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Application of Strain-Promoted Azide-Alkyne Cycloaddition and Tetrazine Ligation to Targeted Fc-Drug Conjugates

Joshua D. Thomas[1], **Huiting Cui**[2], **Patrick J North**[2], **Thomas Hofer**[2], **Christoph Rader**[2], and **Terrence R. Burke Jr.**[1,*]

[1]Chemical Biology Laboratory, Center for Cancer Research, National Cancer Institute, National Institutes of Health, Frederick, MD, U.S.A.

[2]Experimental Transplantation and Immunology Branch, Center for Cancer Research, National Cancer Institute, National Institutes of Health, Bethesda, MD, U.S.A.

Abstract

We have previously described an approach whereby antibody Fc fragments harboring a single C-terminal selenocysteine residue (Fc-Sec) are directed against a variety of targets by changing the peptide or small molecule to which they are conjugated. In the present work we describe methodology for improving the efficacy of these Fc-Sec conjugates by incorporating cytotoxic drugs. The Fc-Sec protein is first programmed to target specific tumor cell types by attachment of a bifunctional linker that contains a "clickable" handle (e.g. cyclobutane or cyclooctyne) in addition to a tumor cell-binding peptide or small molecule. Following Fc-Sec conjugation, a cytotoxic warhead is then attached by cycloaddition reactions of tetrazine or azide-containing linker. To validate this approach we used a model system in which folic acid (FA) is the targeting moiety and a disulfide-linked biotin moiety serves as a cytotoxic drug surrogate. We demonstrated successful targeting of Fc-Sec proteins to folate-receptor expressing tumor cells. Tetrazine ligation was found to be an efficient method for biotin "arming" of the folate-targeted Fc-Sec proteins. We also report novel bioconjugation methodologies that use [4+2] cycloaddition reactions between tetrazines and cyclootynes.

The therapeutic value of monoclonal antibodies (mAbs) in cancer therapy is well established. One reason for the success of mAbs is that they intrinsically exhibit many ideal drug properties, including high target specificity and affinity and long circulatory half-life. However, therapeutic mAbs often lack the potency and tissue penetration (particularly against solid tumors) required to eradicate target cell populations.[1, 2] A common strategy for improving antibody potency involves attaching powerful cytotoxic drugs that are too poisonous to be safely administered as single agents.[3] Antibody-drug conjugates (ADCs) function as "smart bombs" that allow the toxin to bypass healthy tissue and hit only the targets of interest. The remaining problem with mAbs of poor solid tumor penetration has spurred research into alternative antibody-like proteins that retain high affinity and selectivity for tumor cells but are smaller than full-size antibodies.[4] Therapeutic proteins based on antibody Fc (fragment crystallizable) fragments are attractive examples of this latter class, since they often exhibit serum half-lives close to those of full-size antibodies, yet they are much smaller in size.[5] An additional property of Fc proteins is that they can

*To whom correspondence should be addressed, tburke@helix.nih.gov.

recruit elements of the immune system to attack targets of interest via interaction with immune effector cells (antibody-dependent cellular cytotoxicity, ADCC) and complement (complement-dependent cytotoxicity, CDC).[6]

Once they are separated from their antigen binding (Fab) regions, Fc proteins lose their ability to attack specific cell populations. One method for restoring antigen-recognition to the Fc moiety involves genetically fusing tumor cell-binding peptides or proteins.[7] While this can be an effective strategy (five Fc-fusion proteins are currently on the market[5]), introducing specificity for each new target requires its own protein engineering project. We have previously described an alternative approach for grafting antigen-binding sites onto Fc proteins that does not rely on genetic engineering.[8, 9] This involves initial protein engineering to introduce a single selenocysteine (Sec) residue into the C-terminus of an IgG Fc fragment (Fc-Sec). A tumor cell-binding peptide or small molecule can then be attached using standard protein alkylation methodologies (e.g. via maleimide-containing linkers) to generate conjugates having a 1:1 ratio of targeting agent to protein. This approach allows a single generic Fc-Sec protein to be directed against a wide variety of targets by simply changing the peptide or small molecule to which it is conjugated.

Although targeted Fc-Sec proteins may be therapeutically valuable in their own right, their efficacy may be improved by incorporating cytotoxic drugs. Several drug conjugate linker systems are designed to release their cargos under acidic (e.g. hydrazones) or reductive (e.g. disulfides) conditions. However, because Fc-Sec-conjugates are prepared under just such conditions, the use of these linker modalities is potentially contraindicated. In order to avoid the loss of drug cargo during conjugation to Fc-Sec proteins (Figure 1, Route A), we are examining two-step "program and arm" approaches (Figure 1, Route B). Here the Fc-Sec proteins are first programmed to target specific tumor cell types by attachment of bifunctional linkers that contain "clickable" handles (e.g. cyclobutane or cyclooctyne) in addition to tumor cell-binding peptides or small molecules. These linkers are conjugated to Fc-Sec proteins using previously established conditions (Figure 1).[8] Following appropriate Fc-Sec conjugation, a tetrazine or azide-containing linker can be used to attach a cytotoxic warhead by means of cycloaddition reactions. Our current paper presents a model version of this "program and arm" concept in which folic acid (FA) serves as the targeting moiety, and a disulfide-linked biotin group is employed as a cytotoxic drug surrogate. We also report the use of [4+2] cycloaddition reactions between tetrazines and cyclooytnes as novel bioconjugation methodologies.

