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Discovery of Pyrophosphate Diesters as Tunable, Soluble, and Bioorthogonal Linkers for Site-Specific Antibody−Drug Conjugates

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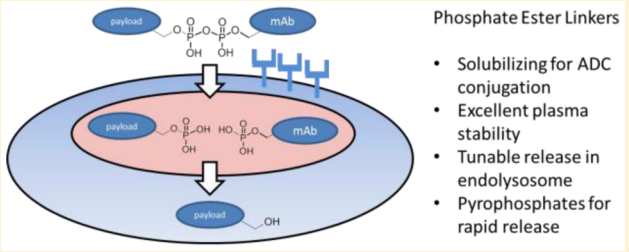
Supporting Information

ABSTRACT: As part of an effort to examine the utility of antibody−drug conjugates (ADCs) beyond oncology indications, a novel pyrophosphate ester linker was discovered to enable the targeted delivery of glucocorticoids. As small molecules, these highly soluble phosphate ester drug linkers were found to have ideal orthogonal properties: robust plasma stability coupled with rapid release of payload in a lysosomal environment. Building upon these findings, site-specific ADCs were made between this drug linker combination and an antibody against human CD70, a receptor specifically expressed in immune cells but also found aberrantly expressed in multiple human carcinomas. Full characterization of these ADCs enabled procession to in vitro proof of concept, wherein ADCs **1**−**22** and **1**−**37** were demonstrated to afford potent, targeted delivery of glucocorticoids to a representative cell line, as measured by changes in glucocorticoid receptor-mediated gene mRNA levels. These activities were found to be antibody-, linker-, and payload-dependent. Preliminary mechanistic studies support the notion that lysosomal trafficking and enzymatic linker cleavage are required for activity and that the utility for the pyrophosphate linker may be general for internalizing ADCs as well as other targeted delivery platforms.



INTRODUCTION

The antibody−drug conjugate (ADC) modality is enjoying a renewal of interest and success because of advances in expression, conjugation, and linker−payload technology that have paved the path for the recent approvals of Adcetris and Kadcyla for Hodgkin lymphoma and Her-2-positive metastatic breast cancer, respectively.[1,2] In all, nearly 30 ADCs are reported to be in clinical development, all of which are aimed at addressing unmet medical needs in oncology.[3] As a result, while there is significant diversity in the monoclonal antibodies (mAbs) employed in these candidates, the options among the varied linkers and payloads are limited to a select few that are used repeatedly because of the clinical experience and common purpose of targeted cytotoxicity. To fully explore the potential of this modality, particularly in its applicability beyond oncology, there is a clear need to develop alternative linker designs that address present limitations and facilitate the use of the larger payload pharmacopeia.

As part of an effort to test the ADC modality beyond oncology, a project was initiated to achieve the targeted delivery of glucocorticoids to immune cells. Glucocorticoids are potent steroid hormones that have powerful anti-inflammatory properties with important utility in a range of immunological diseases.[4a] Unfortunately, glucocorticoids also suffer from an array of side effects (e.g., effects on glucose, bone, mood) because of their pleiotropic pharmacology and wide biodistribution as small molecule therapeutics.[4b] Decades of research in the medicinal chemistry community have been devoted to the concepts of dissociated or tissue-selective synthetic glucocorticoids to overcome their limited therapeutic index, and, arguably, the most successful approaches have come through topical or inhaled delivery methods, where biodistribution can be physically limited.[5] Despite this focus, the goal of achieving a systemic glucocorticoid devoid of these dose-limiting side effects has remained elusive. This problem of therapeutic index has recently been revisited through the lens of the ADC modality using heterogeneous conjugation with E-

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selectin[6] and anti-CD163[7] using ester-based linkers for the glucocorticoid. Our project aimed to leverage site-specific conjugation technology using unnatural amino acid incorporation[8] to enable clear structure−activity relationship (SAR) assessment for an optimal drug−linker combination and, as a preliminary goal, proof-of-concept in a human immune cell.

Within the ADC modality, the linker functions to stably attach a potent payload to a circulating mAb while at the same time acting as a trigger for payload release following internalization of that mAb within its target cell. This bioorthogonal property relies on differences in the physiological environment of the endosomal−lysosomal pathway relative to the extracellular matrix. In taking advantage of these physiological differences, four distinct linker designs (and physiological triggers) have been successfully advanced to clinical trials as part of the current wave of oncology ADCs: hydrazones (acid), disulfides (reducing thiols), cathepsin B-cleavable dipeptides (enzymes), and noncleavable (mAb catabolism). This toolset has performed well for a limited set of cytotoxic payloads, but we recognized limitations in each design as we looked to leverage them for the glucocorticoid payloads. Given the nuances of glucocorticoid pharmacology with the potential for partial agonism/antagonism, we sought to design an ADC that released dexamethasone in its parental form without any residual linker.[9] Early publications with a similar goal employed an ester-based linker to dexamethasone that we evaluated and found to be rapidly hydrolyzed in blood, suggesting that circulatory stability for this linker design would be suboptimal. Thus, we aimed to identify a novel linker for the delivery of glucocorticoids that would provide stable attachment to a circulating mAb as well as efficient release of the payload once internalized into an antigen-positive cell. This work led to the discovery of a family of highly soluble phosphate ester linkers that have tunable and optimal properties for this specific goal and that may have wide applicability in the ADC and bioconjugate fields (Figure 1).

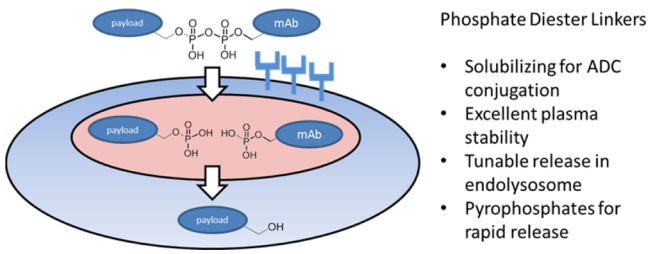

Figure 1. Phosphate diester linkers for antibody−drug conjugates.

Importantly, in vitro proof of concept was established using anti-human CD70 (α-hCD70) conjugates **1**−**22** and **1**−**37** through site-specific conjugation technology coupled with strain-promoted 3 + 2 click chemistry.[10] α-hCD70 was chosen as a model antibody for achieving proof of concept based on its precedent in the oncology ADC literature as a targeting vector, and the validated cell lines used to test it are also responsive to glucocorticoids. The resulting conjugates have robust SAR with clear evidence of antibody-, linker-, and payload-dependent activity and highlight the potential of the novel class of bioorthogonal linkers for targeted delivery.

CHEMISTRY

The desired properties adopted for a linker that would enable the targeted delivery of glucocorticoids to CD70+ cells using an ADC approach were as follows: soluble, stable in extracellular circulation, and rapid payload release in a lysosomal environment. It was hypothesized that phosphate diester linkers could provide these properties and be applicable to a range of glucocorticoids with varying potency (see Results and Discussion for further explanation).

Synthesis of Glucocorticoid−Phosphate Ester Linkers. The synthesis of dexamethasone phosphate ester linkers (**4**, **7**, and **10**) was accomplished according to Scheme 1. Dexamethasone (**1**) was reacted with pyrophosphate tetrachloride at low temperature to selectively generate the C-21-reactive phosphorodichloridate species in situ with no reaction observed at the more hindered C-11 and C-17 positions. Upon consumption of the steroid, corresponding alcohol **2** or **5** was introduced along with triethylamine at low temperature to produce **3** and **6**, respectively, in a one-pot manner, with good yields after aqueous workup. Acidic deprotection of **3** and **6** followed by EDC coupling with 2-(cyclooct-2-yn-1-yloxy)acetic acid[10] provided target dexamethasone linkers **4** and **7** in low yields.[11] To obtain phosphoramidate **10**, dexamethasone-21-phosphate (**8**) was isolated upon aqueous workup under the conditions described above. EDC coupling of 2-(cyclooct-2-yn-1-yloxy)acetic acid with ethylene diamine yielded intermediate **9**. A coupling reaction of **8** and **9** was carried out in aqueous *t*-butanol using DCC to give phosphoramidate **10**.

The synthesis of dexamethasone and budesonide pyrophosphate diester linkers (**14**, **18**, and **22**) was accomplished according to Scheme 2. This approach[12] to pyrophosphate synthesis involved the coupling of two terminal phosphates. First, alcohols **11** and **15** were phosphorylated to create the necessary intermediates **12** and **16**, respectively. Dexamethasone phosphate (**8**) was first treated with CDI in DMF to form an active phosphorimidazolidate species. To this were added either **12** or **16** and $ZnCl_2$, which yielded dexamethasone pyrophosphates **13** and **17**. Deprotection of the Fmoc group with piperidine followed by EDC coupling with 2-(cyclooct-2-yn-1-yloxy)acetic acid provided target pyrophosphate linkers **14** and **18**. Budesonide phosphate **20** was activated to its phosphorimidazolidate and coupled with **12** as above to provide budesonide pyrophosphate **21**. **21** was deprotected in the same manner, but instead of EDC coupling, **21** was alternatively reacted with the preformed phthalate ester of the cyclooctyne, providing a cleaner reaction with superior yield to give pyrophosphate linker **22**.

The synthesis of a triphosphate diester (**28**) was accomplished using sequential couplings according to Scheme 3. To achieve the triphosphate linkage, fluorenyl-methyl phosphate **24** was derived by phosphorylation of fluorenyl methanol **23** and coupled to dexamethasone phosphate (**8**) as described above. Deprotection of the resulting intermediate pyrophosphate ester **25** yielded terminal pyrophosphate **26**. A second coupling reaction of **26** with the active phosphorimidazolidate species of **12** provided Fmoc-protected triphosphate **27**. Deprotection of the Fmoc group followed by reaction with the preformed phthalate ester of the cyclooctyne provided the desired triphosphate diester **28**.

To include fluticasone propionate as a payload alternative, it was necessary to construct the pyrophosphate linker from the 11-position because it contains the only hydroxyl handle in the steroid (Scheme 4). Because of the steric hindrance around the 11-position of the steroid from the flanking 18- and 19-position methyl groups, direct phosphorylation was not possible under the conditions demonstrated for dexamethasone and budeso-

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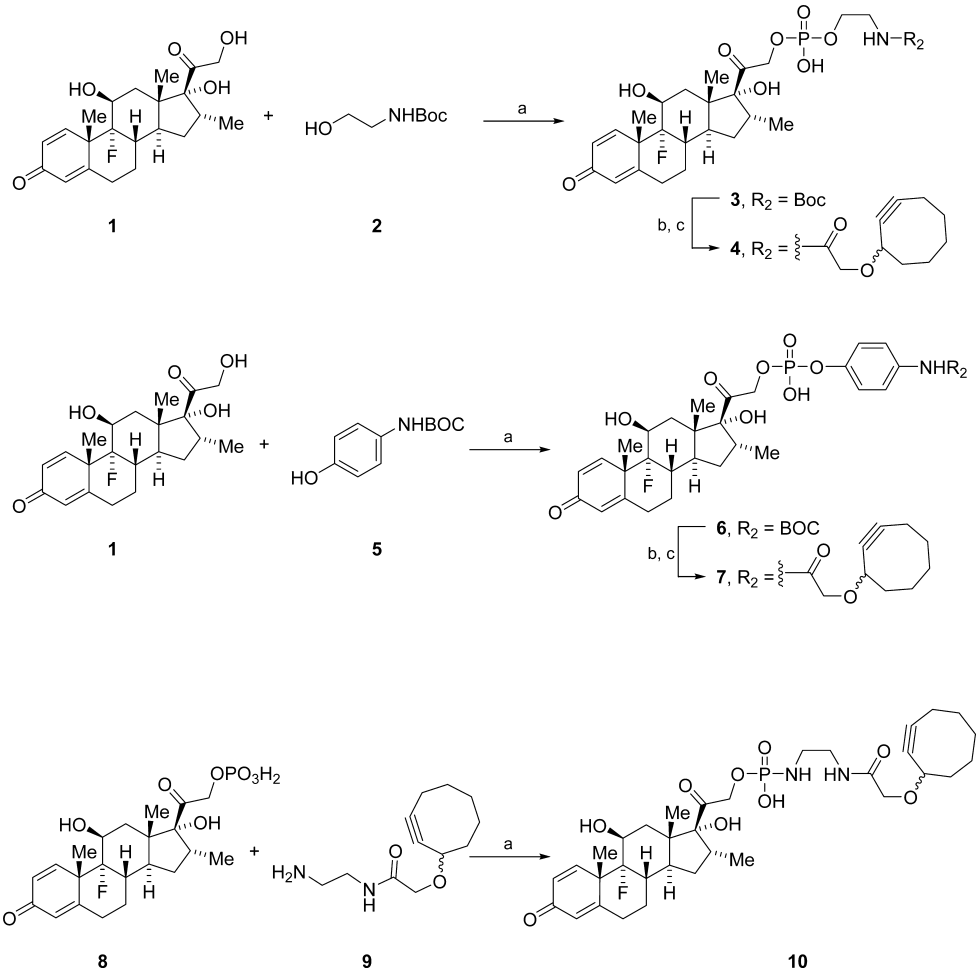
Scheme 1. Synthesis of Series of Dexamethasone Phosphate Linker Molecules 4, 7, and 10[a]



[a]Reagents and conditions. For **4**: (a) $P_2O_3Cl_4$, THF, −40 °C, 1 h, then Et_3N and **2**, 4 h, aq. workup, 73%; (b) HCl, EtOAc, 1 h, rt, 100%; (c) 2-(cyclooct-2-yn-1-yloxy)acetic acid, EDCI, HOAt, Et_3N, CH_2Cl_2, 37%. For **7**: (a) $P_2O_3Cl_4$, THF, −40 °C, 75 min, then Et_3N and **5**, 30 min, aq. workup, 88%; (b) HCl, EtOAc, rt, 1 h, 100%; (c) 2-(cyclooct-2-yn-1-yloxy)acetic acid, EDCI, HOAt, Et_3N, CH_2Cl_2, 9%. For **10**: (a) DCC, *t*-BuOH/water, 100 °C, 4 h, 30%.

nide. Instead, a stepwise approach was discovered. Fluticasone propionate (**29**) was first reacted with PCl_3 at low temperature to install the phosphite,[13] giving compound **30** following aqueous workup. Phosphite **30** was then oxidized and trapped as the phosphorimidazolidate species **31**.[14] Coupling of **31** with phosphate **12** provided the 11-position pyrophosphate ester intermediate **32**. Deprotection of **32** using piperidine followed by 1-[bis(dimethylamino)methylene]-1*H*-1,2,3-triazolo[4,5-*b*]-pyridinium 3-oxid hexafluorophosphate (HATU) coupling with 2-(cyclooct-2-yn-1-yloxy)acetic acid provided the desired 11-position pyrophosphate fluticasone propionate linker **33** in good overall yield.

As mitigation for the potential for poor phosphate hydrolysis on the hindered secondary alcohol of fluticasone propionate, an acetal spacer[15] was synthesized to facilitate efficient release of the parent drug in the acidic lysosomal environment (Scheme 5). Because of the steric hindrance around the hydroxyl group, Sn2 alkylations with sp^3 electrophiles were unsuccessful at introducing the acetal. The introduction of thio ether to give compound **34** was achieved by reaction of fluticasone propionate (**29**) with the sp^2 sulfonium species generated from the mixture of dimethyl sulfide and benzoyl peroxide.[16] This reaction proceeded in low yield, with oxidation of the 11-position hydroxyl as the major competing side reaction. Activation of **34** with *N*-iodosuccinimide (NIS) and reaction with crystalline phosphoric acid under strict anhydrous conditions provided key phosphate acetal intermediate **35**. Activation of Fmoc phosphate **12** to its phosphorimidazolidate species followed by coupling with **35** yielded pyrophosphate diester **36**. Fmoc deprotection with DBU followed by coupling 2-(cyclooct-2-yn-1-yloxy)acetic acid using HATU provided the desired acetal pyrophosphate diester linker **37**.

Additional details on the synthesis and characterization of the small molecules are contained within the experimental and Supporting Information.

