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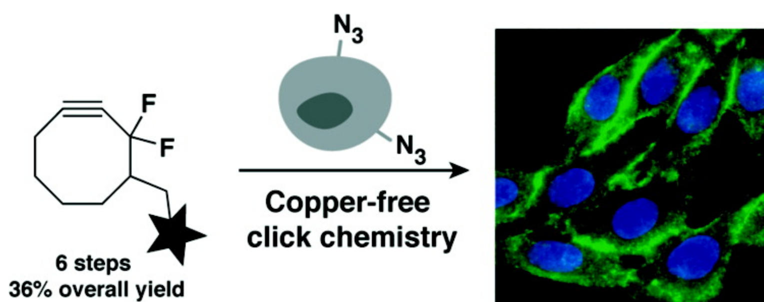

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Second-Generation Difluorinated Cyclooctynes for Copper-Free Click Chemistry

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Abstract: The 1,3-dipolar cycloaddition of azides and activated alkynes has been used for site-selective labeling of biomolecules in vitro and in vivo. While copper catalysis has been widely employed to activate terminal alkynes for [3 + 2] cycloaddition, this method, often termed "click chemistry", is currently incompatible with living systems because of the toxicity of the metal. We recently reported a difluorinated cyclooctyne (DIFO) reagent that rapidly reacts with azides in living cells without the need for copper catalysis. Here we report a novel class of DIFO reagents for copper-free click chemistry that are considerably more synthetically tractable. The new analogues maintained the same elevated rates of [3 + 2] cycloaddition as the parent compound and were used for imaging glycans on live cells. These second-generation DIFO reagents should expand the use of copper-free click chemistry in the hands of biologists.

Introduction

The term "click chemistry" describes a collection of organic reactions that proceed rapidly and selectively under mild conditions to covalently link molecular components.[1] Among the many click reactions described to date, the Huisgen 1,3-dipolar cycloaddition of azides and alkynes[2] has received the most attention. The reaction is highly exergonic ($\Delta G° \approx -61$ kcal/mol),[3] the starting materials are easily prepared, the 1,2,3-triazole products are exceptionally stable, and the reaction occurs readily in both organic and aqueous solvents. However, elevated temperatures or pressures are necessary to accelerate the reaction when simple alkynes and azides are employed, since the activation energy for the cycloaddition is high ($\Delta G^{\ddagger} \approx +26$ kcal/mol).[3]

In the past decade, several strategies have been pursued in order to lower the activation barrier. Sharpless and co-workers[4] and Meldal and co-workers[5] first reported that Cu(I) catalysis dramatically accelerates the reaction of terminal alkynes and azides, regioselectively forming the 1,4-disubstituted triazoles. More recently, ruthenium-based catalysts have been employed to produce the 1,5-disubstituted isomer.[6] These metal-catalyzed variants of the Huisgen [3 + 2] cycloaddition are now central tools for combinatorial library synthesis,[7] construction of peptidomimetics and glycomimetics,[8] supramolecular synthesis,[9] and labeling of biomolecules in vitro or in fixed samples.[10−14] However, the reliance of these chemistries on cytotoxic transition metals has largely precluded their use for applications in vivo, despite parallel growth in the use of azides as chemical reporters in biological systems.[15]

As an alternative approach to lower the activation barrier for [3 + 2] cycloaddition, we have employed the intrinsic ring strain of cyclooctynes, following the precedent of Wittig and Krebs.[16,17] These highly strained alkynes (18 kcal/mol of ring strain[18]) react selectively with azides to form regioisomeric mixtures of

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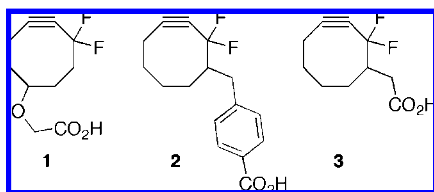
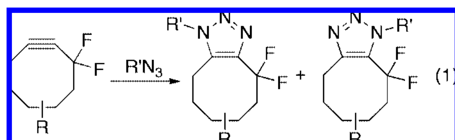


Figure 1. Panel of difluorinated cyclooctyne reagents (**1**−**3**) for copper-free click chemistry.

triazoles at ambient temperatures and pressures without the need for metal catalysis and with no apparent cytotoxicity (eq 1):