Since most previously prepared FA-drug conjugates rely on disulfide linkages for intracellular drug release,[10] we chose this type of linkage for our Fc-Sec conjugates, although it was not clear whether disulfides would survive the reductive conditions previously established for Fc-Sec conjugation.[8] We found that in the absence of Fc-Sec protein, a model disulfide was unstable under anticipated coupling conditions (25 equiv DTT at pH 5.2, 80 min, data not shown). This indicated that undesired reductive cleavage may occur if a disulfide-containing linker were used to alkylate Fc-Sec. Therefore, an alternative approach to Fc-Sec modification was examined in which sensitive functionality could be introduced following Fc-Sec alkylation (Figure 1, Route B).

Chemistries used to attach drug cargos should be both mild and chemoselective in order to preserve the well-defined stoichiometry and residue specificity of Fc-Sec conjugates.[8, 9] A number of bioorthogonal "click" reactions that meet these requirements have been reported recently.[11] Among these, the most widely utilized click reactions employ Cu(I)-catalyzed azide-alkyne [3+2] cycloaddition (CuAAC) chemistries.[12] However, because it can be difficult to remove cytotoxic Cu from the conjugated products,[13, 14] CuAAC may not be appropriate for constructing Fc-Sec drug conjugates. Therefore, we evaluated strain-

promoted azide-alkyne cycloaddition (SPAAC)[15] and tetrazine ligation (TL)[16] as metal-free alternatives to CuAAC.

The synthetic complexity of many reactive cyclooctyne-containing reagents can be a major limitation to their use in SPAAC bioconjugations. This complexity often arises from the difficulty of introducing the required electron-withdrawing groups adjacent to the alkyne moiety.[17] Although synthetic routes have improved in recent years, many of the most reactive cyclooctynes still require lengthy and low-yield procedures.[15] For this reason, our attention was drawn to cyclooctyne **1**,[18] which affords a good balance between synthetic accessibility (3 steps, 47% overall yield for **1** versus 8 steps, ~25% overall yield for **3**[19]) and reactivity (relative reactivity compared to similar reagents: **3**> **1** > **2**[20], Figure 2). Cyclooctyne **1** was prepared according to literature procedures[18] and converted to the water-soluble piperazine salt **6** in 4 - steps (Scheme 1). Although piperazine derivatives of **1** have not been reported, tertiary amines are well-known hydrophilic modifiers that can often improve the water solubility of liphophilic compounds.[21, 22] Conversion to **6** improved the aqueous solubility of the cyclooctyne and rendered it as an easily handled solid.

Although TL has appeared as a bioconjugation strategy only relatively recently,[16] it is quickly becoming an attractive alternative to SPPAC.[23] Tetrazine-based [4 + 2] cycloadditions are generally much faster than SPAAC reactions. They also utilize coupling partners (i.e. dienes and dienophiles) that are usually either commercially available or more synthetically accessible than cyclooctynes.[24] This is exemplified by the dieneophile **8** used in the present work, which is both easily synthesized (Scheme 2) and an efficient TL coupling partner.[25]

Strained alkenes, such as norbornene, trans-cyclooctene, and cyclobutane have traditionally been the dienophiles of choice for TL strategies.[15] Although other potentially suitable alkenes and alkynes, such as cyclooctyne, have been reported,[26, 27] these have not yet been applied in a bioconjugation context. In order to evaluate the performance of cyclooctynes in side-by-side fashion with an established TL dieneophile such as **8**, we included the known cyclooctyne **2** in the present study. The synthesis of **2** proceeded smoothly according to literature procedures,[20] with the exception that the reported NaOMe-mediated bromide elimination in **9** to give **10** proved to be inefficient (Scheme 2). In contrast, the use of DBU at elevated temperature[14] afforded **10** in good yield. Subsequent hydrolysis gave **2**. Cyclooctyne **2** was converted to the corresponding *p*-nitrophenyl ester **11** prior to final incorporation into a folate-containing scaffold (Figure 3).

Before evaluating TL reactions with Fc-Sec constructs containing alkynes **2** and **6**, their reactivities with tetrazines in free form were evaluated (Scheme 3). Reaction of **6** with tetrazine **12** resulted in a 28% combined yield of regioisomers **13** and **14**, while the reaction between **2** and **15** proceeded smoothly to afford a mixture of **16** and **17** in 79% combined yield (Scheme 3). The ability of **2** and **6** to undergo [4 + 2] cycloaddition reactions with tetrazines in nonprotein-bound forms indicated that they would likely retain their reactivity once they were bound to Fc-Sec protein.

Bifunctional FA-containing constructs **18a–c**, employing maleimide for Fc-Sec alkylation and clickable functionality for drug attachment, were prepared using a previously described PEG-SU-Lys-Lys-maleimide linker (Figure 3) (see Supporting Information).[28] The biotin-containing derivative **18d** was also prepared for use as a control in Fc-Sec alkylation and click experiments. In order to show that Fc-Sec programming was possible using **18a–d**, Fc-Sec was first alkylated with **18d** to afford **19** (Figure 4). Conjugate **19** was subsequently incubated with FA receptor-expressing (FR+) HeLa cells and analyzed by flow cytometry (Figure S1). For comparison, Fc-Sec was also alkylated using commercially available biotin-

iodoacetamide to give the Fc-Sec-biotin adduct **20**. It was found that **19** showed strong and selective binding to (FR+) cells (Figure S1A), whereas **20** failed to show binding (Figure S1B). Attempted alkylation of Fc-Stop[8] (a nearly identical protein in which the C-terminal Sec residue is missing) with **18d** resulted in only ~ 10% biotinylation relative to the same reaction with Fc-Sec (Figure S1C). These results demonstrate that alkylation of the Fc protein under our optimized conjugation conditions[8] is very limited unless the protein harbors a Sec residue. This data also serves as indirect evidence that alkylation of Fc-Sec proteins occurs primarily at the C-terminal Sec residue and not at hinge cysteines.