Synthesis of α-hCD70 Antibody Glucocorticoid Conjugates. Drug linkers were conjugated to an α-hCD70 antibody (2h5, IgG1)[17] and an α-RSV antibody (based on Synagis with a H32Y mutation on the heavy chain to further decrease binding to RSV F protein, IgG1/kappa isotype[22]) to produce ADCs. Specifically, the drug linker was conjugated to the unnatural amino acid (UAA) *para*-azido phenylalanine (*p*AF), replacing the alanine at position 1 of CH1 (HA114) of the antibody using copper-free 3 + 2 cycloaddition chemistry as shown in Scheme 6.[23]

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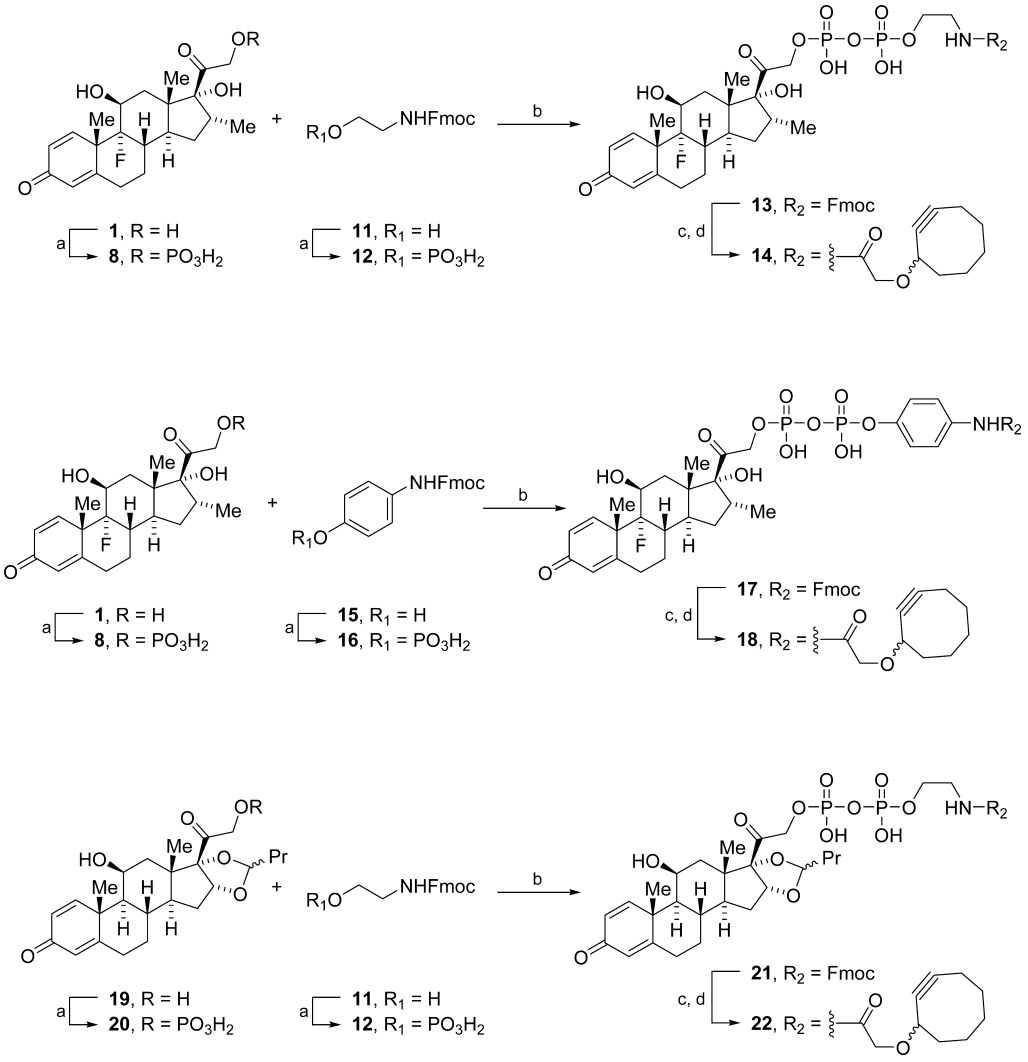
Scheme 2. Synthesis of Dexamethasone and Budesonide Pyrophosphate Diester Linker Molecules 14, 18, and 22[a]



[a]Reagents and conditions. For **14**: (a) $P_2O_3Cl_4$, THF, −40 °C, 30 min, aq. workup, 70−100%; (b) CDI, Et_3N, DMF, rt, 30 min, then **12**, $ZnCl_2$, rt, overnight, 40%; (c) piperidine, CH_2Cl_2, rt, 3 h; (d) 2-(cyclooct-2-yn-1-yloxy)acetic acid, EDCI, HOAt, Et_3N, CH_2Cl_2, 34% (two steps). For **18**: (a) $P_2O_3Cl_4$, THF, −40 °C, 30 min to 3 h, aq. workup, 70−100%; (b) CDI, Et_3N, DMF, rt, 30 min, then **16**, $ZnCl_2$, rt, overnight, 52%; (c) piperidine, CH_2Cl_2, rt, 3 h; (d) 2-(cyclooct-2-yn-1-yloxy)acetic acid, EDCI, HOAt, Et_3N, CH_2Cl_2, 6% (two steps). For **22**: (a) $P_2O_3Cl_4$, THF, −40 °C, 30 min to 2 h, aq. workup, 70−100%; (b) CDI, Et_3N, DMF, rt, 30 min, then **12**, $ZnCl_2$, rt, overnight, 69%; (c) piperidine, CH_2Cl_2, rt, 1.5 h, 69%; (d) 1,3-dioxoisoindolin-2-yl 2-(cyclooct-2-yn-1-yloxy)acetate, Et_3N, DMF, 30 min, 86%.

All of the characterization data for the ADCs has been included in the Supporting Information.

RESULTS AND DISCUSSION

Design and Evaluation of Glucocorticoid Phosphate Ester Linkers. The desired properties adopted for a linker that would enable the targeted delivery of glucocorticoids to CD70+ cells using an ADC approach were as follows: soluble, stable in extracellular circulation, and rapid payload release in a lysosomal environment. Because of the lipophilic nature of glucocorticoids, we reasoned that a solubilizing linker would not only facilitate aqueous bioconjugation to an antibody but also would mitigate against the potential for ADC aggregation seen with other lipophilic payloads.[18] Furthermore, to maximize therapeutic index and provide a prolonged half-life to a potential therapeutic, a well-designed linker should minimize nonspecific release of glucocorticoids in compartments seen by a circulating mAb. Finally, following antigen recognition and internalization into a target cell, the linker should maximize the delivery of its glucocorticoid payload in its parental form as efficiently as possible. Given these design criteria, we chose to focus on strategies that relied on an enzymatic trigger for release wherein the enzyme family used exhibits selective expression in the lysosome (as was done with cathepsin-sensitive linkers).[19] Among the numerous enzyme families present in the lysosomal proteome,[20] we were inspired by phosphodiesterases as a general family and their substrate phosphate diesters for their unique property of stably linking together two molecules and retaining a charged, solubilizing state.[21]

The goal was to synthesize phosphate diesters that connected the payload (e.g., dexamethasone) with a handle that would enable conjugation to a monoclonal antibody. An expression technology[22] was adopted that facilitates this conjugation by the incorporation of UAAs with orthogonal reactivity to create site-specific ADCs with a drug−antibody ratio nearly equal to 2.

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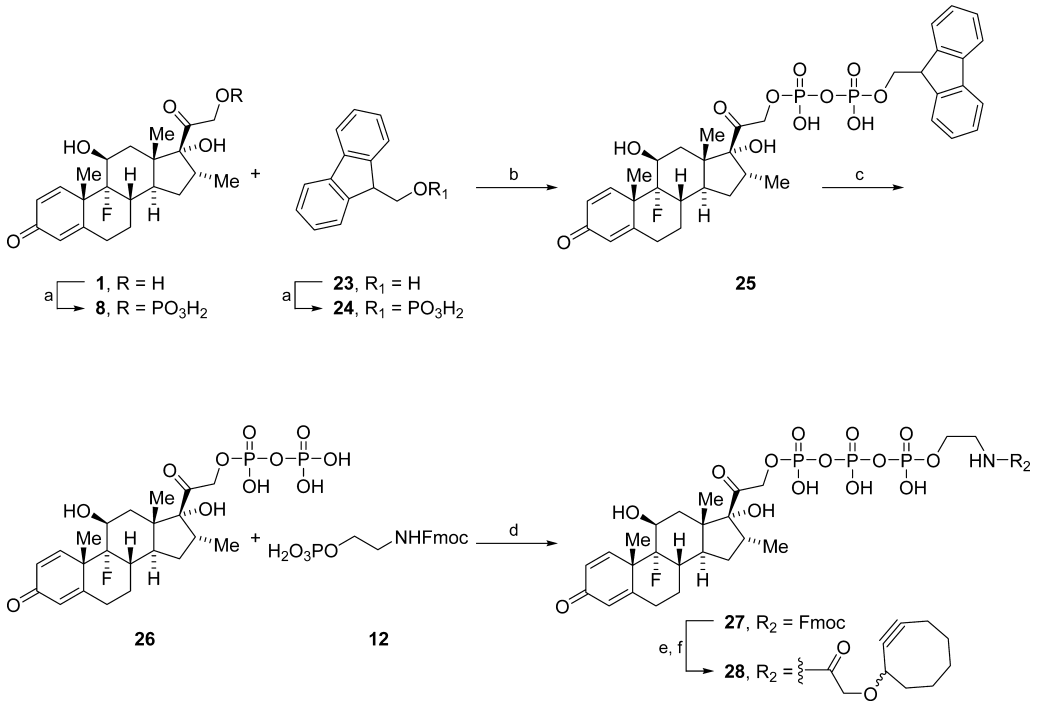
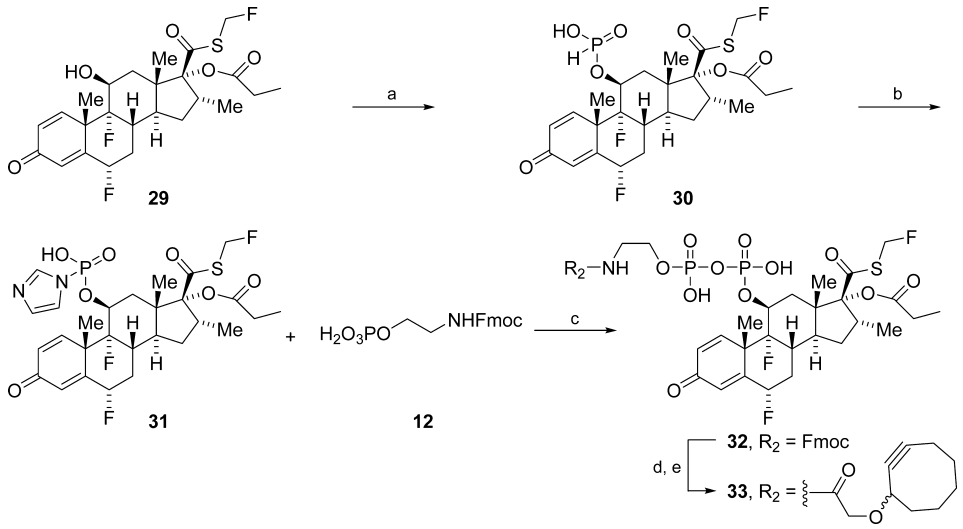
Scheme 3. Synthesis of Dexamethasone Triphosphate Diester Linker Molecule 28[a]



[a]Reagents and conditons: For **28**: (a) $P_2O_3Cl_4$, THF, −40 °C, 30 min to 3 h, aq. workup, 70−100%; (b) CDI, Et_3N, DMF, rt, 30 min, then **24**, $ZnCl_2$, rt, overnight, 66%; (c) piperidine, CH_2Cl_2, rt, 1.5 h, 56%; (d) CDI, Et_3N, DMF, rt, 30 min, then **26**, $ZnCl_2$, rt, overnight, 23%; (e) CH_2Cl_2, rt, 1 h, 80%; (f) 1,3-dioxoisoindolin-2-yl 2-(cyclooct-2-yn-1-yloxy)acetate, Et_3N, DMF, 30 min, 24%.

Scheme 4. Synthesis of Fluticasone Propionate Pyrophosphate Diester Linker Molecule 33[a]



[a]Reagents and conditions. For **33**: (a) PCl_3, Et_3N, THF, −78 °C, aq. workup, 94%; (b) pyridine, TMS-Cl, imidazole, I_2, rt, overnight, 67%; (c) $ZnCl_2$, DMF, rt, 48 h, 51%; (d) piperidine, CH_2Cl_2, rt, 1 h, 80%; (e) 2-(cyclooct-2-yn-1-yloxy)acetic acid, HATU, Et_3N, DMF, rt, 20 min, 71%.

Among the UAAs demonstrated with this platform, *p*-azido phenylalanine is particularly powerful when paired with the strain-induced, copper-free cycloaddition of cycloalkynes.[23] Thus, the design for these drug linkers included a cyclooctyne terminus that could be easily coupled to an UAA-containing mAb. For the payload end of the phosphate ester, clinically utilized dexamethasone has three potential sites of conjugation in the C-11, C-17, and C-21 alcohols. We hypothesized that the most sterically accessible alcohol (C-21) would lead to the most efficient cleavage and produced a series of drug linker molecules that explored the potential of this concept. The synthesis of dexamethasone C-21 phosphate esters **4** and **7**, phosphoramidate **10**, pyrophosphate esters **14** and **18**, and triphosphate ester **28** are described in the Experimental Section, and structures are shown in Figure 2.

As part of the medicinal chemistry program, we also wanted to explore the impact of increasing payload potency within the context of this α-CD70−pyrophosphate ester linker design. Budesonide and fluticasone propionate represent increasingly potent alternatives to dexamethasone that have the added

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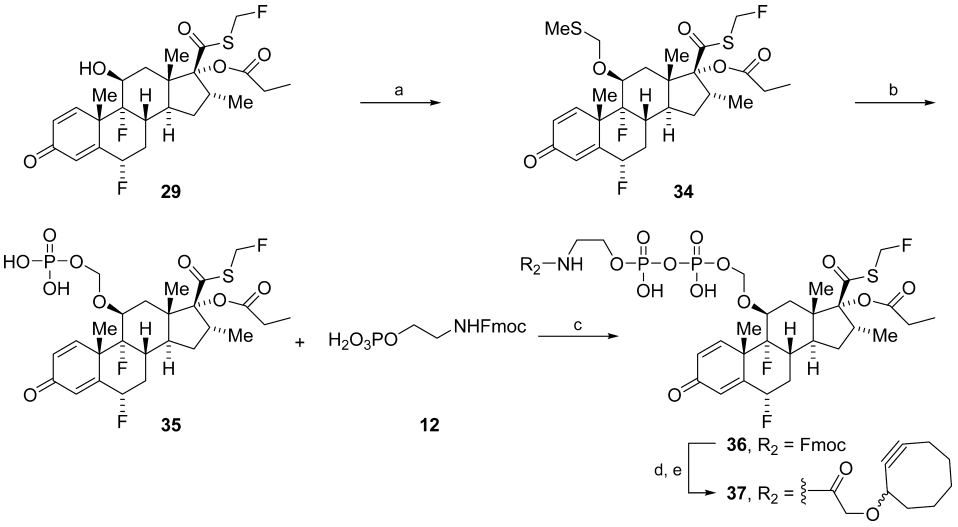
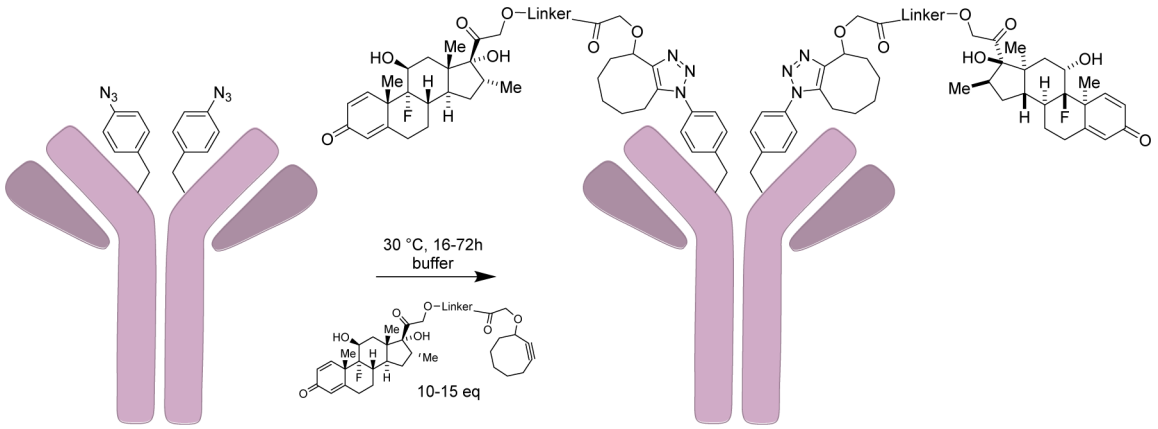
Scheme 5. Synthesis of Acetal-Spaced Fluticasone Propionate Pyrophosphate Diester Linker Molecule 37[a]



[a]Reagents and conditions. For **37**: (a) Me_2S, $(PhCO)_2$, MeCN, 0 °C, 1 h, 13%; (b) NIS, H_3PO_4, THF, 5 Å mol. sieves, rt, overnight, 63%; (c) CDI, Et_3N, DMF, rt, 30 min, then **35**, $ZnCl_2$, rt, overnight, 56%; (d) DBU, CH_2Cl_2, rt, 3 h; (e) 2-(cyclooct-2-yn-1-yloxy)acetic acid, HATU, Et_3N, DMF, 1 h, 70% (two steps).