We further enhanced the cycloaddition rate by installing propargylic fluorine atoms intended to lower the LUMO, thereby increasing its interaction energy with the HOMO of the azide,[19] an effect that was recently studied using density functional theory calculations.[20] The difluorinated cyclooctyne we termed "DIFO" (compound **1** in Figure 1) reacted with azides on intact proteins at a rate comparable to that of Cu-catalyzed click chemistry and has since been employed for dynamic imaging of cell-surface glycans in live cells[19] and in developing zebrafish embryos.[21] Other groups have reported the use of strained oxanorbornadiene[22] and dibenzocyclooctyne[23] reagents as substrates for [3 + 2] cycloaddition with azides, suggesting a rich future for Cu-free click chemistry.

Still, the canonical Cu-catalyzed reaction has the advantage of synthetic convenience. Terminal alkynes can be installed in biomolecules using simple building blocks, such as commercial alkynoic acids. In contrast, the synthesis of DIFO comprised 12 steps and an overall yield of ~1%.[19] The final step of the sequence, elimination of a vinyl triflate to form the cyclooctyne, suffered from significant decomposition and low yield. Thus, synthetically tractable cyclooctynes with the capabilities of DIFO are needed in order to expand the use of this Cu-free click reagent in biological settings.

Here we report the design, synthesis, and biological evaluation of the second-generation DIFO reagents **2** and **3** (Figure 1), which retain the difluorinated cyclooctyne core but possess a C−C bond to a linker substituent at C4 rather than a C−O bond at C6 as in **1** (Figure 1). This structural change dramatically simplified the synthesis without impact on the kinetics or bioorthogonality of [3 + 2] cycloaddition with azides. These synthetically tractable second-generation DIFO reagents should facilitate further applications of Cu-free click chemistry.

Experimental Section

General Materials and Methods. All of the chemical reagents were analytical grade, obtained from commercial suppliers, and used without further purification, unless otherwise noted. All of the reactions were performed in a N_2 atmosphere, unless otherwise noted. Liquid reagents were added with a syringe, unless otherwise noted. THF and CH_2Cl_2 were dried in vacuo over alumina. Flash chromatography was carried out with Merck 60 230−400 mesh silica gel according to the procedure described by Still et al.[24] When necessary, deactivated silica gel was prepared by rinsing silica gel thoroughly with a solution of 1% Et_3N in the starting solvent mixture used for flash chromatography. Reactions and chromatography fractions were analyzed with Analtech 250 μm silica gel G plates and visualized by staining with ceric ammonium molybdate, anisaldehyde, vanillin, or 2,4-dinitrophenylhydrazine or by absorbance of UV light at 245 nm. Solvents were removed using a rotary evaporator at reduced pressure (20 torr). Unless otherwise noted, ^{1}H, ^{13}C{^{1}H}, ^{19}F, and ^{31}P{^{1}H} NMR spectra were obtained with 300, 400, or 500 MHz Bruker spectrometers. Chemical shifts (δ) are reported in parts per million referenced to the solvent peaks for ^{1}H and ^{13}C, to $CFCl_3$ for ^{19}F, and to H_3PO_4 for ^{31}P. Coupling constants (J) are reported in hertz. Low-resolution and high-resolution (HR) fast atom bombardment (FAB), electron impact (EI), and electrospray ionization (ESI) mass spectra were obtained at the UC Berkeley Mass Spectrometry Facility. Elemental analyses were obtained at the UC Berkeley Micro-Mass Facility. Reversed-phase HPLC was performed using a Rainin Dynamax SD-200 HPLC system with 210 nm detection on a Microsorb C18 analytical or preparative column.

Annexin V-PE was purchased from BD Biosciences. Dulbecco's phosphate-buffered saline (PBS) and fetal bovine serum (FBS) were purchased from HyClone Laboratory, and RPMI-1640 and F12 media were obtained from Invitrogen Life Technologies, Inc. Fluorescein isothiocyanate (FITC)-labeled avidin was purchased from Sigma-Aldrich. Flow cytometry analysis was performed on a BD FACSCalibur flow cytometer using a 488 nm argon laser. At least 2×10^4 cells were analyzed for each sample. Cell viability was ascertained on the basis of forward scatter (to sort by size) and side scatter (to sort by granularity). The average fluorescence intensity was calculated from each of three replicate experiments in order to obtain a representative value in arbitrary units. For all of the flow cytometry experiments, data points were collected in triplicate and represent at least two separate experiments. Fluorescence microscopy was performed on a Zeiss 200 M epifluorescence microscope, and the images were deconvolved using the nearest-neighbor algorithm of Slidebook 4.2 (Intelligent Imaging Innovations, Inc.).