Having demonstrated successful programming with **18d**, we next examined SPAAC and TL chemistries for "arming" Fc-Sec proteins (**21–23**) with either biotin-azide **24a**[29] or biotin-tetrazine **24b** (Figure 5).[29] The cycloaddition products (**25–28**) produced by these reactions were then analyzed by flow cytometry to determine the extent of biotinylation as described above for **19**. Since conjugation occurs only at the single Sec residue, **19** consists of a 1:1:1 ratio of biotin:FA:Fc-Sec. Consequently, it represents the maximum level of biotinylation that can be achieved by the two-step approach shown in Figure 5 and could serve as a positive control for evaluating the efficiency of the SPAAC and TL reactions.

The SPAAC reaction of **21** with **24a** was the least efficient method for arming Fc-Sec (Figure 5B). At the same reactant stoichiometry as used to prepare **19** (1:10 protein : small molecule modifier), the SPAAC reaction gave only 5% of the biotin load as was obtained for **19** (Figure 5B). At a 50-fold excess of **24a**, the biotin load increased to approximately 15% of maximum. However, it did not improve substantially at higher concentrations of **24a** (Figure 5B). The reaction of **22** with **24a** was not examined. However, it would be expected to be even less efficient, based on the previously determined reaction rates of benzyl azide with **1**[18] and **2**.[20]

Of the three reagents tested with **24b** in TL reactions, reagent **21** was the least reactive (Figure 6). Although this result was not unexpected given the electron deficient character of **1**,[26] it is noteworthy when compared to the analogous SPAAC reaction with **24a** (Figure 5B). In this latter case, approximately the same levels of biotinylation were obtained with SPAAC as with TL, but at a 5-fold lower concentration of biotinylation reagent **24b** (Figure 5). Protein **22** was much more effective reacting with **24b** as compared with **21**. Differences in steric crowding around the alkyne moieties in **6** and **2** may be partly responsible for the observed differences in reactivity between **21** and **22**.[26] Biotinylation efficiencies with the cyclobutane construct **23** approached 80% of the maximum, which made **23** the most reactive TL coupling partner tested. This is an important finding, since the dieneophile used in **23** (i.e. compound **8**) was the simplest to prepare. It should also be noted that although fused cyclobutane-norborene compounds, such as **8**, have been used previously in bioconjugation contexts,[25, 30] this is the first example of their use in protein modification.

The reactivity ranking observed in our initial experiments (**23** > **22** > **21**) was confirmed in subsequent titration binding experiments with **19**, and **26–28** (Figure 6C). Among the three TL conjugates, **28** consistently gave higher MFI across the concentration range studied. These results suggest that the differences in biotin load observed in **26–28** accurately reflect differences in biotinylation efficiency of the various TL reactions and are not artifacts of the flow cytometry protocol used for their analysis.

In conclusion, reported herein is methodology whereby Fc-Sec proteins can be sequentially programmed to target cancer cells and then armed with cytotoxic payload using SPAAC or TL chemistries. This "program and arm" approach should be more compatible with cleaveable linkers, such as disulfides and hydrazones that may not otherwise survive the acidic, reducing conditions previously used to prepare Fc-Sec conjugates. Our work