Scheme 6. Representative Conjugation of an α-hCD70 Antibody



theoretical benefit of longer off-rates from the glucocorticoid receptor (GR) as a mechanism for cellular retention.[24] The linker designs synthesized above were easily adapted to the free C-21 alcohol within the budesonide structure, but extension to fluticasone propionate and its hindered C-11 alcohol proved to be problematic (Figure 3). With the development of unique chemistry to overcome the poor steric accessibility to this alcohol, both the pyrophosphate of fluticasone propionate and an acetal-spaced variant were produced to mitigate the potential for slow bioconversion of the hindered phosphate.

All of the above phosphate linker molecules were found to be chemically stable, to be amenable to reverse phase purification, and to have outstanding aqueous solubility (>5 mg/mL), thus meeting our first design criteria.

Stability Screening of Dexamethasone Phosphate Ester Linkers. An evaluation of the bioorthogonal stability of this series of drug linkers was conducted under the following assumption: if, as a small molecule, a drug linker was stable in blood and reactive in a lysosomal environment, then it would retain those properties as an antibody conjugate. Put simply, if this series of drug linkers did not release dexamethasone in a lysosomal environment as small molecules, then it was assumed that they would not perform well as antibody conjugates. To test this approach, stability assays that measured the disappearance of the parent and appearance of dexamethasone were conducted in blood (mouse and human) and purified lysosomal lysates (rat). These lysosomal lysates have been used previously to evaluate ADC stability for cathepsin-sensitive linkers.[25]

The results of these tests are summarized in Figure 4. Dexamethasone itself was shown to be completely stable for the 6 h duration of the assay in all matrices examined. Not surprisingly, for the phosphate diester series of drug linkers, **4**, **7**, **10**, **14**, **18**, and **28**, all compounds tested were determined to be completely stable in mouse and human blood up to 6 h (data in Supporting Information). While terminal phosphates are well-known prodrugs that convert readily in the blood compartment, phosphate esters are generally stable molecules. However, to our surprise, these phosphate diester drug linkers displayed a wide range of reactivity in the lysosomal lysate stability assay from slow release of dexamethasone (linkers **4**, **7**, **10**, **18**) to rapid release (linkers **14** and **28**), attesting to the tunable and bioorthogonal nature of their design. An important and unexpected conclusion from this small molecule work was

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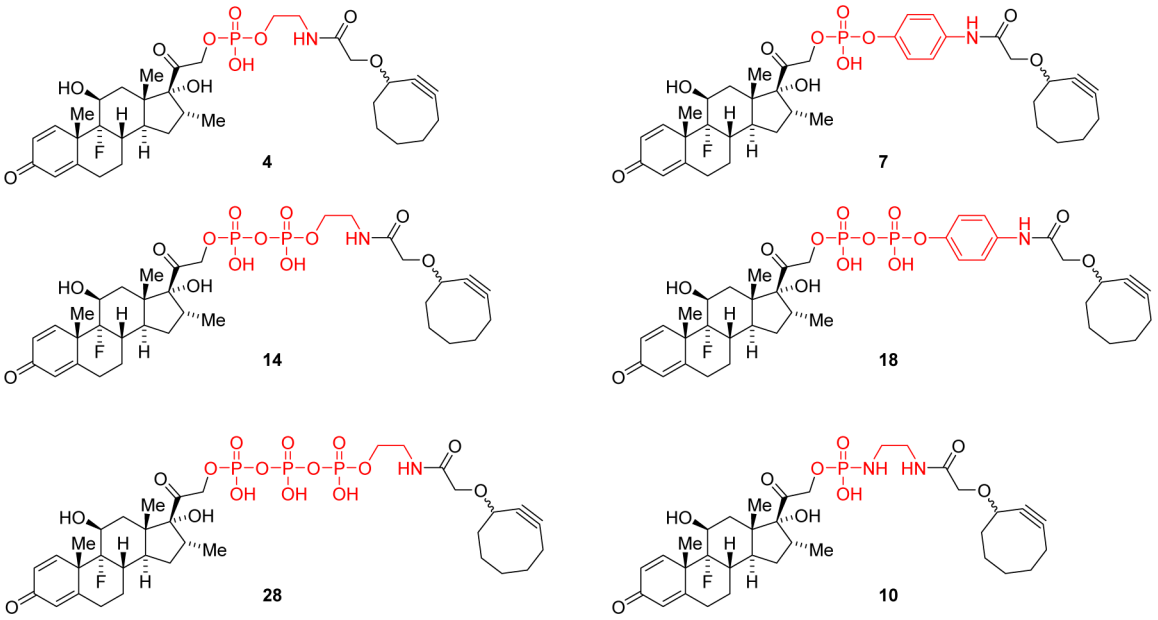

Figure 2. Series of dexamethasone phosphate linker molecules.

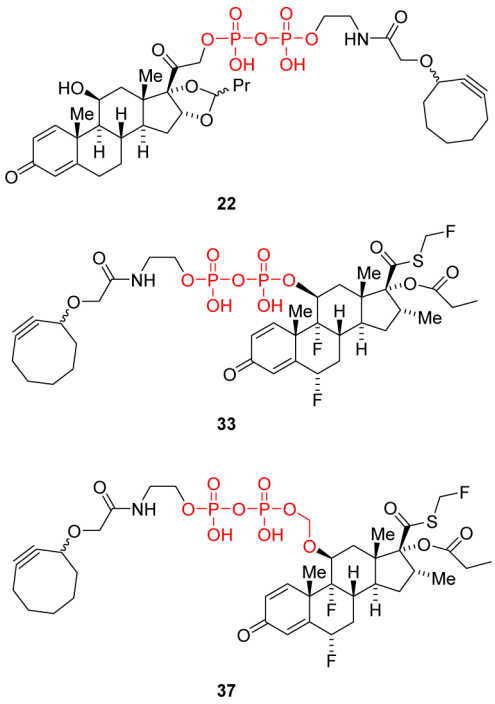

Figure 3. Series of budesonide and fluticasone propionate pyrophosphate linker molecules **22**, **33**, and **37**.

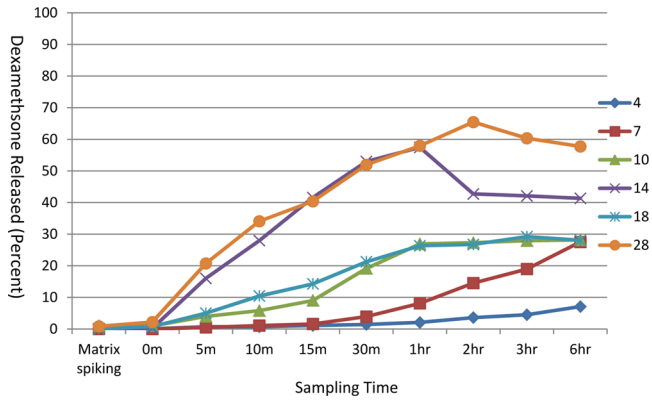

Figure 4. Rat lysosomal lysate conversion of dexamethasone drug linkers to dexamethasone.

that pyrophosphate and triphosphate diesters **14** and **28** had a unique reactivity profile with rapid conversion to payload relative to that of the monophosphate diesters. Interestingly, the phenolic pyrophosphate gave an intermediate reactivity profile, and this suggests that the rate of release may be further tuned by substitution proximal to the pyrophosphate.

Drug linkers for budesonide and fluticasone were also examined in this assay. Budesonide pyrophosphate diester **22** demonstrated a similar profile as that of the dexamethasone comparator **14** (data in Supporting Information), but the C-11-connected fluticasone propionate pyrophosphate diester **33** did not convert to the parent fluticasone propionate but instead stalled at intermediate phosphate **38** (Scheme 7). The incubation in lysosomes revealed rapid conversion to the phosphate intermediate for which further conversion to payload was not seen. Given the steric hindrance of the C-11 alcohol encountered in the synthesis of this compound, it is hypothesized that the intermediate phosphate is a poor substrate for further conversion. In contrast, acetal-spaced fluticasone pyrophosphate diester **37** was observed to convert to the intermediate phosphate and to proceed further with possible acidic hydrolysis to the parent fluticasone propionate. Thus, the introduction of the acetal spacer was required for this linker strategy to be extended to the C-11 locus available for the most potent glucocorticoid examined.

The stable profile in blood coupled with the efficient release in a lysosomal environment met the criteria for linker design within the ADC modality. Thus, a representative mAb was sought to express with an UAA in the CH1 domain, to make site-specific conjugates, and to test the functional utility of these novel linker designs in an in vitro system. At a high level, the goal of the project was to effect targeted delivery of glucocorticoids using model cell lines. For this purpose, α-CD70 was selected given the expression of CD70 on relevant

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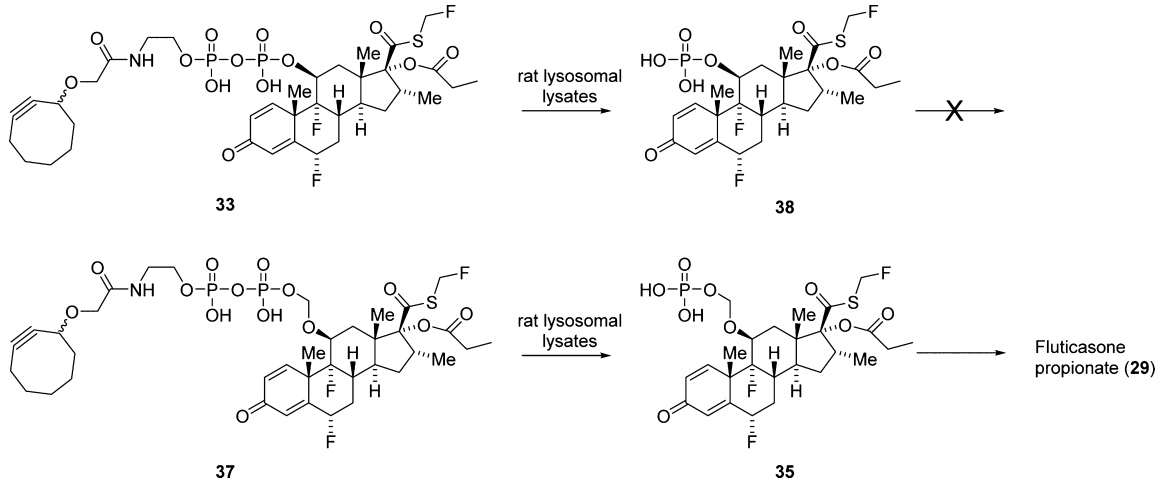
Scheme 7. Lysosomal Lysate Conversion of Fluticasone Propionate Linkers 33 and 37



human T-cells coupled with its precedent in the literature as an ADC. α-hCD70 clones have been demonstrated to be efficient, internalizing vectors for the targeted delivery of cytotoxic molecules like the auristatins[26] and PBD.[27]

Synthesis and Characterization of α-hCD70 (2h5) Conjugates. In order to test the functional performance of these novel linker designs, the team identified α-hCD70 (2h5)[28] as a fit-for-purpose system. The α-hCD70 mAb was engineered with a mutation in the heavy chain (HA114) to include *p*-azido Phe. Conjugates were produced by combining this mAb (denoted **1**−***x*** below) with excess drug linker molecules **4**, **7**, **10**, **14**, **18**, **22**, **28**, **33**, and **37** to drive the reaction to completion and produce homogeneous conjugates with a drug-to-antibody ratio (DAR) > 1.8 (see Supporting Information). These conjugates displayed high monomeric content (>98%) and were found to have <1% residual drug linker following purification. An analogous set of conjugates was produced with α-RSV (IgG1-HA114-pAF3) using the same positional mutation to create the opportunity for a matched negative control (denoted **2**−***x*** below).

In Vitro Stability of α-hCD70 Conjugates. One of the benefits of working with site-specific ADCs is the ability to fully leverage mass spectrometry to understand their metabolism. To firmly establish the stability of this linker design in plasma and to gain insight into its mechanism of cleavage in a lysosomal environment, α-hCD70 conjugates **1**−**22** and **1**−**37** with the pyrophosphate linker were studied in these matrices (Figure 5).

α-hCD70 ADCs **1**−**22** and **1**−**37** were incubated in mouse (C57BL/6) and human plasma for up to 7 days, affinity purified, and analyzed by LC-MS for intact mass, with no observed loss of budesonide (**19**) or fluticasone propionate (**29**), respectively. ADCs **1**−**22** and **1**−**37** were found to be intact in vitro up to 7 days, and the DAR calculated for time points through day 7 was unchanged (see Supporting Information). In contrast, incubation of this ADC in lysosomal lysates for 6 h did result in the release of budesonide monophosphate (**20**) followed by conversion to budesonide (**19**) for **1**−**22** (Figure 5). For **1**−**37**, fluticasone propionate acetal monophosphate (**35**) was also released as a first step, followed by rapid conversion to fluticasone propionate (**29**) itself. As a control, these lysates were boiled prior to incubation, and no payload release was observed, consistent with an enzymatic mechanism of release and not acidic release. The identity of the enzyme(s) involved in the linker cleavage is

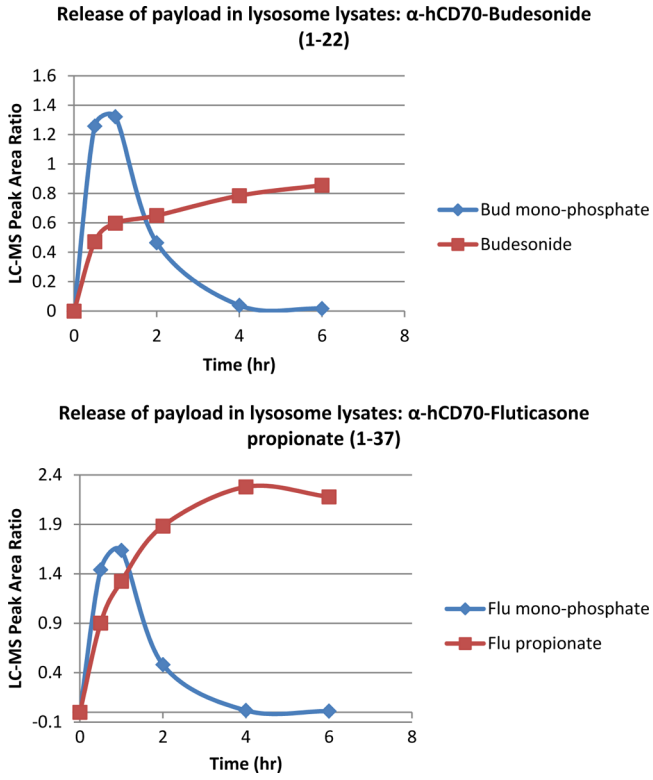


Figure 5. Stability profile of ADCs (top) **1**−**22** and (bottom) **1**−**37** in lysosomal lysates (6 h).

unknown, although it is hypothesized that two different enzymes are involved: one to clip the pyrophosphate ester and the second (likely a phosphatase) to hydrolyze the remaining terminal phosphate. Final acetal breakdown is likely to be fast and acid-mediated. Consistent with the properties observed with small molecule drug linkers **22** and **37**, analogous α-hCD70 ADCs **1**−**22** and **1**−**37** were found to be stable in plasma and reactive in a lysosomal environment.