Synthesis of New Compounds. 2,2-Difluoro-1,3-cyclooctanedione (4). A flame-dried round-bottom flask was charged with 1,3-cyclooctanedione (5.10 g, 36.4 mmol) and MeCN (260 mL). Cs_2CO_3 (24.3 g, 74.6 mmol) was added, and the reaction mixture was stirred at room temperature (rt) for 30 min. The reaction mixture was cooled to 0 °C, and Selectfluor (31.0 g, 87.4 mmol) was added, after which the mixture was stirred for an additional 15 min. The system was allowed to warm to rt, stirred for 1.5 h, concentrated under reduced pressure, diluted with 1 M HCl (200 mL), and extracted with diethyl ether (4 × 200 mL). The combined organic layers were washed with brine (2 × 100 mL), dried over $MgSO_4$, and filtered through a glass frit. The solution was concentrated under reduced pressure and purified by flash chromatography (9:1 hexanes/EtOAc) to yield a white solid (4.70 g, 73%). R_f = 0.40 (4:1 hexanes/EtOAc); mp 42.7−43.7 °C. ^{1}H NMR (500 MHz, $CDCl_3$): δ 2.67 (m, 4H), 1.81 (m, 4H), 1.63 (m, 2H). ^{13}C NMR (125 MHz, $CDCl_3$): δ 197.9 (t, J = 25.1 Hz), 109.5 (t, J = 261.5 Hz), 38.7, 26.2, 24.7. ^{19}F NMR (376 MHz, $CDCl_3$): δ −118.35 (s). IR (thin film, cm^{-1}): 3468, 2946, 2867, 1732. HRMS (EI$^+$): calcd for $C_8H_{10}O_2F_2$, 176.0649; found, 176.0646.

Compound 6. To a flame-dried round-bottom flask were added diketone **4** (1.57 g, 8.92 mmol), phosphonium bromide **5** (4.60

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g, 9.37 mmol), and THF (200 mL). The system was cooled to 0 °C, and DBU (1.34 mL, 8.92 mmol) was added, after which the reaction mixture was stirred for 20 min at 0 °C. After the reaction mixture was warmed to rt, it was stirred for an additional 48 h; the reaction was quenched with AcOH (1.5 mL), and the mixture was diluted with MeOH (20 mL), concentrated under reduced pressure, and purified by flash chromatography (0−5% EtOAc in a 2:1 mixture of hexanes/toluene) to yield a white solid (2.63 g, 96%). R_f = 0.55 (4:1 hexanes/EtOAc); mp 58.8−61.1 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.05 (d, 2H, J = 8.4 Hz), 7.38 (d, 2H, J = 8.2 Hz), 7.23 (s, 1H), 3.92 (s, 3H), 2.70 (t, 2H, J = 6.6 Hz), 2.52 (app t, 2H, J = 6.2 Hz), 1.86 (m, 2H), 1.53 (m, 4H). ^{13}C NMR (125 MHz, $CDCl_3$): δ 202.1 (t, J = 28.9 Hz), 166.8, 140.0, 134.6 (t, J = 19.6 Hz), 131.2 (t, J = 10.3 Hz), 130.0, 129.7, 129.0, 115.2 (t, J = 253.4 Hz), 52.4, 37.5, 27.3, 26.0, 25.7, 25.3 (t, J = 2.5 Hz). ^{19}F NMR (376 MHz, $CDCl_3$): δ −111.09 (s). HRMS (FAB): calcd for $C_{17}H_{18}O_3F_2$ $[M + H]^+$, 309.1302; found, 309.1302.