demonstrates for the first time that cyclooctynes can be effective dienophiles in TL bioconjugation contexts. We are currently using these approaches to prepare Fc-Sec conjugates that are armed with cytotoxic drugs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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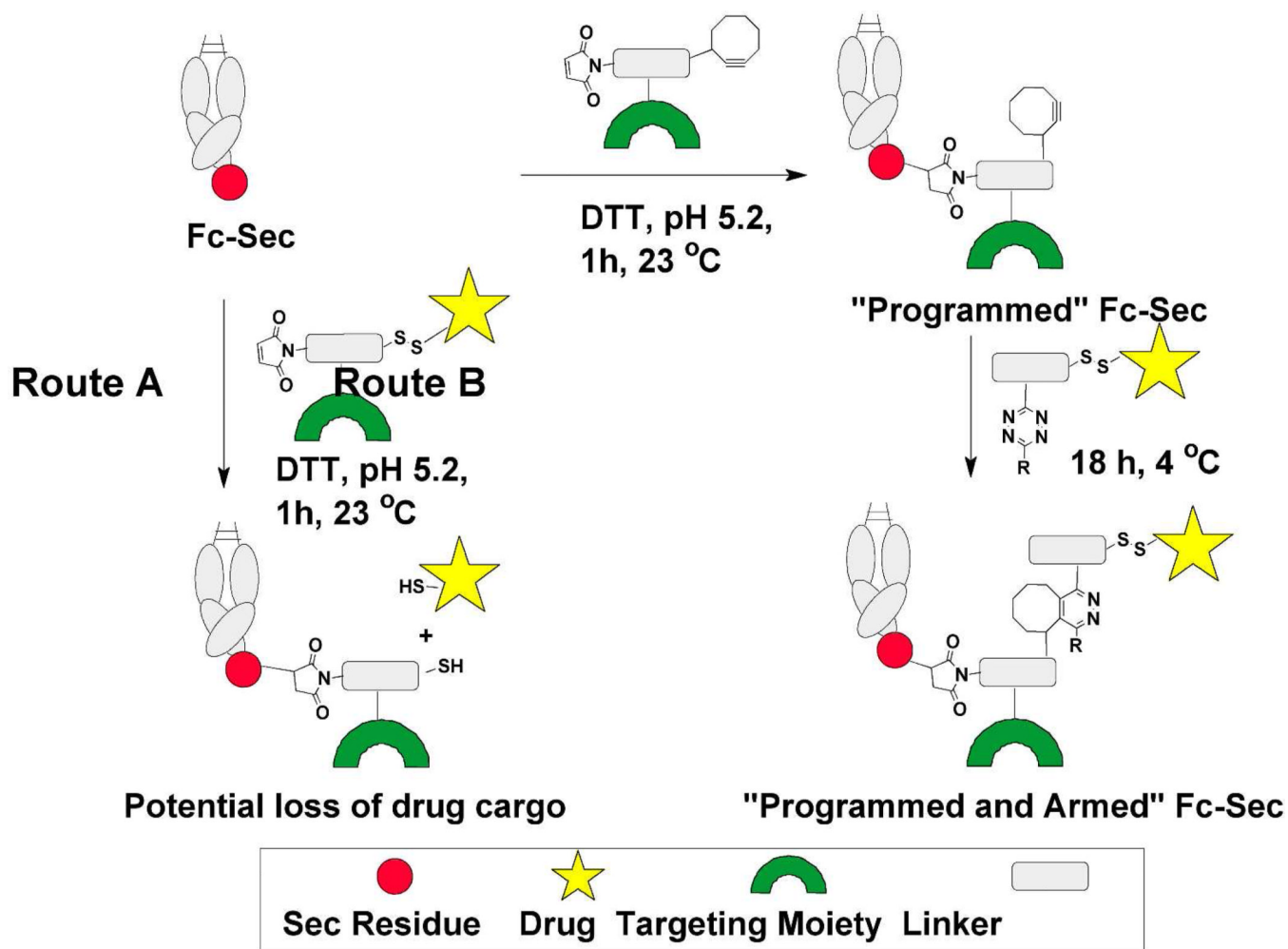
Figure 1.