In Vitro Potency of α-hCD70 (2h5) and α-RSV Conjugates. Cellular assays were developed to test the performance of these site-specific ADCs carrying phosphate ester-linked glucocorticoid payload. As a proximal readout of target engagement with the GR, a panel of GR-targeted genes was evaluated for their change in mRNA expression in these

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cells as a function of dexamethasone exposure and time. GILZ mRNA was identified as providing the most optimal signal for GR target engagement and was chosen as the main readout for glucocorticoid response in these cells. The ADCs were evaluated for their ability to increase GILZ mRNA expression in target-, linker-, and payload-dependent manners, with the matched negative controls included.

As shown in Figure 6, there were linker-dependent GILZ activities induced by these α-hCD70 ADCs carrying phosphate

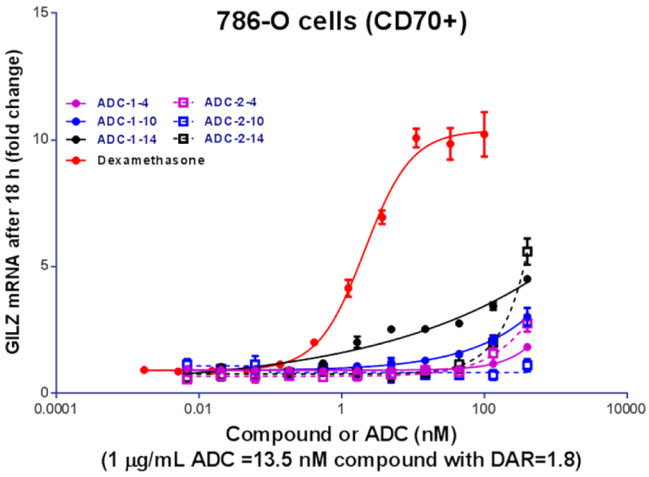

Figure 6. Representative cellular activity of α-hCD70 (**1**−***x***) or αRSV-ADCs (**2**−***x***) carrying phosphate ester-linked dexamethasone in 786-O cells.

ester-linked dexamethasone in 786-O cells,[27] a cell line with high CD70 expression on the cell surface (flow cytometry, data not shown). Overall, there was a clear relationship between the dexamethasone release rate of the drug linkers in the lysosomal lysate assay (Figure 4) and the GILZ activity in 786-O cells. For example, drug linker **4**, which was observed to show slow or negligible dexamethasone release in the lysosomal lysates, was only weakly active or inactive as an ADC in the GILZ assay in 786-O cells (ADC **1**−**4**). In contrast, drug linker **14**, which demonstrated rapid dexamethasone release in the lysosomal lysates, was among the most active as an ADC in the GILZ assay in 786-O cells (ADC **1**−**14**). The same drug linkers as conjugates with negative control α-RSV were inactive in 786-O cells except at the highest concentration. While we regard this diminished activity of the negative controls (~200×) to support our proof of concept for targeted delivery, it is possible that these α-RSV conjugates are taken up by the cells but traffic unproductively or are rapidly recycled to the surface. We interpret the weak activity of the α-RSV conjugates to reflect nonspecific uptake of the ADCs at high concentration, as has been generally observed in other ADC publications.[27] Taken together, the data support the conclusion that targeted delivery has been achieved in CD70-positive cells with an α-hCD70 mAb using these novel phosphate ester linkers and that cellular activity of these ADC correlates with the ability of the drug linkers to be cleaved in a lysosomal environment.

Although they were active in the GILZ assay in 786-O cells, the E_{max} for the most active α-hCD70−dexamethasone conjugates was only approximately 50% of that produced by free dexamethasone (Figure 6). This may reflect kinetic differences in the delivery of the small molecule and ADC modalities and/or the lack of effective accumulation of free dexamethasone inside the cells by ADC because of limited delivery, slow processing, or fast efflux. α-hCD70 ADCs with a glucocorticoid payload of higher potency such as budesonide and fluticasone propionate are expected to display increasing potency and higher E_{max} in cells. To test this hypothesis, select α-hCD70 ADCs with a rapid-cleaving pyrophosphate linker and budesonide or fluticasone propionate payload were made and tested in this same assay. As shown in Figure 7, the α-

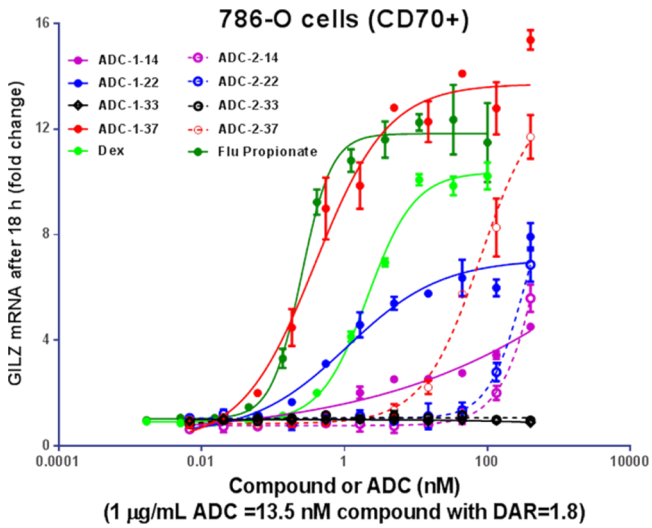

Figure 7. Cellular activity of α-hCD70 (**1**−***x***) or α-RSV (**2**−***x***) ADCs carrying phosphate ester-linked budesonide or fluticasone propionate in 786-O cells.

hCD70 ADCs with the same pyrophosphate linker but with a budesonide (**1**−**22**) or fluticasone propionate (**1**−**37**) payload displayed much higher potency and E_{max} than the dexamethasone counterpart ADC (**1**−**14**) in 786-O cells, which correlated with the enhanced potency of the payload. Again, the GILZ activity of the ADC also correlated with drug linker cleavage in the lysosome, as ADC **1**−**33** was completely inactive in the GILZ assay in 786-O cells because of the lack of conversion to free fluticasone propionate in lysosomal lysates (see Scheme 7 and Table 1). Remarkably, the E_{max} of ADC **1**−**37** equaled or exceeded that of free fluticasone propionate. Similarly, the same

Table 1. Potency of α-hCD70 Conjugates (1−*x*) and α-RSV Controls (2−*x*)

compound	potency in 786-0 cells	
	EC_{50} (IP)[a]	E_{max}
dexamethasone	2.01 nM	10.23
budesonide	0.58 nM	12.03
fluticasone propionate (**29**)	0.25 nM	11.49
α-hCD70 **1**−**14**	1.05 μg/mL (14.18 nM)[a]	4.52
α-hCD70 **1**−**22**	0.082 μg/mL (1.11 nM)	7.93
α-hCD70 **1**−**33**	inactive	1.01
α-hCD70 **1**−**37**	0.029 μg/mL (0.39 nM)	15.37
α-RSV **2**−**14**	12.28 μg/mL (165.78 nM)	5.60
α-RSV **2**−**22**	28.08 μg/mL (378.08 nM)	6.87
α-RSV **2**−**33**	inactive	1.17
α-RSV **2**−**37**	6.056 μg/mL (81.76 nM)	11.7

[a]All EC_{50} (IP) values were calculated in GraphPad Prism with nonlinear regression (curve fit). [a]Assume 1 μg/mL ADC = 13.5 nM compound (DAR = 1.8).

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drug linkers were inactive in 786-O cells except at the highest concentration as conjugates with α-RSV, further supporting the notion that targeted delivery has been achieved in CD70-positive cells with an α-hCD70 mAb using the novel pyrophosphate diester linkers.

CONCLUSIONS

In order to fully leverage the ability of antibodies to effect the targeted delivery of small molecules and widen their therapeutic index, novel linkers and payloads must be examined. In this preliminary report to assess the ADC modality beyond oncology, an effort was initiated to accomplish the targeted delivery of glucocorticoids. As an alternative to fitting existing linker strategies to this payload, a novel series of phosphate ester linkers was designed, synthesized, and evaluated to understand their properties. These phosphate linker−glucocorticoid molecules were examined as small molecules and found to be highly soluble in water, to be stable in blood, and to have a range of reactivities in a lysosomal environment dependent on their structure. These drug linker properties were retained when site-specific ADCs were made with an α-hCD70 antibody, and linkers that were observed to cleave in a lysosomal environment were also found to be active in a cell-based readout of GR target engagement. Importantly, the pyrophosphate and triphosphate diester linkers cleave much more rapidly than do the monophosphate diesters, and this correlates with the activity of their respective ADCs. Furthermore, correlation of payload potency to ADC potency was established with the budesonide and fluticasone propionate payloads, attesting to the generality of the design. Taken together with the matched negative controls, the data support the conclusion that targeted delivery of glucocorticoids has been achieved in vitro and that this novel linker design may facilitate the future development of a targeted glucocorticoid therapeutic.

From a linker standpoint, these phosphate ester linkers can be designed to release their payload at different rates as measured by a lysosomal lysate assay. This tunability for payload release may prove to be an important property of this linker design and is analogous to the different rates of cleavage seen for dipeptides sensitive to the cathepsins. When considered along with their advantageous properties including excellent solubility and robust stability in plasma, these phosphate ester linkers should find additional utility not only within the ADC modality but also in the wider targeted delivery and bioconjugate fields as well.

Several interesting observations were made in the mechanistic aspects of this effort that will require further investigation. The metabolism studies of ADCs **1**−**22** and **1**−**37** revealed a stepwise mechanism for release of the pyrophosphate linker that suggests the potential involvement of two distinct enzymes in the release of the glucocorticoid payload. It is hypothesized that a pyrophosphatase initiates the initial linker cleavage to release dexamethasone monophosphate and that a phosphatase can then rapidly convert the monophosphate to the free dexamethasone. At the time of this publication, the identity of these enzymes has not been investigated, and it is not known how the identity of this trigger impacts the broad potential of this linker design.

This article highlights the potential of the phosphate ester linker design for use in ADCs, as demonstrated through the evaluation of site-specific conjugates using the site-specific conjugation technology, and places special emphasis on pyrophosphate linkers. Nevertheless, it is recognized that validation of this linker design must be established beyond these in vitro studies limited to α-hCD70 as a delivery vehicle and glucocorticoids as payloads. Furthermore, the utility of this linker design and the potential of achieving a targeted glucocorticoid ADC will have to be examined using primary immune cells and in an in vivo setting. Work toward these goals is in progress and will be reported in due course.

EXPERIMENTAL SECTION

Chemical Synthesis of Glucocorticoid Drug Linkers. *tert-Butyl (2-(((2-((8S,9R,10S,11S,13S,14S,16R,17R)-9-Fluoro-11,17-dihydroxy-10,13,16-trimethyl-3-oxo-6,7,8,9,10,11,12,13,14,15,16,17-dodecahydro-3H-cyclopenta[a]phenanthren-17-yl)-2-oxoethoxy)(hydroxy)phosphoryl)oxy)ethyl)carbamate (**3**).* To a stirred solution of dexamethasone (**1**) (0.10 g, 0.26 mmol) in THF (0.5 mL) at −40 °C was added diphosphoryl chloride (0.12 g, 0.48 mmol), and the resulting mixture was stirred at −40 °C for 1 h. To this were added *tert*-butyl *N*-(2-hydroxyethyl)carbamate (**2**) (0.12 g, 0.76 mmol) and triethylamine (0.14 mL, 1.0 mmol). The resulting mixture was stirred at −40 °C for 4 h. The reaction was quenched with water and treated with saturated sodium bicarbonate solution until pH ∼ 8. The solution was made acidic using a 1 N HCl solution and extracted several times with ethyl acetate. The combined organic phase was concentrated onto silica gel. Flash column separation using a 0−10% isopropanol/dichloromethane gradient gave **3** (115 mg, 73%). LRMS (ES) $(M + H)^+$: calcd, 616.6; found, 616.5. Possible mixture (NMR signals were complex); see the Supporting Information.

*2-(2-(Cyclooct-2-yn-1-yloxy)acetamido)ethyl (2-((8S,9R,10S,11S,13S,14S,16R,17R)-9-Fluoro-11,17-dihydroxy-10,13,16-trimethyl-3-oxo-6,7,8,9,10,11,12,13,14,15,16,17-dodecahydro-3H-cyclopenta[a]phenanthren-17-yl)-2-oxoethyl) hydrogen phosphate (**4**).* A stirred solution of **3** (0.11 g, 0.18 mmol) in ethyl acetate (1 mL) at 0 °C was bubbled in HCl gas until it was saturated. The resulting solution was stirred at 0 °C for 1 h and concentrated. To the crude residue was added a solution of 2-(cyclooct-2-yn-1-yloxy)acetic acid (0.036 g, 0.20 mmol), HOAT (0.027 g, 0.20 mmol), EDC (0.041 g, 0.22 mmol), and triethylamine (0.05 mL, 0.36 mmol) in dichloromethane (1 mL) that was prestirred for 40 min at room temperature. Additional 2-(cyclooct-2-yn-1-yloxy)acetic acid activated with HOAT/EDC in DCM was added as necessary to complete the reaction. Upon completion, the mixture was concentrated, and reverse phase preparative chromatography (Phenomenex Gemini NX C18 OBD 5 μM, 30 × 100 mm; 10−50% MeCN/water with 0.1% NH_4OH modifier over 20 min) gave **4** (45 mg, 37%). ^1H NMR (DMSO-d_6 with 0.1% v/v TMS, 500 MHz): δ_H 0.76 (3H, d, J = 7.1 Hz), 0.85 (3H, s), 1.05 (1H, m), 1.47−1.30 (∼2H, complex), 1.49 (3H, s), 1.65−1.51 (∼3H, complex), 1.81−1.69 (∼3H, complex), 1.94−1.83 (∼2H, complex), 2.25−2.08 (∼5H, complex), 2.39−2.27 (∼2H, complex), 2.62 (1H, m), 2.92 (1H, m), 3.23 (1H, m), 3.67 (∼2H, m), 3.74 (1H, d, J = 14.7 Hz), 3.88 (1H, d, J = 14.7 Hz), 4.16−4.11 (∼2H, m), 4.29 (1H, t, J = 5.5 Hz), 4.70 (1H, dd, J = 17.2, 6.6 Hz), 5.37 (1H, dd, J = 4.1, 1.9 Hz), 5.55 (1H, br d, J = 4.3 Hz), 6.00 (1H, dd, J = 2.0, 1.4 Hz), 6.22 (1H, dd, J = 10.1, 1.9 Hz), 7.17 (∼3H, br s), 7.30 (1H, d, J = 10.2 Hz), 8.05 (1H, br t, J = 5.3 Hz). HRMS calcd for $C_{34}H_{48}FNO_{10}P$ $(M + H)^+$, 680.3000; found, 680.3007.

*tert-Butyl (4-(((2-((8S,9R,10S,11S,13S,14S,16R,17R)-9-Fluoro-11,17-dihydroxy-10,13,16-trimethyl-3-oxo-6,7,8,9,10,11,12,13,14,15,16,17-dodecahydro-3H-cyclopenta[a]-phenanthren-17-yl)-2-oxoethoxy) (hydroxy)phosphoryl)oxy)-phenyl)carbamate (**6**).* To a stirred solution of dexamethasone (**1**) (0.20 g, 0.51 mmol) in THF (1.0 mL) at −40 °C was added diphosphoryl chloride (0.24 g, 0.97 mmol), and the resulting mixture was stirred at −40 °C for 1 h 15 min. To this were added N-Boc-4-aminophenol (**5**) (0.32 g, 1.53 mmol) and triethylamine (0.56 mL, 4.0 mmol). The resulting mixture was stirred at −40 °C for 30 min. The reaction was quenched with water and treated with saturated sodium bicarbonate solution until pH ∼ 8. The solution was made acidic using a 1 N HCl solution and extracted several times with ethyl acetate. The combined organic phase was concentrated onto silica gel. Flash

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column separation using a 0−70% isopropanol/dichloromethane gradient gave **6** (370 mg, 88%). LRMS (ES) $(M + H)^+$: calcd, 664.6; found, 664.5. Used without further characterization.