Compound 7. To a round-bottom flask were added olefin **6** (2.63 g, 8.53 mmol) and MeOH (100 mL). The system was flushed with N_2, and a catalytic amount of Pd/C was added. The system was again flushed with N_2 followed by H_2, and the reaction mixture was stirred under an H_2 atmosphere (using a balloon) for 24 h. The system was then flushed thoroughly with N_2, after which the reaction mixture was diluted with CH_2Cl_2 (100 mL), filtered through Celite, and concentrated under reduced pressure. The crude product was purified by flash chromatography (0−2% EtOAc in a 2:1 mixture of hexanes/toluene) to yield a white solid (2.47 g, 93%). R_f = 0.60 (4:1 hexanes/EtOAc); mp 70.1−71.4 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.99 (dm, 2H, J = 8.3 Hz), 7.27 (d, 2H, J = 8.14 Hz), 3.92 (s, 3H), 3.31 (dd, 1H, J = 13.6, 2.9 Hz), 2.82−2.73 (m, 1H), 2.66−2.48 (m, 2H), 2.45−2.28 (m, 1H), 2.17−2.02 (m, 1H), 1.97−1.84 (m, 1H), 1.66−1.42 (m, 4H), 1.41−1.29 (m, 1H), 1.29−1.11 (m, 1H). ^{13}C NMR (125 MHz, $CDCl_3$): δ 205.7 (dd, J = 30.4, 25.1 Hz), 167.1, 144.7, 130.0, 129.4, 128.6, 119.4 (dd, J = 258.0, 250.7 Hz), 52.2, 46.4 (t, J = 21.6), 39.1, 33.8 (t, J = 4.8 Hz), 27.2, 24.3 (d, J = 6.9 Hz), 24.1 (d, J = 3.4 Hz), 22.9. ^{19}F NMR (376 MHz, $CDCl_3$): δ −102.72 (d, 1F, J = 245.5 Hz), −122.67 (d, 1F, J = 251.7 Hz). HRMS (FAB): calcd for $C_{17}H_{20}O_3F_2$ $[M + H]^+$, 311.1459; found, 311.1467.

Compound 8. To a flame-dried round-bottom flask was added THF (175 mL) followed by KHMDS (14.81 mL of a 0.5 M solution in toluene, 7.40 mmol). The reaction mixture was cooled to −78 °C with stirring, and ketone **7** (2.19 g, 7.05 mmol) in THF (70 mL) was added dropwise over 20 min. The reaction mixture was stirred for an additional 40 min, and then a solution of Tf_2NPh (2.65 g, 7.40 mmol) in THF (70 mL) was added via syringe. The system was warmed to rt with stirring over 21 h, and the reaction was then quenched with MeOH (10 mL). The reaction mixture was concentrated under reduced pressure and purified by flash chromatography (0−5% EtOAc in 4:1 hexanes/toluene with 1% Et_3N) to yield a pale-yellow oil (2.74 g, 88%). R_f = 0.60 (4:1 hexanes/EtOAc). ^{1}H NMR (400 MHz, CD_3CN): δ 7.93 (d, 2H, J = 8.2 Hz), 7.36 (d, 2H, J = 8.2 Hz), 6.25 (t, 1H, J = 9.4 Hz), 3.85 (s, 3H), 3.24 (dd, 1H, J = 13.5, 3.8 Hz), 2.88−2.70 (m, 1H), 2.61 (dd, 1H, J = 13.5, 10.2 Hz), 2.56−2.42 (m, 1H), 2.41−2.30 (m, 1H), 1.68−1.52 (m, 3H), 1.51−1.44 (m, 2H), 1.43−1.34 (m, 1H). ^{13}C NMR (125 MHz, CD_3CN): δ 167.6, 145.8, 143.6 (t, J = 29.4 Hz), 130.5, 130.4, 129.5, 129.1 (t, J = 4.0 Hz), 120.5 (dd, J = 246.2, 243.1 Hz), 119.5 (q, J = 319.1 Hz), 52.7, 46.8 (app t, J = 22.0 Hz), 35.2 (app d, J = 5.4 Hz), 27.0, 26.2, 23.0, 21.8. ^{19}F NMR (376 MHz, CD_3CN): δ −74.45 (s, 3F), −93.93 (d, 1F, J = 272.2 Hz), −105.18 (d, 1F, J = 266.9 Hz). HRMS (FAB): calcd for $C_{18}H_{19}O_5F_5S$ $[M + H]^+$, 443.0952; found, 443.0960.