Two methods of producing Fc-Sec drug conjugates. Route A; a one-step approach that may result in loss of drug cargo and Route B; a two-step program and arm protocol that preserves drug cargo.
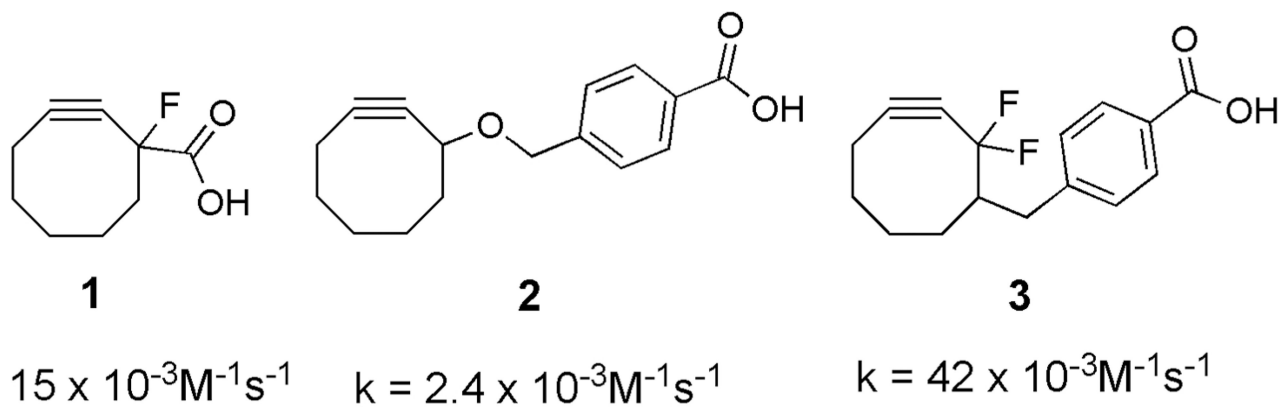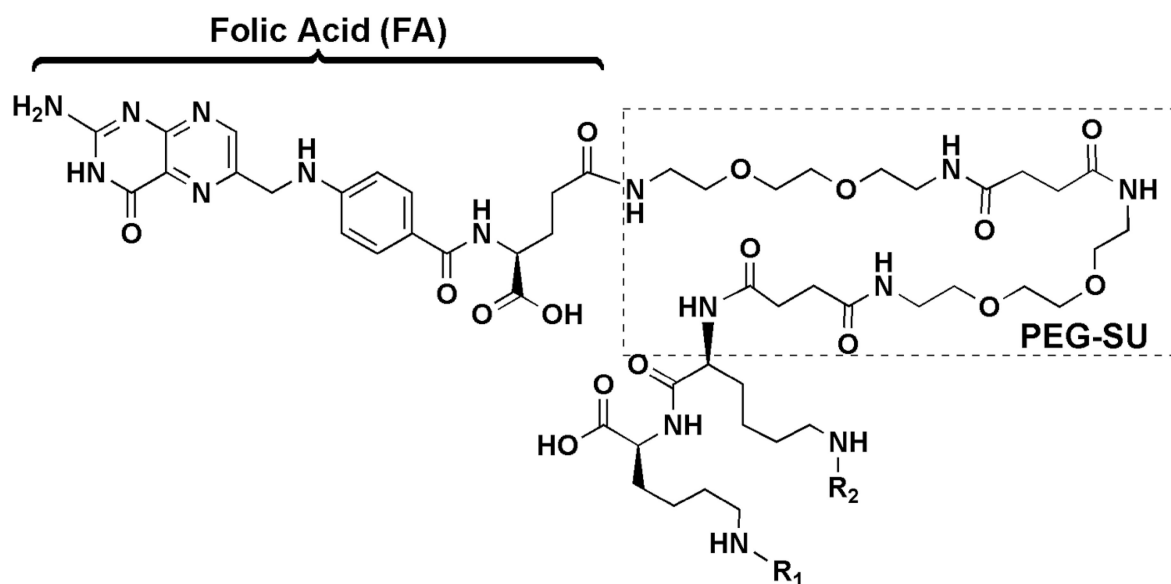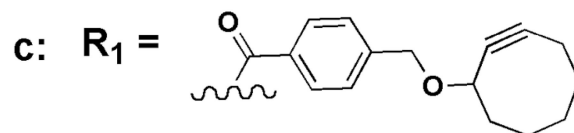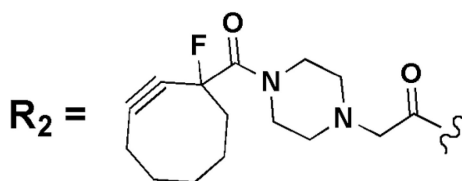
$$k = 15 \times 10^{-3} M^{-1} s^{-1} \qquad k = 2.4 \times 10^{-3} M^{-1} s^{-1} \qquad k = 42 \times 10^{-3} M^{-1} s^{-1}$$