*4-(2-(Cyclooct-2-yn-1-yloxy)acetamido)phenyl (2-((8S,9R,10S,11S,13S,14S,16R,17R)-9-Fluoro-11,17-dihydroxy-10,13,16-trimethyl-3-oxo-6,7,8,9,10,11,12,13,14,15,16,17-dodecahydro-3H-cyclopenta[a]phenanthren-17-yl)-2-oxoethyl) hydrogen phosphate (**7**).* A stirred solution of **6** (0.37 g, 0.55 mmol) in ethyl acetate (4 mL) at 0 °C was bubbled in HCl gas until it was saturated. The resulting solution was stirred at 0 °C for 1 h and concentrated. To the crude residue was added a solution of 2-(cyclooct-2-yn-1-yloxy)acetic acid (0.10 g, 0.56 mmol), HOAT (0.077 g, 0.56 mmol), EDC (0.13 g, 0.67 mmol), and triethylamine (0.22 mL, 1.54 mmol) in dichloromethane (4 mL) that was prestirred for 20 min at room temperature. Additional 2-(cyclooct-2-yn-1-yloxy)acetic acid activated with HOAT/EDC in DCM was added as necessary to complete the reaction. Upon completion, the mixture was washed with 1 N HCl and back extracted with ethyl acetate. The combined organic layers were concentrated purified by flash column separation using a 0−100% isopropanol/dichloromethane gradient, and reverse phase preparative chromatography (Phenomenex Gemini NX C18 OBD 5 μM, 30 × 100 mm; 10−50% MeCN/water with 0.1% NH_4OH modifier over 20 min) gave **7** (33 mg, 9%). ^{1}H NMR (DMSO-d_6 with 0.1% v/v TMS, 500 MHz): δ_H 0.76 (3H, d, J = 7.1 Hz), 0.84 (3H, s), 1.05 (1H, m), 1.45−1.33 (3H, complex), 1.48 (3H, s), 1.63−1.54 (3H, complex), 1.81−1.72 (3H, complex), 1.92−1.83 (1H, m), 2.02−1.93 (1H, m), 2.40−2.04 (∼7H, complex), 2.61 (1H, m), 2.91 (1H, m), 3.17 (1H, d, J = 5.1 Hz), 3.94 (1H, d, J = 14.6 Hz), 4.06 (1H, d, J = 14.6 Hz), 4.15 (1H, m), 4.27 (1H, dd, J = 17.5, 9.0 Hz), 4.37 (1H, m), 4.79 (1H, dd, J = 17.5, 6.3 Hz), 5.37 (1H, d, J = 4.2 Hz), 5.41 (1H, s), 6.00 (1H, dd, J = 2.0, 1.4 Hz), 6.22 (1H, dd, J = 10.1, 1.9 Hz), 7.04 (2H, d, J = 8.5 Hz), 7.15 (∼3H, br s), 7.30 (1H, d, J = 10.1 Hz), 7.43 (2H, d, J = 8.5 Hz), 9.53 (1H, s). HRMS calcd for $C_{38}H_{48}FNO_{10}P$ $(M + H)^+$, 728.3000; found, 728.3016.

*2-((8S,9R,10S,11S,13S,14S,16R,17R)-9-Fluoro-11,17-dihydroxy-10,13,16-trimethyl-3-oxo-6,7,8,9,10,11,12,13,14,15,16,17-dodecahydro-3H-cyclopenta[a]phenanthren-17-yl)-2-oxoethyl Dihydrogen Phosphate (**8**).* To a stirred solution of dexamethasone (0.50 g, 1.27 mmol) in THF (2.5 mL) at −40 °C was added diphosphoryl chloride (0.53 mL, 3.82 mmol), and the resulting mixture was stirred at −40 °C for 30 min. The reaction was quenched with water and treated with saturated sodium bicarbonate solution until pH ∼ 8. The solution was made acidic using a 1 N HCl solution and extracted several times with ethyl acetate. The combined organic phase washed with brine, dried over sodium sulfate, and concentrated to give **8** as a solid (555 mg, 92%). LRMS (ES) $(M + H)^+$: calcd, 472.4; found, 473.3. ^{1}H NMR (DMSO-d_6 with 0.1% v/v TMS, 500 MHz): δ_H 0.79 (3H, d, J = 7.2 Hz), 0.88 (3H, s), 1.10−1.05 (1H, m), 1.35 (1H, m), 1.48−1.43 (4H, m), 1.63 (1H, q, J = 11.8 Hz), 1.79−1.75 (1H, m), 2.16−2.07 (2H, m), 2.38−2.30 (2H, m), 2.62 (1H, td, J = 13.2, 5.8 Hz), 2.93 (1H, ddd, J = 11.4, 7.3, 4.3 Hz), 4.15 (1H, d, J = 10.4 Hz), 4.51 (1H, dd, J = 18.0, 6.3 Hz), 4.92 (1H, dd, J = 18.0, 7.4 Hz), 5.14 (1H, br s), 5.37 (1H, br s), 6.01 (1H, s), 6.23 (1H, dd, J = 10.1, 1.9 Hz), 7.29 (1H, d, J = 10.1 Hz).

*N-(2-Aminoethyl)-2-(cyclooct-2-yn-1-yloxy)acetamide (**9**).* To a solution of 2-(cyclooct-2-yn-1-yloxy)acetic acid (0.10 g, 0.56 mmol) in DCM (2 mL) were added HOAT (0.075 g, 0.55 mmol) and EDC (0.13 g, 0.67 mmol), and the resulting mixture was stirred for 20 min at room temperature. The mixture was then added to a solution of ethylene diamine (0.49 g, 8.23 mmol) in DCM (1 mL). Upon completion, the mixture was purified by reverse phase preparative chromatography (Phenomenex Gemini NX C18 OBD 5 μM, 20 × 50 mm; 20−60% MeCN/water with 0.1% NH_4OH modifier over 7 min) to give **9** (50 mg, 40%). LRMS (ES) $(M + H)^+$: calcd, 224.2; found, 225.2. ^{1}H NMR (DMSO-d_6 with 0.1% v/v TMS, 500 MHz): δ_H 1.43−1.37 (1H, m), 1.67−1.53 (2H, m), 1.82−1.72 (2H, m), 1.99−1.83 (2H, m), 2.19−2.07 (2H, m), 2.27−2.21 (1H, m), 2.58 (2H, t, J = 6.5 Hz), 3.08 (2H, q, J = 6.3 Hz), 3.76 (1H, d, J = 15.0 Hz), 3.87 (1H, d, J = 14.7 Hz), 4.29 (1H, dd, J = 6.8, 4.9 Hz), 7.61 (1H, t, J = 6.0 Hz).

*2-((8S,9R,10S,11S,13S,14S,16R,17R)-9-Fluoro-11,17-dihydroxy-10,13,16-trimethyl-3-oxo-6,7,8,9,10,11,12,13,14,15,16,17-dodecahydro-3H-cyclopenta[a]phenanthren-17-yl)-2-oxoethyl hydrogen (2-(2-(Cyclooct-2-yn-1-yloxy)acetamido)ethyl)phosphoramidate (**10**).* To a stirred solution of **9** (0.05 g, 0.22 mmol) and **8** (0.035 g, 0.074 mmol) in a solution of *t*-butanol (1.2 mL) and water (0.25 mL) was added DCC (0.06 g, 0.30 mmol), and the resulting mixture was heated at 100 °C for 4 h. The reaction mixture was allowed to cool and concentrated. The residue was dissolved in a 1:1:1 MeOH/water/MeCN solution and syringe filtered. The mixture was purified using reverse phase preparative chromatography (Phenomenex Gemini NX C18 OBD 5 μM, 30 × 100 mm; 5−45% MeCN/water with 0.1% NH_4OH modifier over 20 min) to give **10** (15 mg, 30%). ^{1}H NMR (DMSO-d_6 with 0.1% v/v TMS, 500 MHz): δ_H 0.76 (3H, d, J = 7.2 Hz), 0.85 (3H, s), 1.05 (1H, m), 1.37 (2H, m), 1.49 (3H, s), 1.63−1.5 (∼4H, complex), 1.76 (3H, complex), 1.85 (1H, m), 1.93 (1H, m), 2.40−2.20 (7H, complex), 2.62 (1H, m), 2.74 (2H, m), 2.92 (1H, m), 3.07 (2H, m), 3.17 (2H, s), 3.73 (1H, d, J = 14.6 Hz), 3.86 (1H, d, J = 14.5 Hz), 4.19−4.03 (3H, complex), 4.29 (1H, br t, J = 5.5 Hz), 4.64 (1H, br dd, J = 16.6, 7.4 Hz), 6.00 (1H, s), 6.21 (1H, dd, J = 10.0, 1.9 Hz), 7.31 (1H, d, J = 10.1 Hz), 8.25 (1H, br s). HRMS calcd for $C_{34}H_{49}FN_2O_9P$ $(M + H)^+$, 679.3159; found, 679.3163.

*(9H-Fluoren-9-yl)methyl (2-(phosphonooxy)ethyl)carbamate (**12**).* To a stirred solution of (9*H*-fluoren-9-yl)methyl (2-hydroxyethyl)carbamate (0.25 g, 0.88 mmol) in THF (1.7 mL) at −40 °C was added diphosphoryl chloride (0.30 mL, 2.20 mmol), and the resulting mixture was stirred at −40 °C for 30 min. The reaction was quenched with water and treated with saturated sodium bicarbonate solution until pH ∼ 8. The solution was made acidic using a 1 N HCl solution and extracted several times with dichloromethane. The combined organic phase was allowed to sit for 48 h, during which time a solid precipitated. The solid was filtered to give **12** as a solid (265 mg, 83%). LRMS (ES) $(M + H)^+$: calcd, 363.3; found, 364.2. ^{1}H NMR (DMSO-d_6 with 0.1% v/v TMS, 500 MHz): δ_H 3.21 (2H, q, J ∼ 5.9 Hz), 3.80 (2H, q, J ∼ 6.7 Hz), 4.21 (1H, t, J = 7.1 Hz), 4.29 (2H, d, J = 6.8 Hz), 7.33 (2H, t, J = 7.4 Hz), 7.45−7.40 (3H, m), 7.70 (2H, d, J = 7.5 Hz), 7.89 (2H, d, J = 7.6 Hz).

*(9H-Fluoren-9-yl)methyl (2-(((((2-((8S,9R,10S,11S,13S,14S,16R,-17R)-9-Fluoro-11,17-dihydroxy-10,13,16-trimethyl-3-oxo-6,7,8,9,10,11,12,13,14,15,16,17-dodecahydro-3H-cyclopenta[a]-phenanthren-17-yl)-2-oxoethoxy) (hydroxy)phosphoryl)oxy)-(hydroxy)phosphoryl)oxy)ethyl)carbamate (**13**).* To a stirred solution of **12** (0.15 g, 0.41 mmol) in DMF (1.2 mL) were added triethylamine (0.06 mL, 0.41 mmol) and CDI (0.17 g, 1.03 mmol). The resulting solution was stirred at room temperature for 30 min. To this mixture were added **8** (0.19 g, 0.41 mmol) and $ZnCl_2$ (0.45 g, 3.31 mmol), and the mixture was allowed to stir at room temperature overnight. The reaction was diluted with 1 N HCl and extracted several times with ethyl acetate. The combined organic layers were concentrated, and reverse phase preparative chromatography (Phenomenex Gemini NX C18 OBD 5 μM, 30 × 100 mm; 5−35% MeCN/water with 0.1% NH_4OH modifier over 20 min) gave **13** (134 mg, 40%). LRMS (ES) $(M + H)^+$: calcd, 818.7; found, 818.6. Possible mixture (NMR signals were complex); see the Supporting Information.

*2-(2-(Cyclooct-2-yn-1-yloxy)acetamido)ethyl (2-((8S,9R,10S,11S,-13S,14S,16R,17R)-9-Fluoro-11,17-dihydroxy-10,13,16-trimethyl-3-oxo-6,7,8,9,10,11,12,13,14,15,16,17-dodecahydro-3H-cyclopenta-[a]phenanthren-17-yl)-2-oxoethyl) Dihydrogen Pyrophosphate (**14**).* To a stirred solution of **13** (0.19 g, 0.23 mmol) in DCM (3 mL) was added piperidine (0.15 mL, 1.51 mmol), and the resulting mixture was stirred at room temperature for 3 h. The solution was concentrated to dryness and redissolved in DCM (2 mL). To a stirred solution of 2-(cyclooct-2-yn-1-yloxy)acetic acid (0.045 g, 0.25 mmol) in dichloromethane (1 mL) in a separate vial was added HOAT (0.034 g, 0.25 mmol), EDC (0.056 g, 0.30 mmol), and triethylamine (0.1 mL, 0.68 mmol). The resulting solution was stirred at room temperature for 40 min. The two solutions were combined and stirred at room temperature. Additional 2-(cyclooct-2-yn-1-yloxy)acetic acid activated with HOAT/EDC was added as necessary to complete the reaction. Upon completion, the mixture was concentrated, and reverse phase preparative chromatography (Phenomenex Gemini NX C18 OBD 5 μM, 30 × 100 mm; 5−30% MeCN/water with 0.1% NH_4OH modifier

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J. Am. Chem. Soc. 2016, 138, 1430−1445

over 20 min) gave **14** (59 mg, 34%). ^{1}H NMR (DMSO-d_6 with 0.1% v/v TMS, 500 MHz): δ_H 0.77 (3H, d, J = 7.2 Hz), 0.88 (3H, s), 1.05 (1H, m), 1.18 (1H, m), 1.43−1.31 (2H, m), 1.50 (3H, s), 1.67−1.52 (∼3H, complex), 1.81−1.69 (∼2H, m), 1.96−1.81 (∼2H, complex), 2.17−2.02 (∼3H, complex), 2.4−2.19 (∼2H, complex), 2.62 (1H, m), 3.12−2.89 (∼2H, complex), ∼3.23 (2H, m), 3.79−3.76 (3H, m), 3.92 (1H, dd, J = 14.4, 8.6 Hz), 4.12 (1H, br d, J = 11.2 Hz), 4.31 (1H, br t, J = 5.4 Hz), 4.57 (2H, br d, J = 8.3 Hz), 6.00 (1H, s), 6.21 (1H, dd, J = 10.1, 1.9 Hz), 7.29 (1H, d, J = 9.2 Hz), ∼7.29 (∼3H, br s), 8.64 (1H, br s). HRMS calcd for $C_{34}H_{49}FNO_{13}P_2$ $(M + H)^+$, 758.2507; found, 758.2511.

*(9H-Fluoren-9-yl)methyl (4-Hydroxyphenyl)carbamate (**15**).* To a stirred solution of 4-aminophenol (0.30 g, 2.75 mmol) in DCM (9 mL) was added (9*H*-fluoren-9-yl)methyl carbonochloridate (0.71 g, 2.75 mmol), and the resulting mixture was stirred at room temperature for 2 h. The solution was directly purified by flash column separation using a 0−100% ethyl acetate/hexane gradient to give the title compound (634 mg, 70%). ^{1}H NMR DMSO-d_6 with 0.1% v/v TMS, 500 MHz): δ_H 4.28 (1H, t, J = 6.7 Hz), 4.43 (2H, br d, J = 6.9 Hz), 6.66 (2H, br d, J = 8.3 Hz), 7.24 (2H, br d, J = 8.8 Hz), 7.35 (2H, t, J = 7.5 Hz), 7.43 (2H, t, J = 7.5 Hz), 7.74 (2H, br d, J = 7.5 Hz), 7.91 (2H, d, J = 7.5 Hz), 9.09 (1H, s), 9.38 (1H, br s).

*(9H-Fluoren-9-yl)methyl (4-(Phosphonooxy)phenyl)carbamate (**16**).* To a stirred solution of (9*H*-fluoren-9-yl)methyl (4-hydroxyphenyl)carbamate (0.31 g, 0.95 mmol) in THF (1.9 mL) at −40 °C was added diphosphoryl chloride (0.33 mL, 2.38 mmol), and the resulting mixture was stirred at −40 °C for 3 h. The reaction was quenched with water and treated with saturated sodium bicarbonate solution until pH ∼ 8. The solution was made acidic using a 1 N HCl solution and extracted several times with ethyl acetate. The combined organic phase washed with brine, dried over sodium sulfate, and concentrated to give **16** (342 mg, 87%). LRMS (ES) $(M + H)^+$: calcd, 411.3; found, 412.3. ^{1}H NMR (DMSO-d_6 with 0.1% v/v TMS, 500 MHz): δ_H 4.31 (1H, t, J = 6.6 Hz), 4.48 (2H, br d, J = 6.7 Hz), 7.06 (2H, br d, J = 8.4 Hz), 7.35 (2H, t, J = 7.6 Hz), ∼7.39 (2H, br), 7.43 (2H, t, J = 7.7 Hz), 7.75 (2H, d, J = 7.5 Hz), 7.91 (2H, d, J = 7.6 Hz), 9.66 (1H, br s).