Compound 9. To a flame-dried round-bottom flask was added vinyl triflate **8** (2.74 g, 6.20 mmol) in THF (160 mL). The mixture was cooled to −20 °C with stirring. In a separate flame-dried round-bottom flask, a 0.199 M solution of LDA was made by adding *n*-butyllithium (8.46 mL of a 2.5 M solution in hexanes, 21.2 mmol) dropwise to a solution of diisopropylamine (3.59 mL, 25.4 mmol) in THF (93.8 mL) while stirring at −78 °C. A portion of the LDA solution (37.4 mL, 7.44 mmol) was added dropwise to the first mixture over 1 h using a syringe pump at −20 °C. The reaction mixture was then brought to rt over 20 min, and the reaction was quenched with MeOH (10 mL); the mixture was then concentrated under reduced pressure and purified by flash chromatography (0−3% EtOAc in 2:1 hexanes/toluene) to yield a white solid (1.58 g, 87%). R_f = 0.50 (1:2 hexanes/toluene); mp 78.5−82.7 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.99 (d, 2H, J = 8.1 Hz), 7.27 (d, 2H, J = 7.9 Hz), 3.92 (s, 3H), 3.16 (app d, 1H, J = 11.2 Hz), 2.60−2.43 (m, 2H), 2.41−2.24 (m, 2H), 2.10−1.88 (m, 2H), 1.87−1.68 (m, 2H), 1.62−1.44 (m, 1H), 1.21−1.08 (m, 1H). ^{13}C NMR (125 MHz, $CDCl_3$): δ 167.2, 145.5, 130.0, 129.4, 128.4, 119.5 (t, J = 238.6 Hz), 109.9 (t, J = 11.1 Hz), 85.1 (dd, J = 47.2, 41.6 Hz), 58.2 (t, J = 24.3 Hz), 52.2, 34.5 (d, J = 4.7 Hz), 32.6, 30.8 (d, J = 4.4 Hz), 28.0, 20.4. ^{19}F NMR (376 MHz, $CDCl_3$): δ −94.32 (d, 1F, J = 260.2 Hz), −101.36 (dm, 1F, J = 259.8 Hz). HRMS (FAB): calcd for $C_{17}H_{18}O_2F_2$ $[M + H]^+$, 293.1353; found, 293.1357.

Compound 2. To a round-bottom flask fitted with a reflux condenser were added cyclooctyne methyl ester **9** (1.58 g, 5.42 mmol), LiOH (2.59 g, 108 mmol), water (6 mL), and dioxane (24 mL) under an air atmosphere. The reaction mixture was heated to 55 °C and stirred for 3 h. The mixture was then cooled to rt and diluted with 1 M HCl until the pH of the solution was <2. The solution was extracted with CH_2Cl_2 (4 × 50 mL). The combined organic layers were then washed (1:1 1 M HCl/brine, 50 mL), dried over $MgSO_4$, filtered through a glass frit, and concentrated under reduced pressure. The crude product was then purified by flash chromatography (9:1 to 3:1 hexanes/EtOAc with 1−2% AcOH) to yield a white solid (1.08 g, 72%). R_f = 0.45 (4:1 hexanes/EtOAc with 1% AcOH); mp 181.0−182.0 (dec). ^{1}H NMR (400 MHz, CD_3CN): δ 10.10−8.80 (br s, 1H), 7.94 (d, 2H, J = 8.2 Hz), 7.35 (d, 2H, J = 8.1 Hz), 3.10 (d, 1H, J = 10.9 Hz), 2.70−2.50 (m, 2H), 2.43−2.24 (m, 2H), 2.04−1.84 (m, 2H) 1.83−1.67 (m, 2H), 1.55−1.41 (m, 1H), 1.22−1.11 (m, 1H). ^{13}C NMR (125 MHz, acetone-d_6): δ 167.6, 146.2, 130.8, 130.2, 129.6, 120.2 (dd, J = 239.1, 235.4 Hz), 111.6 (app t, J = 11.3 Hz), 85.5 (dd, J = 46.7, 41.9 Hz), 58.6 (t, J = 24.2 Hz), 34.9 (d, J = 4.9 Hz), 33.3 (d, J = 2.1 Hz), 31.6 (d, J = 4.7 Hz), 28.4, 20.5. ^{19}F NMR (376 MHz, CD_3CN): δ −93.81 (d, 1F, J = 258.7 Hz), −101.32 (ddt, 1F, J = 258.8, 20.3, 7.0 Hz). HRMS (FAB): calcd for $C_{16}H_{16}O_2F_2$ $[M + H]^+$, 279.1197; found, 279.1190.