Figure 2.
Examples of readily prepared cyclooctynes (**1** and **2**) and the synthetically more challenging **3**. Rate constants refer to cycloaddition reactions with benzyl azide in CD_3CN.[18–20]
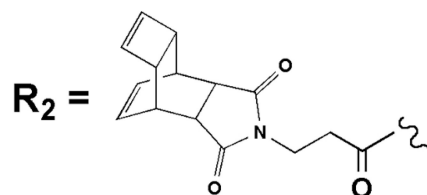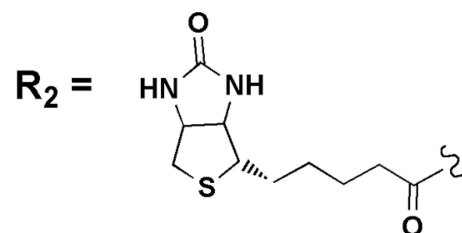
Figure 3.
Bifunctional FA conjugates **18a–d** for Fc-Sec programming.
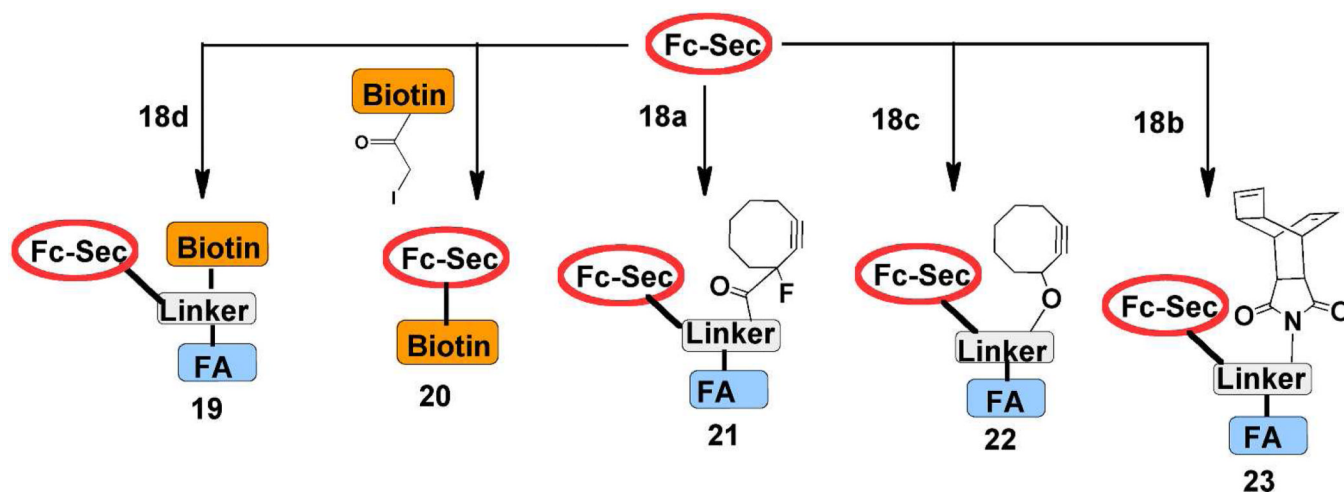
Figure 4.
Fc-Sec was programmed with either **18d** or biotin iodoacetamide to afford biotinylated constructs **19** and **20**, respectively. Additional programming experiments were conducted with **18a, 18c**, and **18b** to afford "clickable" proteins **21–23**.
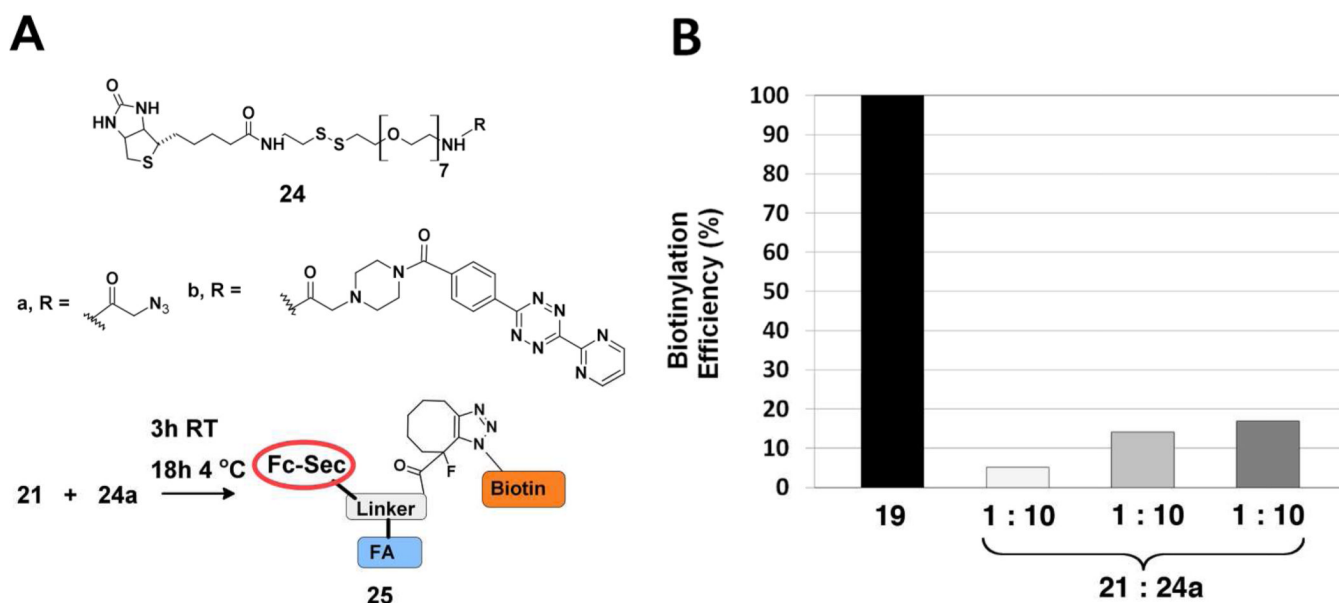
Figure 5.
Biotinylation reagents **24a** and **24b** used to "arm" Fc-Sec proteins **21–23**. (A) SPAAC reaction of Fc-Sec protein **21** (5 μg/mL) with