*(9H-Fluoren-9-yl)methyl (4-(((((2-((8S,9R,10S,11S,13S,14S,16R,-17R)-9-fluoro-11,17-dihydroxy-10,13,16-trimethyl-3-oxo-6,7,8,9,10,11,12,13,14,15,16,17-dodecahydro-3H-cyclopenta[a]-phenanthren-17-yl)-2-oxoethoxy) (hydroxy)phosphoryl)oxy)-(hydroxy)phosphoryl)oxy)phenyl)carbamate (**17**).* To a stirred solution of **16** (0.15 g, 0.37 mmol) in DMF (1.0 mL) were added triethylamine (0.05 mL, 0.37 mmol) and CDI (0.15 g, 0.94 mmol). The resulting solution was stirred at room temperature for 30 min. To this mixture were added **8** (0.18 g, 0.38 mmol) and $ZnCl_2$ (0.41 g, 3.05 mmol), and the mixture was allowed to stir at room temperature overnight. The reaction was diluted with 1 N HCl and extracted several times with ethyl acetate. The combined organic layers were concentrated. The residue was dissolved in a 1:1:1 MeOH/water/MeCN solution and syringe filtered. The mixture was purified using reverse phase preparative chromatography (Phenomenex Gemini NX C18 OBD 5 μM, 30 × 100 mm; 5−40% MeCN/water with 0.1% NH_4OH modifier over 20 min) to give **17** (170 mg, 52%). LRMS (ES) $(M + H)^+$: calcd, 865.7; found, 866.5. Possible mixture (NMR signals were complex); see the Supporting Information.

*4-(2-(Cyclooct-2-yn-1-yloxy)acetamido)phenyl (2-((8S,9R,10S,-11S,13S,14S,16R,17R)-9-Fluoro-11,17-dihydroxy-10,13,16-trimethyl-3-oxo-6,7,8,9,10,11,12,13,14,15,16,17-dodecahydro-3H-cyclopenta-[a]phenanthren-17-yl)-2-oxoethyl) Dihydrogen Pyrophosphate (**18**).* To a stirred solution of **17** (0.17 g, 0.19 mmol) in DCM (3 mL) was added piperidine (0.29 mL, 2.95 mmol), and the resulting mixture was stirred at room temperature for 1 h and concentrated. The residue was dissolved in a 1:1:1 MeOH/water/MeCN solution and syringe filtered. The mixture was purified using reverse phase preparative chromatography (Phenomenex Gemini NX C18 OBD 5 μM, 30 × 100 mm; 3−30% MeCN/water with 0.1% NH_4OH modifier over 20 min) and then redissolved in DCM (1 mL). To a stirred solution of 2-(cyclooct-2-yn-1-yloxy)acetic acid (0.07 g, 0.39 mmol) in dichloromethane (1 mL) in a separate vial were added HOAT (0.053 g, 0.39 mmol) and EDC (0.094 g, 0.49 mmol). The resulting solution was stirred at room temperature for 20 min. The two solutions were combined and stirred at room temperature. Upon completion, the mixture was concentrated, and reverse phase preparative chromatography (Phenomenex Gemini NX C18 OBD 5 μM, 30 × 100 mm; 5−35% MeCN/water with 0.1% NH_4OH modifier over 20 min) gave **18** (10 mg, 6%). ^{1}H NMR (DMSO-d_6 with 0.1% v/v TMS, 500 MHz): δ_H 0.76 (3H, d, J = 7.1 Hz), 0.85 (3H, s), 1.05 (1H, m), 1.48 (3H, s), 1.9−1.3 (∼15H, complex), 2.38−1.93 (∼5H, complex), 2.62 (1H, m), 2.93 (1H, m), 3.94 (1H, d, J = 14.6 Hz), 4.06 (1H, d, J = 14.6 Hz), 4.06 (1H, obs m), 4.37 (1H, br t, J = 5.6 Hz), 4.47 (1H, dd, J = 17.3, 9.4 Hz), 4.66 (1H, dd, J = 17.6, 7.0 Hz), 5.99 (1H, s), 6.20 (1H, d, J = 10.1 Hz), 7.11 (2H, d, J = 8.3 Hz), 7.26 (1H, d, J = 10.2 Hz), 7.45 (2H, d, J = 8.6 Hz), 9.53 (1H, s). HRMS calcd for $C_{38}H_{49}FNO_{13}P_2$ $(M + H)^+$, 808.2663; found, 808.2697.

*2-((6aR,6bS,7S,8aS,8bS,11aR,12aS,12bS)-7-Hydroxy-6a,8a-dimethyl-4-oxo-10-propyl-2,4,6a,6b,7,8,8a,8b,11a,12,12a,12b-dodecahydro-1H-naphtho[2′,1′:4,5]indeno[1,2-d][1,3]dioxol-8b-yl)-2-oxoethyl Dihydrogen Phosphate (**20**).* To a stirred solution of budesonide (**19**) (4.0 g, 9.29 mmol) in THF (18.5 mL) at −40 °C was added diphosphoryl chloride (2.57 mL, 18.58 mmol), and the resulting mixture was stirred at −40 °C for 2 h. The reaction was quenched with water and treated with saturated sodium bicarbonate solution until pH ∼ 8. The solution was made acidic using a 1 N HCl solution and extracted several times with ethyl acetate. The combined organic phase washed with brine, dried over sodium sulfate, and concentrated to give **20** (3.55 g, 75%). LRMS (ES) (M + H)+: calcd, 510.5; found, 511.2. ^{1}H NMR (DMSO- d_6 with 0.1% v/v TMS, 500 MHz): δ_H 0.89−0.82 (6H, m), 1.04−0.90 (∼1.5H, m), 1.10 (0.5H, m), 1.46−1.23 (∼6H, complex), 1.62−1.49 (∼3.5H, m), 1.83−1.71 (∼2.5H, m), 2.13−1.92 (∼2H, complex), 2.29 (1H, br d, J = 13.2 Hz), 2.55−2.50 (∼1H, obs m), 4.30 (1H, m), 4.48 (0.5H, dd, J = 18.2, 7.0 Hz), 4.55 (0.5H, dd, J = 17.8, 7.2 Hz), 4.59 (0.5H, t, J = 4.6 Hz), 4.75 (0.5H, d, J = 4.3 Hz), 4.78 (0.5H, dd, J = 18.1, 7.9 Hz), 4.85 (0.5H, dd, J = 18.5, 8.4 Hz), 5.07 (0.5H, d, J = 7.3 Hz), 5.19 (0.5H, t, J = 4.8 Hz), 5.92 (1H, s), 6.17 (1H, d, J = 10.1 Hz), 7.31 (1H, dd, J = 10.1, 3.9 Hz).

*(9H-Fluoren-9-yl)methyl (2-((Hydroxy((hydroxy(2-((6aR,6bS,7-S,8aS,8bS,11aR,12aS,12bS)-7-hydroxy-6a,8a-dimethyl-4-oxo-10-propyl-2,4,6a,6b,7,8,8a,8b,11a,12,12a,12b-dodecahydro-1H-naphtho[2′,1′:4,5]indeno[1,2-d][1,3]dioxol-8b-yl)-2-oxoethoxy)-phosphoryl)oxy)phosphoryl)oxy)ethyl)carbamate (**21**).* To a stirred solution of **12** (0.125 g, 0.34 mmol) in DMF (1 mL) were added triethylamine (0.05 mL, 0.35 mmol) and CDI (0.14 g, 0.86 mmol). The resulting solution was stirred at room temperature for 30 min. To this mixture were added **19** (0.17 g, 0.34 mmol) and $ZnCl_2$ (0.37 g, 2.73 mmol), and the mixture was allowed to stir at room temperature overnight. The reaction was diluted with methanol and filtered. The crude was purified by reverse phase preparative chromatography (Phenomenex Gemini NX C18 OBD 5 μM, 30 × 100 mm; 5−45% MeCN/water with 0.1% NH_4OH modifier over 20 min) to give **21** (200 mg, 69%). LRMS (ES) $(M + H)^+$: calcd, 855.8; found, 856.3. Possible mixture (NMR signals were complex); see the Supporting Information.

*2-(2-(Cyclooct-2-yn-1-yloxy)acetamido)ethyl (2-((6aR,6bS,7-S,8aS,8bS,11aR,12aS,12bS)-7-Hydroxy-6a,8a-dimethyl-4-oxo-10-propyl-2,4,6a,6b,7,8,8a,8b,11a,12,12a,12b-dodecahydro-1H-naphtho[2′,1′:4,5]indeno[1,2-d][1,3]dioxol-8b-yl)-2-oxoethyl) Dihydrogen Pyrophosphate (**22**).* To a stirred solution of **21** (0.20 g, 0.23 mmol) in DCM (3 mL) was added piperidine (0.16 mL, 1.61 mmol), and the resulting mixture was stirred at room temperature for 1.5 h and concentrated. The residue was dissolved in a 2:1:1 MeOH/water/MeCN solution and syringe filtered. The mixture was purified using reverse phase preparative chromatography (Phenomenex Gemini NX C18 OBD 5 μM, 30 × 100 mm; 5−35% MeCN/water with 0.1% NH_4OH modifier over 20 min) and then redissolved in DMF (0.8 mL). To this solution were added triethylamine (0.04 mL, 0.31 mmol) and 1,3-dioxoisoindolin-2-yl 2-(cyclooct-2-yn-1-yloxy)acetate (0.08 g, 0.24 mmol). The reaction was stirred for 30 min at room temperature and then directly purified using reverse phase preparative chromatography (Phenomenex Gemini NX C18 OBD 5 μM, 30 × 100 mm; 5−45% MeCN/water with 0.1% NH_4OH modifier over 20 min) to give **22** (54 mg, 86%). NMR signals indicated a complex mixture of

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isomers; see the Supporting Information. HRMS calcd for $C_{37}H_{54}NO_{14}P_2$ $(M + H)^+$, 798.3019; found, 798.3028.

*(9H-Fluoren-9-yl)methyl Dihydrogen Phosphate (**24**).* To a stirred solution of (9*H*-fluoren-9-yl)methanol (0.30 g, 1.53 mmol) in THF (3.0 mL) at −40 °C was added diphosphoryl chloride (0.53 mL, 3.82 mmol), and the resulting mixture was stirred at −40 °C for 2 h. The reaction was quenched with water and treated with saturated sodium bicarbonate solution until pH ∼ 8. The solution was made acidic using a 1 N HCl solution and extracted several times with ethyl acetate. The combined organic phase washed with brine, dried over sodium sulfate, and concentrated to give **24** (0.42 g, >95%). LRMS (ES) $(M + H)^+$: calcd, 276.2; found, 277.1. ^{1}H NMR (DMSO-d_6 with 0.1% v/v TMS, 500 MHz): δ_H 4.16 (2H, t, J = 6.5 Hz), 4.24 (1H, t, J = 6.8 Hz), 7.34 (2H, t, J = 7.4 Hz), 7.42 (2H, t, J = 7.5 Hz), 7.67 (2H, d, J = 7.5 Hz), 7.89 (2H, d, J = 7.5 Hz).

*((9H-Fluoren-9-yl)methyl) (2-((8S,9R,10S,11S,13S,14S,16R,17R)-9-Fluoro-11,17-dihydroxy-10,13,16-trimethyl-3-oxo-6,7,8,9,10,11,12,13,14,15,16,17-dodecahydro-3H-cyclopenta[a]-phenanthren-17-yl)-2-oxoethyl) Dihydrogen Pyrophosphate (**25**).* To a stirred solution of **24** (0.20 g, 0.72 mmol) in DMF (2 mL) were added triethylamine (0.10 mL, 0.72 mmol) and CDI (0.29 g, 1.81 mmol). The resulting solution was stirred at room temperature for 30 min. To this mixture were added **8** (0.34 g, 0.72 mmol) and $ZnCl_2$ (0.79 g, 5.79 mmol), and the mixture was allowed to stir at room temperature overnight. The reaction was diluted with 1 N HCl and extracted several times with ethyl acetate. The combined organic layers were concentrated to give **25** (348 mg, 66%). LRMS (ES) $(M + H)^+$: calcd, 730.6; found, 731.2. Possible mixture (NMR signals were complex); see the Supporting Information.

*2-((8S,9R,10S,11S,13S,14S,16R,17R)-9-Fluoro-11,17-dihydroxy-10,13,16-trimethyl-3-oxo-6,7,8,9,10,11,12,13,14,15,16,17-dodecahydro-3H-cyclopenta[a]phenanthren-17-yl)-2-oxoethyl Trihydrogen Diphosphate (**26**).* To a stirred solution of **25** (0.29 g, 0.39 mmol) in DCM (2 mL) was added piperidine (0.23 mL, 2.36 mmol), and the resulting mixture was stirred at room temperature for 1.5 h and concentrated. The mixture was purified using reverse phase preparative chromatography (Phenomenex Gemini NX C18 OBD 5 μM, 30 × 100 mm; 3−25% MeCN/water with 0.1% NH_4OH modifier over 20 min) to give **26** (123 mg, 56%). LRMS (ES) $(M + H)^+$: calcd, 552.4; found, 553.2. ^{1}H NMR (DMSO-d_6 with 0.1% v/v TMS, 500 MHz): δ_H 0.77 (3H, d, J = 7.1 Hz), 0.87 (3H, s), 1.06−1.02 (1H, m), 1.39−1.31 (1H, m), 1.50 (3H, s), 1.59 (1H, q, J = 12.2 Hz), 1.76−1.73 (1H, m), 1.93−1.90 (1H, m), 2.06−2.03 (2H, m), 2.36−2.29 (2H, m), 2.64−2.59 (1H, m), 2.94 (1H, ddd, J = 11.4, 7.1, 4.2 Hz), 4.13 (1H, d, J = 11.6 Hz), 4.60−4.57 (2H, m), 5.99 (1H, s), 6.19 (1H, dd, J = 10.0, 1.9 Hz), 7.31 (1H, d, J = 10.2 Hz).

*(9H-Fluoren-9-yl)methyl (2-(((((((2-((8S,9R,10S,11S,13S,14S,16R,-17R)-9-Fluoro-11,17-dihydroxy-10,13,16-trimethyl-3-oxo-6,7,8,9,10,11,12,13,14,15,16,17-dodecahydro-3H-cyclopenta[a]-phenanthren-17-yl)-2-oxoethoxy)(hydroxy)phosphoryl)oxy)-(hydroxy)phosphoryl)oxy)(hydroxy)phosphoryl)oxy)ethyl)-carbamate (**27**).* To a stirred solution of **12** (0.08 g, 0.22 mmol) in DMF (1.0 mL) were added triethylamine (0.03 mL, 0.22 mmol) and CDI (0.90 g, 0.56 mmol). The resulting solution was stirred at room temperature for 30 min. To this mixture were added **26** (0.12 g, 0.22 mmol) and $ZnCl_2$ (0.24 g, 1.78 mmol), and the mixture was allowed to stir at room temperature overnight. The reaction was diluted with 1 N HCl and extracted several times with ethyl acetate. The combined organic layers were concentrated, and reverse phase preparative chromatography (Phenomenex Gemini NX C18 OBD 5 μM, 30 × 100 mm; 5−40% MeCN/water with 0.1% NH_4OH modifier over 20 min) gave **27** (45 mg, 23%). LRMS (ES) $(M + H)^+$: calcd, 897.7; found, 898.3. ^{1}H NMR (DMSO-d_6 with 0.1% v/v TMS, 500 MHz): δ_H 0.77 (3H, d, J = 7.2 Hz), 0.89 (3H, s), 1.04 (1H, m), 1.35 (1H, m), 1.48 (3H, s), 1.58 (1H, m), 1.74 (1H, m), 1.90 (1H, m), 2.10−2.02 (2H, m), 2.38−2.26 (2H, m), 2.60 (1H, m), 2.94 (1H, m), ∼3.20 (∼2H, m), 3.82 (2H, m), 4.15 (1H, d, J = 12.1 Hz), 4.26−4.18 (3H, m), 4.59 (1H, dd, J = 17.8, 7.8 Hz), 4.73 (1H, br d, J = 17.1 Hz), 5.98 (1H, s), 6.17 (1H, d, J = 10.2 Hz), 7.28 (1H, d, J = 10.4 Hz), 7.34 (2H, t, J = 7.3 Hz), 7.40 (2H, t, J = 7.3 Hz), 7.73 (2H, d, J = 7.2 Hz), 7.87 (2H, d, J = 7.4 Hz), 7.99 (1H, br s).

