: present and future. *MAbs 6*, 15−17.

(3) Junutula, J. R., Raab, H., Clark, S., Bhakta, S., Leipold, D. D., Weir, S., Chen, Y., Simpson, M., Tsai, S. P., Dennis, M. S., et al. (2008) Site-specific conjugation of a cytotoxic drug to an antibody improves the therapeutic index. *Nat. Biotechnol. 26*, 925−932.

(4) Agarwal, P., and Bertozzi, C. R. (2015) Site-specific antibody-drug conjugates: the nexus of biorthogonal chemistry, protein engineering, and drug development. *Bioconjugate Chem. 26*, 176−192.

(5) Behrens, C. R., and Liu, B. (2014) Methods for site-specific drug conjugation to antibodies. *MAbs 6*, 46−53.

(6) Panowski, S., Bhakta, S., Raab, H., Polakis, P., and Junutula, J. R. (2014) Site-specific antibody drug conjugates for cancer therapy. *MAbs 6*, 34−45.

(7) Zhou, Q., Stefano, J. E., Manning, C., Kyazike, J., Chen, B., Gianolio, D. A., Park, A., Busch, M., Bird, J., Zheng, X., et al. (2014) Site-specific antibody-drug conjugation through glycoengineering. *Bioconjugate Chem. 25*, 510−520.

(8) Li, X., Fang, T., and Boons, G. J. (2014) Preparation of well-defined antibody-drug conjugates through glycan remodeling and strain-promoted azide-alkyne cycloadditions. *Angew. Chem., Int. Ed. 53*, 7179−7182.

(9) Van Geel, R., Wijdeven, M. A., Heesbeen, R., Verkade, J. M. M., Wasiel, A. A., van Berkel, S. S., and van Delft, F. L. (2015) Chemoenzymatic conjugation of toxic payloads to the globally conserved N-glycan of native mAbs provides homogeneous and highly efficacious antibody-drug conjugates. *Bioconjugate Chem.*, DOI: 10.1021/acs.bioconjchem.5b00224.

(10) Okeley, N. M., Toki, B. E., Zhang, X., Jeffrey, S. C., Burke, P. J., Alley, S. C., and Senter, P. D. (2013) Metabolic engineering of monoclonal antibody carbohydrates for antibody-drug conjugation. *Bioconjugate Chem. 24*, 1650−1655.

(11) Sutherland, M. S. K., Walter, R. B., Jeffrey, S. C., Burke, P. J., Yu, C., Kostner, H., Stone, I., Ryan, M. C., Sussman, D., Lyon, R. P., et al. (2013) SGN-CD33A: a novel CD33-targeting antibody-drug conjugate using a pyrrolobenzodiazepine dimer is active in models of drug-resistant AML. *Blood 122*, 1455−1463.

(12) Jeffrey, S. C., Burke, P. J., Lyon, R. P., Meyer, D. W., Sussman, D., Anderson, M., Hunter, J. H., Leiske, C. I., Miyamoto, J. B., Nicholas, N. D., et al. (2013) A potent anti-CD70 antibody-drug conjugate combining a dimeric pyrrolobenzodiazepine drug with site-specific conjugation technology. *Bioconjugate Chem. 24*, 1256−1263.

(13) Lyon, R. P., Setter, J. R., Bovee, T. D., Doronina, S. O., Hunter, J. H., Anderson, M. E., Balasubramanian, C. L., Duniho, S. M., Leiske, C. I., Li, F., and Senter, P. D. (2014) Self-hydrolyzing maleimides improve the stability and pharmacological properties of antibody-drug conjugates. *Nat. Biotechnol. 32*, 1059−1062.