biotin azide **24a**. Reactions were conducted at room temperature (3 h) then overnight (4 °C) in PBS, pH 7.4. (B) SPAAC reaction of **21** with **24a**. Protein **21** was mixed with 10-, 50- and 100-fold excess **24a** as described above and then incubated with FR(+) HeLa cells. The cells were stained with PE-coupled avidin and then analyzed by flow cytometry. The upper limit of biotinylation is defined as the mean fluorescence intensity (MFI) exhibited by **19**. Biotinylation efficiency is expressed as $100 \times (MFI_{25} / MFI_{19})$.
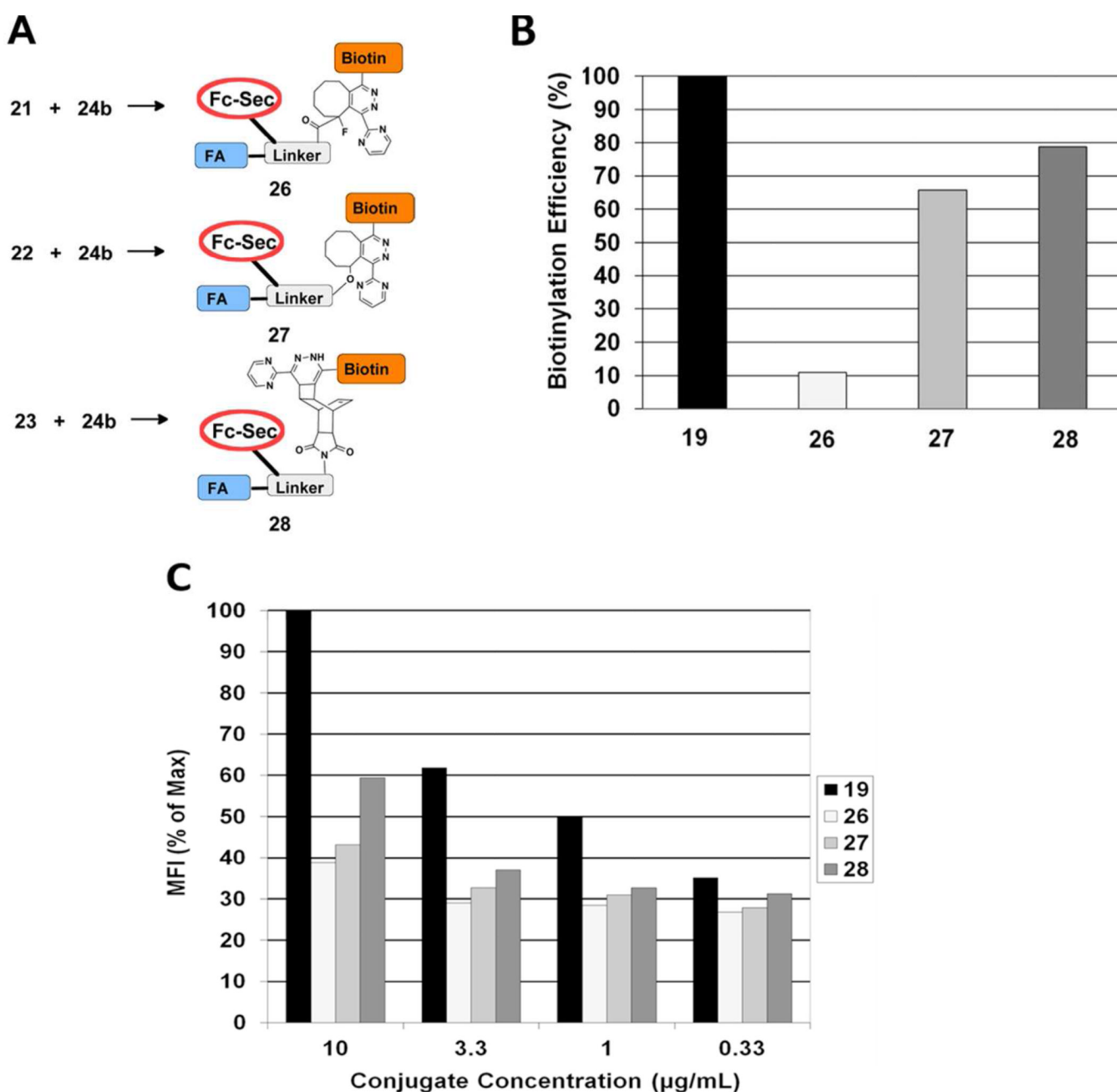
Figure 6.
(A) TL reactions of proteins **21–23** with biotin tetrazine **24b** to generate **26–28** respectively. All reactions included 10-fold excess **24b** and were conducted at room temperature (3 h) then overnight (4 °C) in PBS, pH 7.4. (B and C) Reaction