*2-(2-(Cyclooct-2-yn-1-yloxy)acetamido)ethyl (2-((8S,9R,10S,11S,13S,14S,16R,17R)-9-Fluoro-11,17-dihydroxy-10,13,16-trimethyl-3-oxo-6,7,8,9,10,11,12,13,14,15,16,17-dodecahydro-3H-cyclopenta[a]phenanthren-17-yl)-2-oxoethyl) Trihydrogen Triphosphate (**28**).* To a stirred solution of **27** (0.048 g, 0.053 mmol) in DCM (1 mL) was added piperidine (0.04 mL, 0.38 mmol), and the resulting mixture was stirred at room temperature for 1 h and concentrated. The residue was dissolved in a 2:1:1 MeOH/water/MeCN solution and syringe filtered. The mixture was purified using reverse phase preparative chromatography (Phenomenex Gemini NX C18 OBD 5 μM, 30 × 100 mm; 3−30% MeCN/water with 0.1% NH_4OH modifier over 20 min) and then redissolved in DMF (0.8 mL). To this solution were added triethylamine (0.02 mL, 0.16 mmol) and 1,3-dioxoisoindolin-2-yl 2-(cyclooct-2-yn-1-yloxy)acetate (0.04 g, 0.12 mmol). The reaction was stirred for 30 min at room temperature and then directly purified using reverse phase preparative chromatography (Phenomenex Gemini NX C18 OBD 5 μM, 30 × 100 mm; 5−40% MeCN/water with 0.1% NH_4OH modifier over 20 min) to give **28** (8 mg, 24%). ^{1}H NMR (DMSO-d_6 with 0.1% v/v TMS, 500 MHz, presat): δ_H 0.78 (3H, d, J = 7.1 Hz), 0.88 (3H, s), 1.07−1.02 (1 H, m), 1.42−1.32 (2H, m), 1.50 (3H, s) 1.67−1.48 (5H, m), 1.81−1.68 (3H, m), 1.98−1.81 (3H, m), 2.16−2.02 (3.5H, m), 2.40−2.19 (2.5H, m), 2.62 (1H, m), 3.01−2.92 (2H, m), ∼3.25 (∼2H, m overlap with H_2O signal), 3.78 (1H, d, J = 14.5 Hz), 3.80 (2H, m), 3.91 (1H, d, J = 14.5 Hz), 4.14 (1H, br d, J = 11.4 Hz), 4.31 (1H, br t, J = 5.1 Hz), 4.56 (1H, dd, J = 18.0, 8.1 Hz), 4.71 (1H, dd, J = 17.9, 7.1 Hz), 5.99 (1H, s), 6.20 (1H, d, J = 10.1 Hz), 7.30 (2H, d, J = 10.7 Hz), 7.37 (3H, br s), 8.48 (1H, br s). HRMS calcd for $C_{34}H_{49}FNO_{16}P_3$ $(M + H)^+$, 838.2170; found, 838.2150.

*(6S,8S,9R,10S,11S,13S,14S,16R,17R)-6,9-Difluoro-17-(((fluoromethyl)thio)carbonyl)-11-((hydroxyhydrophosphoryl)oxy)-10,13,16-trimethyl-3-oxo-6,7,8,9,10,11,12,13,14,15,16,17-dodecahydro-3H-cyclopenta[a]phenanthren-17-yl Propionate (**30**).* To a stirred solution of fluticasone propionate (**29**) (0.50 g, 1.00 mmol) in THF (10.0 mL) at −78 °C was added phosphorus trichloride (0.175 mL, 2.0 mmol) dissolved in THF (1.0 mL) followed by triethylamine (0.70 mL, 5.00 mmol) dissolved in THF (1.0 mL). The resulting mixture was stirred at −78 °C for 10 min and allowed to warm to room temperature for 26 h. The reaction was chilled in an ice bath and quenched with water (0.50 mL). The solution was allowed to warm to room temperature, and saturated sodium bicarbonate solution was added until pH 9 and stirred for 10 min. The mixture was acidified with 1 N HCl and extracted several times with ethyl acetate. The combined organic phase was dried over sodium sulfate and concentrated to give **30** (0.53 g, 94%). LRMS (ES) $(M + H)^+$: calcd, 564.5; found, 565.3. ^{1}H NMR (DMSO-d_6 with 0.1% v/v TMS, 500 MHz): δ_H 0.90 (3H, d, J = 7.3 Hz), 0.96 (3H, s), 1.02 (3H, t, J = 7.6 Hz), 1.30 (1H, m), 1.47(3H, s), 1.54−1.47 (1H, m), 1.94−1.84 (1H, m), 2.21−2.09 (3H, m), 2.27 (1H, m), 2.38 (2H, q, J = 7.6 Hz), 2.70−2.52 (1H, m), 4.85 (1H, br t, J = 8.3 Hz), 5.63 (1H, ddd, J = 48.6, 11.2, 6.6 Hz), 5.93 (2H, d, J = 50.1 Hz), 6.13 (1H, br s), 6.34 (1H, d, J = 10.2 Hz), 7.35 (1H, d, J = 10.2 Hz).

*(6S,8S,9R,10S,11S,13S,14S,16R,17R)-6,9-Difluoro-17-(((fluoromethyl)thio)carbonyl)-11-((hydroxy(1H-imidazol-1-yl)-phosphoryl)oxy)-10,13,16-trimethyl-3-oxo-6,7,8,9,10,11,12,13,14,15,16,17-dodecahydro-3H-cyclopenta[a]-phenanthren-17-yl Propionate (**31**).* To a stirred solution of **30** (0.03 g, 0.05 mmol) and imidazole (0.018 g, 0.26 mmol) in pyridine (0.5 mL) at room temperature was added TMS-Cl (0.066 mL, 0.51 mmol), and the resulting solution was stirred for 10 min. To this mixture was added iodine (0.026 g, 0.103 mmol) dissolved in pyridine (0.1 mL), and the mixture was stirred at room temperature overnight. The reaction was then cooled in an ice bath and quenched with water (0.5 mL). The reaction was concentrated, dissolved in aqueous acetonitrile, and purified using reverse phase preparative chromatography (Phenomenex Gemini NX C18 OBD 5 μM, 30 × 100 mm; 10−50% MeCN/water with 0.1% NH4OH modifier over 20 min) to give **31** (22 mg, 67%). LRMS (ES) (M + H)+: calcd, 630.6; found, 631.3. ^{1}H NMR (DMSO- d_6 with 0.1% v/v TMS, 500 MHz): δ_H 0.61 (3H, s), 0.83 (3H, d, J = 7.2 Hz), 0.99 (3H, t, J = 7.5 Hz), 1.21 (1H, br t, J = 10.3 Hz), 1.32 (1H, d, J = 14.5 Hz), 1.52−1.42 (2H, m), 1.54 (3H, s),

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J. Am. Chem. Soc. 2016, 138, 1430−1445

1.80 (1H, br q, J = 12 Hz), 1.88 (1H, br d, J = 14.6 Hz), 2.03 (1H, td, J = 12.2, 7.6 Hz), 2.23 (1H, m), 2.33 (2H, q, J = 7.7 Hz), 2.59−2.50 (1H, m), 4.81 (1H, br t, J = 8.9 Hz), 5.63 (1H, ddd, J = 48.6, 11.2, 6.6 Hz), 5.82 (1H, dd, J = 50.2, 10.0 Hz), 5.85 (1H, dd, J = 50.2, 9.7 Hz), 6.11 (1H, dd, J = 2.3, 1.6 Hz), 6.30 (1H, dd, J = 10.1, 1.8 Hz), 7.16 (1H, s), 7.26 (1H, s), 7.77 (1H, dd, J = 10.2, 1.6 Hz), 8.28 (1H, br s).

*(6S,8S,9R,10S,11S,13S,14S,16R,17R)-11-((((((2-((((9H-Fluoren-9-yl)-methoxy)carbonyl)amino)ethoxy) (hydroxy)phosphoryl)oxy)-(hydroxy)phosphoryl)oxy)-6,9-difluoro-17-(((fluoromethyl)thio)-carbonyl)-10,13,16-trimethyl-3-oxo-6,7,8,9,10,11,12,13,14,15,16,17-dodecahydro-3H-cyclopenta[a]phenanthren-17-yl Propionate (**32**).* To a stirred solution of **31** (0.15 g, 0.238 mmol) and **12** (0.086 g, 0.238 mmol) in DMF (1.0 mL) was added $ZnCl_2$ (0.26 g, 1.90 mmol), and the mixure was allowed to stir at room temperature 48 h. The reaction was diluted with 1 N HCl and extracted several times with ethyl acetate. The combined organic layers were concentrated, dissolved in aqueous acetonitrile, and purified using reverse phase preparative chromatography (Phenomenex Gemini NX C18 OBD 5 μM, 30 × 100 mm; 10−50% MeCN/water with 0.1% NH_4OH modifier over 20 min) to give **32** (112 mg, 51%). LRMS (ES) $(M + H)^+$: calcd, 925.8; found, 943.41. ^{1}H NMR (DMSO-d_6 with 0.1% v/v TMS, 500 MHz): δ_H 0.87 (3H, d, J = 7.6 Hz), ∼0.95 (3H, t, J = 7 Hz), 0.98 (3H, s), 1.02−0.93 (2H, m), 1.23 (1H, m), 1.53−1.45 (2H, m), 1.53 (3H, s), 1.84 (1H, m), 1.93 (1H, m), 2.08 (1H, m), ∼2.34−2.20 (3H, m), 3.18 (2H, br s), 3.80 (2H, br s), 4.2−4.1 (3H, m), 4.74 (1H, br t, J = 7.9 Hz), 5.61 (1H, ddd, J = 48.8, 11.0, 6.4 Hz), 5.85 (1H, dd, J = 50.7, 10.1 Hz), 5.89 (1H, dd, J = 50.5, 9.9 Hz), 6.08 (1H, s), 6.13 (1H, d, J = 10.2 Hz), 7.32 (2H, t, J = 8.0 Hz), 7.40 (2H, t, J = 7.6 Hz), 7.73 (2H, d, J = 7.5 Hz), 7.78 (1H, d, J = 10.3 Hz), 7.88 (2H, d, J = 7.6 Hz), 8.39 (1H, s).

*(6S,8S,9R,10S,11S,13S,14S,16R,17R)-11-((((((2-(2-(Cyclooct-2-yn-1-yloxy)acetamido)ethoxy)(hydroxy)phosphoryl)oxy) (hydroxy)-phosphoryl)oxy)-6,9-difluoro-17-(((fluoromethyl)thio)carbonyl)-10,13,16-trimethyl-3-oxo-6,7,8,9,10,11,12,13,14,15,16,17-dodeca-hydro-3H-cyclopenta[a]phenanthren-17-yl Propionate (**33**).* To a stirred solution of **32** (0.11 g, 0.12 mmol) in DCM (1 mL) was added piperidine (0.12 mL, 1.20 mmol), and the resulting mixture was stirred at room temperature for 1 h and concentrated. The residue was dissolved in a 2:1:1 MeOH/water/MeCN solution and syringe filtered. The mixture was purified using reverse phase preparative chromatography (Phenomenex Gemini NX C18 OBD 5 μM, 30 × 100 mm; 5−45% MeCN/water with 0.1% NH_4OH modifier over 20 min) and then redissolved in DMF (0.8 mL). To this solution were added 2-(cyclooct-2-yn-1-yloxy)acetic acid (0.055 g, 0.305 mmol), HATU (0.116 g, 0.305 mmol), and triethylamine (0.053 mL, 0.38 mmol). The reaction was stirred at room temperature for 20 min and then purified directly using reverse phase preparative chromatography (Phenomenex Gemini NX C18 OBD 5 μM, 30 × 100 mm; 10−50% MeCN/water with 0.1% NH_4OH modifier over 20 min) to give **33** (59 mg, 71%). ^{1}H NMR (DMSO-d_6 with 0.1% v/v TMS, 500 MHz, presat): δ_H 0.89 (3H, d, J = 7.2 Hz), 0.96 (3H, s), 1.03 (3H, t, J = 7.6 Hz), 1.15 (2H, t, J = 7.3 Hz), 1.25 (1H, br t, J = 9.7 Hz), 1.37 (1H, m), 1.60−1.44 (2H, complex), 1.52 (3H, s), 1.94−1.68 (∼6H, complex), 2.29−2.05 (∼5H, complex), 2.37 (2H, q, J = 7.6 Hz), 3.08 (1H, m), 3.18 (1H, m), 3.74 (1H, m), 3.77 (1H, d, J = 14.6 Hz), 3.91 (1H, d, J = 14.5 Hz), 4.31 (1H, br t, J = 5.3 Hz), 4.64 (1H, br t, J = 8.4 Hz), 5.62 (1H, ddd, J = 48.7, 10.5, 6.2 Hz), 5.87 (1H, dd, J = 50.6, 9.8 Hz), 5.91 (1H, dd, J = 50.5, 9.8 Hz), 6.10 (1H, dd, J = 2.4, 1.5 Hz), 6.22 (1H, dd, J = 10.1, 2.0 Hz), 7.79 (1H, t, J = 8.7 Hz), 8.85 (1H, br s). HRMS calcd for $C_{37}H_{51}F_3NO_{13}P_2S$ $(M + H)^+$, 868.2508; found, 868.2525.

*(6S,8S,9R,10S,11S,13S,14S,16R,17R)-6,9-Difluoro-17-(((fluoromethyl)thio)carbonyl)-10,13,16-trimethyl-11-((methylthio)-methoxy)-3-oxo-6,7,8,9,10,11,12,13,14,15,16,17-dodecahydro-3H-cyclopenta[a]phenanthren-17-yl Propionate (**34**).* To a stirred solution of fluticasone propionate (**29**) (0.50 g, 1.00 mmol) in MeCN (5.0 mL) at 0 °C was added dimethyl sulfide (0.59 mL, 8.00 mmol) followed by benzoyl peroxide (0.97 g, 4.00 mmol) added in four portions over 20 min. The resulting mixture was stirred at 0 °C for 1 h. The reaction was concentrated, taken up in ethyl acetate, and washed with saturated sodium bicarbonate. The combined organic phase was concentrated. The crude was purified directly using reverse phase preparative chromatography (Phenomenex Gemini NX C18 OBD 5 μM, 30 × 100 mm; 40−80% MeCN/water with 0.1% NH_4OH modifier over 20 min) to give to give **34** (0.07 g, 13%). LRMS (ES) $(M + H)^+$: calcd, 560.6; found, 561.3. ^{1}H NMR (DMSO-d_6 with 0.1% v/v TMS, 500 MHz): δ_H 0.90 (3H, d, J = 7.1 Hz), 0.94 (3H, s), 1.02 (3H, t, J = 7.5 Hz), 1.28 (1H, m), 1.49 (3H, s), 1.54 (1H, s), 1.70−1.46 (∼2H, complex), 1.95−1.83 (∼2H, complex), 2.14 (1H, td, J = 12.3, 7.6 Hz), 2.19 (3H, s), 2.8−2.2 (∼2H, complex), 2.38 (2H, q, J = 7.6 Hz), 4.24 (1H, ddd, J = 8.8, 3.3, 2.2 Hz), 4.73 (1H, d, J = 11.5 Hz), 4.81 (1H, d, J = 11.5 Hz), 5.64 (1H, ddd, J = 48.7, 11.1, 6.6 Hz), 5.93 (1H, dd, J = 50.1, 9.6 Hz), 5.95 (1H, dd, J = 50.2, 9.5 Hz), 6.13 (1H, dd, J = 2.1, 1.4 Hz), 6.32 (1H, dd, J = 10.1, 1.9 Hz), 7.29 (1H, dd, J = 10.1, 1.5 Hz).