Compound 10. To a flame-dried round-bottom flask were added DCC (3.66 g, 17.7 mmol), DMAP (98.6 mg, 0.807 mmol), 3-methyl-3-oxetanemethanol (1.59 mL, 16.1 mmol), and CH_2Cl_2 (20 mL). The mixture was cooled to 0 °C with stirring. A solution of iodoacetic acid (3.00 g, 16.1 mmol) in CH_2Cl_2 (30 mL) was then added via syringe. The reaction mixture was stirred for 1 h at 0 °C and allowed to warm to rt over an additional 1.5 h; the reaction was quenched with acetic acid (1 mL), and the mixture was stirred for an additional 30 min. The mixture was then diluted with CH_2Cl_2 and filtered through Celite. The filtrate (~300 mL total) was then washed with water (200 mL), saturated $NaHCO_3$ (2 × 200 mL), and brine (200 mL). The organic layer was then dried over $MgSO_4$, filtered through a glass frit, and concentrated under reduced pressure. The crude product was diluted with CH_2Cl_2 and again filtered through Celite to remove any residual dicyclohexylurea that had precipitated. The filtrate was again concentrated to yield a yellow oil. This material was then transferred to a new round-bottom flask and dissolved in THF (100 mL). Triphenylphosphine (4.65 g, 17.7 mmol) was added, and the reaction mixture was stirred under N_2 at rt for 40 h and then diluted with diethyl ether (100 mL) and filtered through a glass frit to isolate the precipitated product. Residual solvent was removed under reduced pressure to yield a pale-yellow solid (7.93 g, 92% over two steps). Mp 152.8−154.6 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.93−7.84 (m, 6H), 7.84−7.78 (m, 3H), 7.73−7.65 (m, 6H), 5.51 (d, 2H, J = 13.5 Hz) 4.26 (s, 4H), 4.15 (s, 2H), 1.22 (s, 3H). ^{13}C NMR (125 MHz, $CDCl_3$): δ

164.3 (d, J = 3.5 Hz), 135.4 (d, J = 3.1 Hz), 133.9 (d, J = 10.8 Hz), 130.4 (d, J = 13.2 Hz), 117.4 (d, J = 89.2 Hz), 79.1, 71.1, 38.8, 33.6 (d, J = 56.6 Hz), 21.0. ^{31}P NMR (162 MHz, $CDCl_3$): δ 20.41 (s). HRMS (ESI$^+$): calcd for $C_{25}H_{26}O_3P$, 405.1620; found, 405.1631.

Compound 11. To a flame-dried round-bottom flask were added phosphonium iodide **10** (5.66 g, 10.6 mmol), CH_2Cl_2 (75 mL), and DBU (1.51 mL, 10.1 mmol), and the reaction mixture was stirred for 20 min. In a separate flame-dried round-bottom flask, difluoroketone **4** (1.78 g, 10.1 mmol) was dissolved in CH_2Cl_2 (125 mL), and this solution was then added to the phosphonium iodide solution. The reaction mixture was allowed to stir for 48 h, concentrated under reduced pressure, and purified by flash chromatography (10:1 to 4:1 hexanes/EtOAc), yielding a white solid (2.87 g, 94%). R_f = 0.50 (2:1 hexanes/EtOAc); mp 44.2−46.0 °C. ^{1}H NMR (500 MHz, $CDCl_3$): δ 6.49 (s, 1H), 4.51 (d, 2H, J = 6.0 Hz), 4.40 (d, 2H, J = 6.0 Hz), 4.25 (s, 2H), 2.81 (t, 2H, J = 6.6 Hz), 2.66 (t, 2H, J = 6.7 Hz), 1.82 (m, 2H), 1.72 (m, 2H), 1.52 (m, 2H), 1.35 (s, 3H). ^{13}C NMR (125 MHz, $CDCl_3$): δ 200.2 (t, J = 28.1 Hz), 165.1, 150.4 (t, J = 20.0 Hz), 121.1 (t, J = 9.6 Hz), 114.2 (t, J = 254.6 Hz), 79.7, 69.3, 39.2, 37.5, 26.7, 26.6, 26.0, 25.5, 21.3. ^{19}F NMR (376 MHz, CD_3CN): δ −113.03 (s). HRMS (FAB): calcd for $C_{15}H_{20}O_4F_2$ [M + H]$^+$, 303.1408; found, 303.1404.