products **26–28** were first incubated at the indicated concentrations (5 μg/mL data shown in B) with FR(+) HeLa cells and then analyzed by flow cytometry as described above. The upper limit of biotinylation defined as MFI exhibited by **19**. (B) Biotinylation efficiency expressed as $100 \times$ ($\text{MFI}_{26, 27, \text{ or } 28}$ / MFI_{19}). (C) Maximum fluorescence signal defined as MFI of **19** at 10 μg/mL. All MFI data shown in C was normalized to MFI of **19** at 10 μg/mL.
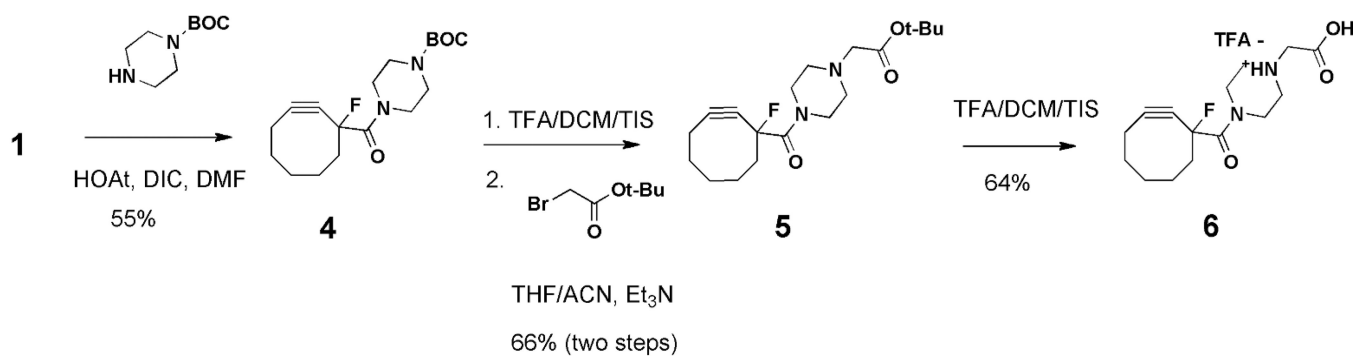
Scheme 1.
Synthesis of the water-soluble cyclooctyne derivative **6**.
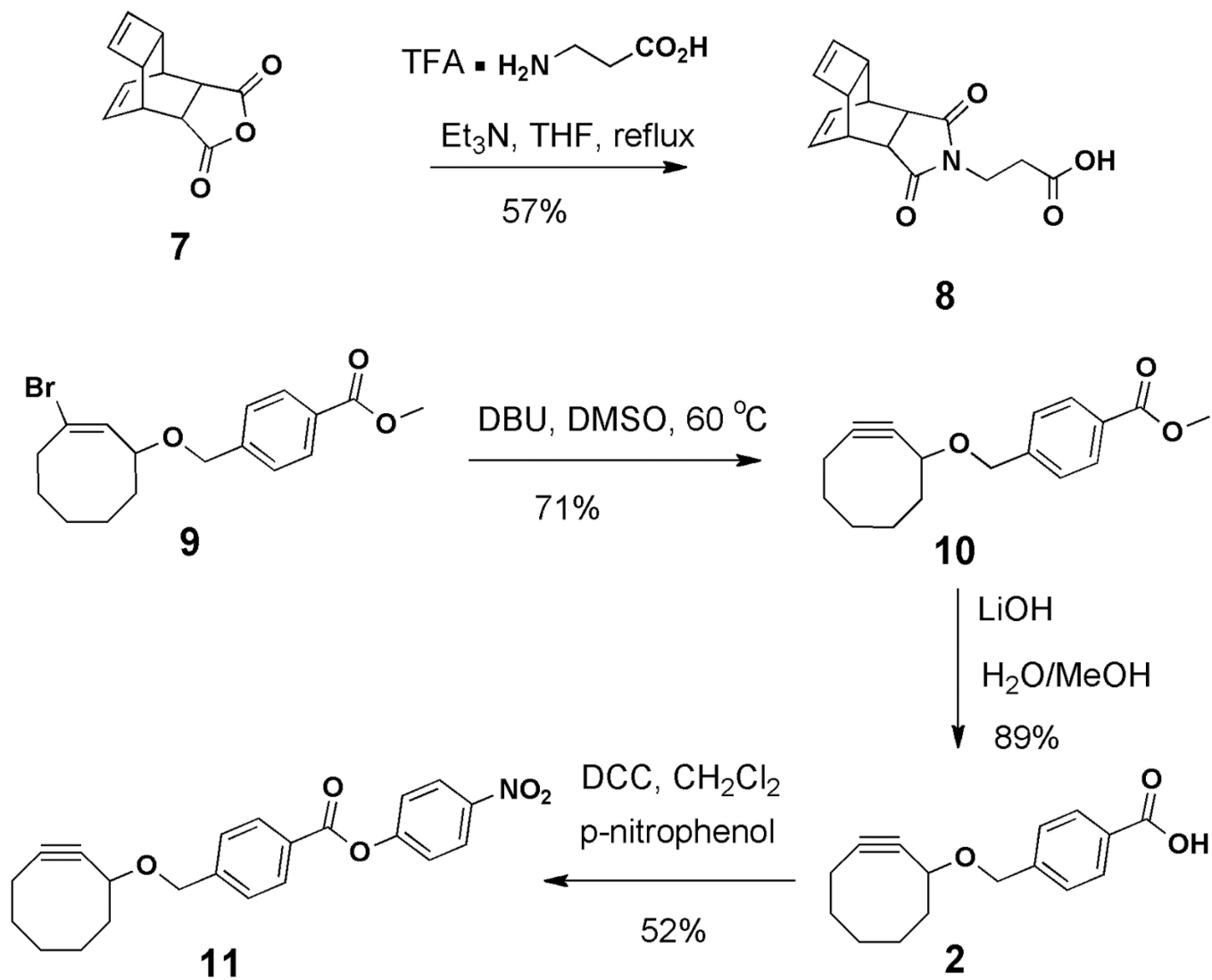
Scheme 2.
Synthesis of TL dieneophiles **11** and **8**.
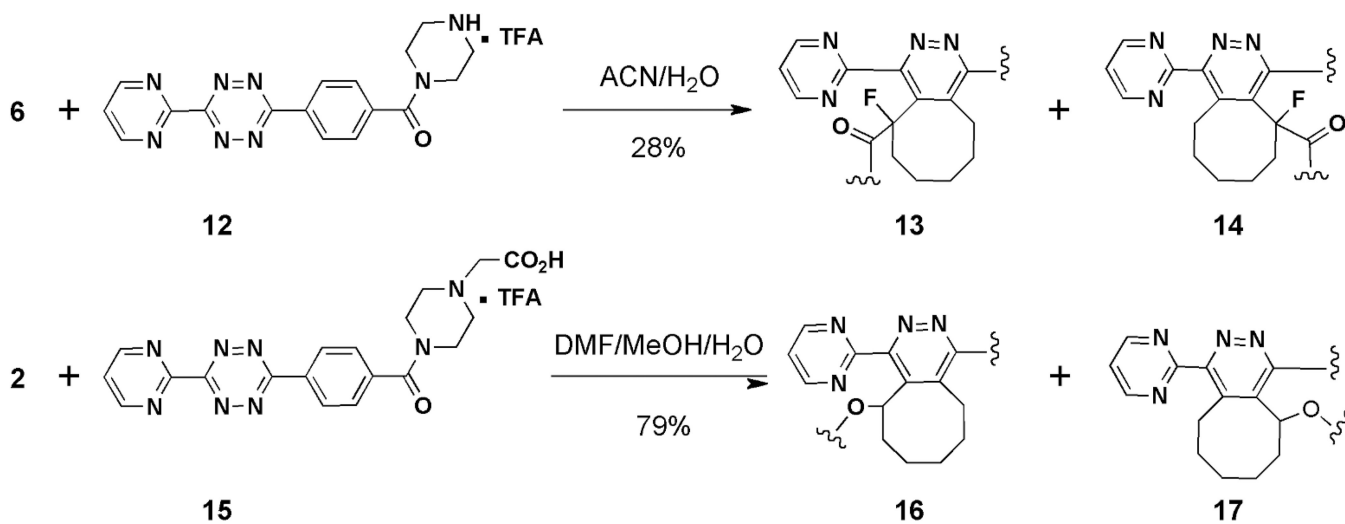
Scheme 3.
[4 + 2] Cycloaddition reactions of free **6** and **2** with tetrazines **12** and **15**, respectively.