*(6S,8S,9R,10S,11S,13S,14S,16R,17R)-6,9-Difluoro-17-(((fluoromethyl)thio)carbonyl)-10,13,16-trimethyl-3-oxo-11-((phosphonooxy)methoxy)-6,7,8,9,10,11,12,13,14,15,16,17-dodeca-hydro-3H-cyclopenta[a]phenanthren-17-yl Propionate (**35**).* Phosphoric acid (0.09 g, 0.89 mmol) was heated under nitrogen at 120 °C for 30 min. This was allowed to cool, and to it were added molecular sieves and **34** (0.07 g, 0.13 mmol). This mixture was dissolved in THF (1.3 mL), and NIS (0.04 g, 0.19 mmol) was added. The resulting solution was allowed to stir overnight at room temperature. The mixture was filtered and concentrated. The crude was purified directly using reverse phase preparative chromatography (Phenomenex Gemini NX C18 OBD 5 μM, 30 × 100 mm; 10−50% MeCN/water with 0.1% NH_4OH modifier over 20 min) to give **35** (0.05 g, 63%). LRMS (ES) $(M + H)^+$: calcd, 610.5; found, 611.3. ^{1}H NMR (DMSO-d_6 with 0.1% v/v TMS, 500 MHz): δ_H 0.89−0.88 (6H, m), 1.02 (4H, t, J = 7.38 Hz), 1.27−1.23 (3H, m), 1.49 (3H, s), 1.90−1.84 (2H, m), 2.16−2.07 (3H, m), 2.27−2.23 (2H, m), 2.38 (3H, q, J = 7.89 Hz), 4.54 (1H, d, J = 7.62 Hz), 4.83−4.81 (1H, m), 4.96−4.95 (1H, m), 5.60 (1H, d, J = 48.77 Hz), 5.93 (2H, d, J = 50.28 Hz), 6.09 (1H, s), 6.21 (1H, d, J = 9.22 Hz), 7.81 (1H, d, J = 9.88 Hz).

*(6S,8S,9R,10S,11S,13S,14S,16R,17R)-11-((((((2-((((9H-Fluoren-9-yl)methoxy)carbonyl)amino)ethoxy)(hydroxy)phosphoryl)oxy)-(hydroxy)phosphoryl)oxy)methoxy)-6,9-difluoro-17-(((fluoromethyl)thio)carbonyl)-10,13,16-trimethyl-3-oxo-6,7,8,9,10,11,12,13,14,15,16,17-dodecahydro-3H-cyclopenta[a]-phenanthren-17-yl Propionate (**36**).* To a stirred solution of **12** (0.03 g, 0.083 mmol) in DMF (0.4 mL) were added triethylamine (0.012 mL, 0.083 mmol) and CDI (0.034 g, 0.20 mmol). The resulting solution was stirred at room temperature for 30 min. To this mixture were added **35** (0.049 g, 0.08 mmol) and $ZnCl_2$ (0.044 g, 0.32 mmol), and the mixture was allowed to stir at room temperature overnight. The reaction was diluted with ethyl acetate and washed with 5% citric acid solution. The organic phase was concentrated, and the crude material was purified by reverse phase preparative chromatography (Phenomenex Gemini NX C18 OBD 5 μM, 30 × 100 mm; 5−45% MeCN/water with 0.1% NH_4OH modifier over 20 min) to give **36** (43 mg, 56%). LRMS (ES) $(M + H)^+$: calcd, 955.8; found, 956.5. ^{1}H NMR (DMSO-d_6 with 0.1% v/v TMS, 500 MHz): δ_H 0.88−0.86 (6H, m), 0.95 (3H, t, J = 7.5 Hz), 1.11−1.08 (1H, m), 1.24 (1H, t, J = 9.0 Hz), 1.50−1.45 (4H, m), 1.89−1.79 (2H, m), 2.15−2.09 (2H, m), 2.35−2.21 (3H, m), 3.18−3.15 (2H, m), 3.79−3.75 (2H, m), 4.19−4.15 (3H, m), 5.03−4.98 (2H, m), 5.59 (1H, ddd, J = 48.8, 11.1, 6.4 Hz), 5.88 (1H, dd, J = 50.2, 9.4 Hz), 5.91 (1H, dd, J = 50.1, 9.0 Hz), 6.09 (1H, t, J = 1.9 Hz), 6.23 (1H, dd, J = 10.1, 2.1 Hz), 7.33−7.30 (3H, m), 7.39 (2H, t, J = 7.6 Hz), 7.75 (2H, t, J = 7.3 Hz), 7.87 (2H, d, J = 7.6 Hz), 8.28 (1H, s).

*(6S,8S,9R,10S,11S,13S,14S,16R,17R)-11-((((((2-(2-(2-(Cyclooct-2-yn-1-yloxy)acetamido)ethoxy)(hydroxy)phosphoryl)oxy)(hydroxy)-phosphoryl)oxy)methoxy)-6,9-difluoro-17-(((fluoromethyl)thio)-carbonyl)-10,13,16-trimethyl-3-oxo-6,7,8,9,10,11,12,13,14,15,16,17-dodecahydro-3H-cyclopenta[a]phenanthren-17-yl Propionate (**37**).* To a stirred solution of **36** (0.043 g, 0.045 mmol) in DCM (1 mL) was added DBU (0.014 mL, 0.094 mmol), and the resulting mixture was stirred at room temperature for 3 h. To this solution were added 2-(cyclooct-2-yn-1-yloxy)acetic acid (0.033 g, 0.18 mmol), HATU (0.055 g, 0.144 mmol), and triethylamine (0.044 mL, 0.32 mmol). The reaction was stirred at room temperature for 1 h and then purified directly using reverse phase preparative chromatography (Phenomen-

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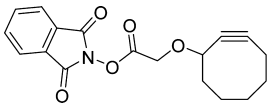
ex Gemini NX C18 OBD 5 μM, 30 × 100 mm; 5−45% MeCN/water with 0.1% NH4OH modifier over 20 min) to give **37** (28 mg, 70%). ^{1}H NMR (DMSO-d_6 with 0.1% v/v TMS, 500 MHz, presat): δ_H 0.89 (3H, s), 0.90 (3H, d, J = 6.7 Hz), 1.03 (3H, t, J = 7.5 Hz), 1.16 (3H, t, J = 7.2 Hz), 1.26 (1H, m), 1.37 (1H, m), 1.51 (3H, s), 1.95−1.45 (∼8H, complex), 2.3−2.0 (∼5H, complex), 2.39 (2H, q, J = 7.5 Hz), 3.02 (2H, q, J = 7.2 Hz), ∼3.21 (1H, m), 3.80−3.73 (3H, complex), 3.90 (1H, d, J = 14.5 Hz), 4.29 (1H, m), 4.50 (1H, m), 4.99 (2H, m), 5.61 (1H, ddd, J = 48.9, 11.1, 6.5 Hz), 5.92 (1H, dd, J = 50.1, 9.8 Hz), 5.95 (1H, dd, J = 50.1, 9.9 Hz), 6.10 (1H, dd, J = 2.2, 1.4 Hz), 6.22 (1H, dd, J = 10.1, 1.9 Hz), 7.71 (1H, d, J = 10.1 Hz), 8.76 (1H, t, J = 5.3 Hz). HRMS calcd for $C_{38}H_{53}F_3NO_{14}P_2S$ $(M + H)^+$, 896.2458; found, 896.2463.



1,3-Dioxoisoindolin-2-yl 2-(Cyclooct-2-yn-1-yloxy)acetate. To a stirred solution of 2-(cyclooct-2-yn-1-yloxy)acetic acid (0.10 g, 0.55 mmol) in DCM (2 mL) were added 2-hydroxyisoindoline-1,3-dione (0.18 g, 1.10 mmol) and EDC (0.21 g 1.10 mmol), and the resulting mixture was stirred at room temperature for 1.5 h. The solution was directly purified by flash column separation using a 0−50% ethyl acetate/hexane gradient to give the title compound (163 mg, 91%).

General Procedure for Antibody Conjugation. To initiate conjugation, 10% v/v DMSO was added to the antibody solution, followed by a 15-fold molar excess of cyclooctyne-functionalized drug linker. The solution was gently mixed and allowed to react at 28 °C for 48 h. Removal of unreacted drug linker and aggregates was performed via cation exchange as previously described. The final cation exchange pool was then concentrated and formulated into 50 mM histidine, 100 mM NaCl, and 2.5% trehalose, pH 6.0, and 0.22 μm filtered.

Stability Screening of Dexamethasone Phosphate Ester Linkers. *Human Blood Incubation.* Human blood was collected the morning of the experiment from at least three individuals using K_2EDTA as the anticoagulant. An equal volume from each individual was combined for use in the experiment. The experiment started no more than 2 h after the blood collection. All drug linker conjugates were solubilized in DMSO to form a 10 mM stock solution of each. Dosing solution for each linker was prepared by serial dilution of each stock solution using 1:3 acetonitrile/water. All solutions were kept on ice during the experiment.

Human blood was prewarmed in a 37 °C water bath in an appropriate volume to collect samples over a time course from 0 to 6 h. Incubating blood was mixed well just prior to sampling to give a homogeneous mixture. Aliquots of blood were removed at appropriate time points, added to cold stopping solution, which was methanol containing an appropriate internal standard, and mixed rigorously. The samples were centrifuged at 4000 rpm for 10 min, after which equal volumes of the supernatant fractions were diluted with cold deionized water. The samples were then ready for analysis by liquid chromatography and triple quadrupole mass spectrometry. A time 0 sample was prepared by spiking blood, which had been pretreated with the same stopping reagent used above, with the drug linker. This sample is referred to in the tables as matrix spiking.

Rat Lysosome Incubation. Rat liver tritosomes (lysosomes) were available commercially as a custom product from a pool of six animals. All linker compounds were solubilized in DMSO to form a 10 mM stock solution of each. Dosing solution for each linker was prepared by serial dilution of each stock solution using 1:3 acetonitrile/water. All solutions were kept on ice during the experiment.

Rat lysosomes were prewarmed in a 37 °C water bath in an appropriate volume to collect samples over a time course from 0 to 6 h. Incubating lysosomes were mixed well just prior to sampling to give a homogeneous mixture. Aliquots of lysosomes were removed at appropriate time points, added to one volume of cold stopping solution, which was acetonitrile containing an appropriate internal standard, and mixed rigorously. The samples were centrifuged at 10 000 rpm for 10 min, and supernatants were ready for analysis by liquid chromatography and time-of-flight mass spectrometry. A time 0 sample was prepared by spiking the drug linker into boiled lysosomes. This sample is referred to as boiled lysosomes in the tables.

Stability Screening of α-CD70 Antibody Glucocorticoid Conjugates. *Plasma Incubation.* To measure in vitro stability, ADCs **1**−**22** (human CD70-2H5-HA114-pAz-AXC 389 IgG1 (DG)) and **1**−**37** (human CD70-2H5-HA114-pAz-AXC 449 IgG1 (DG)) were spiked into C57BL/6 plasma, human plasma, and HBSS/2% FBS buffer at 0.1 μg/μL. Samples was capped under nitrogen and incubated at 37 °C for 6 h and 1, 2, 3, 7, 14, and 21 days. An aliquot was collected right after spiking (time 0), and samples were kept at −80 °C until analysis. The human ADCs were affinity-purified from C57BL/6 plasma and HBSS, 2% FBS buffer using 100 μL of biotinylated human polyclonal IgG (Southern Biotech, 2049-08) coupled to streptavidin beads packed in AssayMap (Agilent Technologies, G5496-60010) cartridges. After polyclonal immobilization, 40 μL of plasma was mixed with 70 μL of TBS buffer and loaded into the AssayMap system. Tips were washed with 1× TBS containing 0.1% Rapigest (Waters, 186001861) and with 1× TBS, and the enriched ADCs were eluted with 40 μL of 0.25% formic acid solution. The human ADCs were affinity-purified from human plasma using an in-house produced and biotinylated CD70 antibody also coupled to streptavidin beads packed in AssayMap cartridges. The affinity purification was performed as described above.

Samples were analyzed in an LC-UV-MS Acquity/Synapt G2-S (Waters, Milford). Five microliters of sample was injected onto an POROS R2/10 (2.1 mm D × 30 mm L, 1-1112-12) column kept at 60 °C, and the LC separation was conducted at a flow rate of 100 μL/min. Mobile phase A was water, 0.1% formic acid, and phase B was acetonitrile, 0.1% formic acid. A linear gradient starting from 30 to 58% phase B in 10 min was applied; the column was washed for 1 min with 100% phase B and re-equilibrated to 30% phase B for 4 min before the next injection. The eluate was analyzed in series in an Acquity UV (280 nm) detector and in the Synapt G2-S system equipped with positive electrospray source. The data acquisition was performed within the MS range of 400−4000 m/z, and the software MassEnt1 (Waters, Milford) was used for deconvolution of the raw data and intact mass analysis.

Rat Lysosome Incubation. Rat liver tritosomes (lysosomes) were available commercially as a custom product from a pool of six animals. All ADC solutions were formulated in buffer and kept on ice during the experiment.

Rat lysosomes were prewarmed in a 37 °C water bath in an appropriate volume to collect samples over a time course from 0 to 6 h. Incubating lysosomes were mixed well just prior to sampling to give a homogeneous mixture. Aliquots of lysosomes were removed at appropriate time points, added to two volumes of cold stopping solution, which was acetonitrile containing an appropriate internal standard, and mixed rigorously. The samples were centrifuged at 10 000 rpm for 10 min, and supernatants were dried under a stream of nitrogen until approximately one-third of the volume remained. Dried supernatants were centrifuged at 4000 rpm for 10 min prior to analysis by liquid chromatography and time-of-flight mass spectrometry. A time 0 sample was prepared by spiking the ADC into boiled lysosomes. This sample is referred to as boiled lysosomes in the tables.

In Vitro Potency Evaluation for α-CD70 Antibody Glucocorticoid Conjugates (GILZ mRNA Readout). The cellular targeted delivery and activity of α-CD70 antibody glucocorticoid conjugates and their isotype-control antibody glucocorticoid conjugates were assessed in 786-O cells, a human renal carcinoma cell line (ATCC CRL-1932), for modulation of glucocorticoid-induced leucine zipper (GILZ) mRNA levels. Although CD70 (TNFSF7), a type II transmembrane receptor, is normally expressed on a subset of B-, T-, and NK cells, where it plays a costimulatory role in immune cell activation, CD70 expression was also found to be aberrantly elevated in multiple human carcinoma types and tumor-derived cell lines.[17] It was previously demonstrated that binding of an α-CD70 antibody to CD70 endogenously expressed on the surface of the 786-O cell line resulted in the rapid internalization of the antibody−receptor complex,[17] indicating that CD70 represents a potential target antigen

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J. Am. Chem. Soc. 2016, 138, 1430−1445

for facilitating payload delivery to specified cells using a conjugated therapeutic antibody.

In brief, 786-O cells were maintained in RPMI/10% FBS culture medium. To quantitate GILZ mRNA expression by RT-PCR, the cells were spun and resuspended in assay buffer (HBSS + 2% FBS) at a density of 1.11×10^6 cells/mL, and 45 μL of the cell suspension was plated per well (5×10^4 cells/well) in 384-well plate(s). Dosing solutions of free drug and ADCs were prepared by 1:3 serial dilution of each stock solution in HBSS + 2% FBS supplemented with 10% ADC buffer (50 mM Histidine, 100 mM NaCl, 5% Trehalose, pH 6.0), and 5 μL of the dosing solution was added into each well (1:10 dilution). The plates were incubated in an incubator at 37 °C for 18 h. The cells were then lysed, and cDNA synthesis and real-time PCR were performed according to the manufacturer's instructions using the TaqMan gene expression cells-to-Ct kit (Invitrogen, Carlsbad, CA). Specific primers against human GILZ and GAPDH were purchased from Life Technologies (Invitrogen, Carlsbad, CA). Real-time PCR reactions were performed on an Applied Biosystems 7900 HT fast real-time PCR system. Thermal cycling conditions consisted of an initial UDG incubation hold (50 °C, 2 min) and denaturing and enzyme activation step (95 °C, 2 min) followed by 40 cycles of denaturing (95 °C, 15 s) and annealing and extending (60 °C, 1 min). The mRNA levels were normalized to GAPDH (internal control) using the formula Δ threshold cycle (C_t) = C_t target − C_t reference. The differential expression signal was expressed as ΔC_t by subtracting the C_t values of the unstimulated samples (containing only assay buffer or DMSO vehicle) from those of the stimulated samples and expressed as relative fold change using the formula $2^{\Delta\Delta C_t}$.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.5b12547.

Experimental procedures for antibody production, analytical characterization of conjugates, stability incubation studies, and cell-based functional assays (PDF)
NMR spectra for small molecules (PDF)

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Notes

The authors declare no competing financial interest.

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