(14) Fontaine, S. D., Reid, R., Robinson, L., Ashley, G. W., and Santi, D. V. (2015) Long-term stabilization of maleimide-thiol conjugates. *Bioconjugate Chem. 26*, 145−152.
(15) Axup, J. Y., Bajjuri, K. M., Ritland, M., Hutchins, B. M., Kim, C. H., Kazane, S. A., Halder, R., Forsyth, J. S., Santidrian, A. F., Stafin, K., et al. (2012) Synthesis of site-specific antibody-drug conjugates using unnatural amino acids. *Proc. Natl. Acad. Sci. U. S. A. 109*, 16101−16106.
(16) Liu, C. C., and Schultz, P. G. (2010) Adding new chemistries to the genetic code. *Annu. Rev. Biochem. 79*, 413−444.
(17) Young, T. S., and Schultz, P. G. (2010) Beyond the canonical 20 amino acids: expanding the genetic lexicon. *J. Biol. Chem. 285*, 11039−11044.
(18) Geoghegan, K. F., and Stroh, J. G. (1992) Site-directed conjugation of nonpeptide groups to peptides and proteins via periodate oxidation of a 2-amino alcohol. Application to modification at N-terminal serine. *Bioconjugate Chem. 3*, 138−146.
(19) Ning, X., Temming, R. P., Dommerholt, J., Guo, J., Ania, D. B., Debets, M. F., Wolfert, M. A., Boons, G. J., and van Delft, F. L. (2010) Protein modification by strain-promoted alkyne−nitrone cycloaddition. *Angew. Chem., Int. Ed. 49*, 3065−3068.
(20) Colombo, M., Sommaruga, S., Mazzucchelli, S., Polito, L., Verderio, P., Galeffi, P., Corsi, F., Tortora, P., and Prosperi, D. (2012) Site-Specific Conjugation of ScFvs Antibodies to Nanoparticles by Bioorthogonal Strain-Promoted Alkyne−Nitrone Cycloaddition. *Angew. Chem. 124*, 511−514.
(21) Jackson, D., Gooya, J., Mao, S., Kinneer, K., Xu, L., Camara, M., Fazenbaker, C., Fleming, R., Swamynathan, S., Meyer, D., et al. (2008) A human antibody-drug conjugate targeting EphA2 inhibits tumor growth in vivo. *Cancer Res. 68*, 9367−9374.
(22) Muller, P. Y., and Milton, M. N. (2012) The determination and interpretation of the therapeutic index in drug development. *Nat. Rev. Drug Discovery 11*, 751−761.
(23) Hinrichs, M. J., and Dixit, R. (2015) Antibody Drug Conjugates: Nonclinical Safety Considerations. *AAPS J. 17*, 1055−1064.
(24) Zuberbuhler, K., Casi, G., Bernardes, G. J. L., and Neri, D. (2012) Fucose-specific conjugation of hydrazide derivatives to a vascular-targeting monoclonal antibody in IgG format. *Chem. Commun. 48*, 7100−7102.
(25) Liu, B., Burdine, L., and Kodadek, T. (2006) Chemistry of periodate-mediated cross-linking of 3,4-dihydroxylphenylalanine (DOPA)-containing molecules to proteins. *J. Am. Chem. Soc. 128*, 15228−15235.
(26) Agarwal, P., van der Weijden, J., Sletten, E. M., Rabuka, D., and Bertozzi, C. R. (2013) A Pictet-Spengler ligation for protein chemical modification. *Proc. Natl. Acad. Sci. U. S. A. 110*, 46−51.
(27) Agarwal, P., Kudirka, R., Albers, A. E., Barfield, R. M., deHart, G. W., Drake, P. M., Jones, C. L., and Rabuka, D. (2013) Hydrazino-Pictet Spengler ligation as a biocompatible method for the generation of stable protein conjugates. *Bioconjugate Chem. 24*, 846−851.
(28) Dimasi, N., Gao, C., Fleming, R., Woods, R. M., Yao, X. T., Shirinian, L., Kiener, P. A., and Wu, H. (2009) The design and characterization of oligospecific antibodies for simultaneous targeting of multiple disease mediators. *J. Mol. Biol. 393*, 672−692.
(29) Wendeler, M., Grinberg, L., Wang, X., Dawson, P. E., and Baca, M. (2014) Enhanced catalysis of oxime-based bioconjugations by substituted anilines. *Bioconjugate Chem. 25*, 93−101.
(30) *Guide for the care and use of laboratory animals* (1996) National Research Council, Washington, DC.

DOI: 10.1021/acs.bioconjchem.5b00355
Bioconjugate Chem. 2015, 26, 2085−2096