Compound 12. To a round-bottom flask were added compound **11** (804 mg, 2.66 mmol) and MeOH (30 mL). The system was flushed with N_2, and a catalytic amount of Pd/C was added. The system was then flushed again with N_2 followed by H_2, and the reaction mixture was stirred under an H_2 atmosphere for 24 h. The system was flushed thoroughly with N_2, and the reaction mixture was diluted with CH_2Cl_2 (30 mL) and filtered through Celite to remove the catalyst. The filtrate was then concentrated under reduced pressure. The crude material was dissolved in CH_2Cl_2 (17 mL) and transferred via syringe to a new flame-dried round-bottom flask, which was under a N_2 atmosphere and contained activated 4 Å molecular sieves. The mixture was then cooled to 0 °C with stirring. In a separate flame-dried conical flask, a 0.20 M solution of $BF_3 \cdot OEt_2$ (100 μL, 0.780 mmol) in CH_2Cl_2 (3.9 mL) was prepared, and a portion of this solution (1.0 mL, 0.20 mmol) was added to the reaction mixture via syringe. The reaction mixture was warmed to rt and stirred for an additional 20 h before the reaction was quenched with Et_3N (0.5 mL). The reaction mixture was then concentrated under reduced pressure and purified by flash chromatography (20:1 hexanes/EtOAc with 1% Et_3N over deactivated silica gel) to yield a white solid (731 mg, 90% over two steps). R_f = 0.70 (2:1 hexanes/EtOAc); mp 80.3−83.3 °C. ^{1}H NMR (500 MHz, $CDCl_3$): δ 3.89 (s, 6H), 2.65 (m, 1H), 2.62−2.46 (m, 2H), 2.21 (d, 1H, J = 14.6 Hz), 2.10−1.94 (m, 2H), 1.90 (br s, 1H), 1.60 (m, 2H), 1.56−1.42 (m, 2H), 1.36 (m, 2H), 0.80 (s, 3H). ^{13}C NMR (125 MHz, $CDCl_3$): δ 205.8 (dd, J = 29.7, 25.7 Hz), 119.3 (dd, J = 257.6, 250.2 Hz), 109.0, 72.8, 39.2 (t, J = 21.2 Hz), 38.9, 34.6 (t, J = 4.7 Hz), 30.5, 27.0, 26.3 (d, J = 7.2 Hz), 24.8 (d, J = 2.7 Hz), 23.3, 14.7. ^{19}F NMR (376 MHz, CD_3CN): δ −103.10 (d, 1F, J = 246.4 Hz), −124.20 (dm, 1F, J = 249.0 Hz). HRMS (FAB): calcd for $C_{15}H_{22}O_4F_2$ [M + H]$^+$, 305.1564; found, 305.1558.

Compound 13. To a flame-dried round-bottom flask was added THF (30 mL) followed by KHMDS (2.66 mL of a 0.5 M solution in toluene, 1.33 mmol). The reaction mixture was cooled to −78 °C with stirring, and ketone **12** (354 mg, 1.16 mmol) in THF (15 mL) was added dropwise via syringe over 15 min. The reaction mixture was stirred for an additional 3 h, and then a solution of Tf_2NPh (457 mg, 1.28 mmol) in THF (15 mL) was added via syringe. The system was allowed to slowly warm to rt with stirring over 19 h. The reaction was then quenched with deactivated silica gel, and the mixture was concentrated under reduced pressure and purified by flash chromatography (20:1 to 15:1 hexanes/EtOAc with 1% Et_3N over deactivated silica gel) to yield a white solid (407 mg, 80%). R_f = 0.72 (2:1 hexanes/EtOAc); mp 81.2−83.3 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ 6.05 (t, 1H, J = 9.6 Hz), 3.89 (s, 6H), 2.74−2.56 (m, 1H), 2.51−2.31 (m, 2H), 2.20 (dd, 1H, J = 14.5, 1.7 Hz), 2.00−1.87 (m, 1H), 1.72−1.49 (m, 6H), 0.80 (s, 3H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 143.2 (t, J = 30.2 Hz), 126.9, 119.2 (app t, J = 246.5 Hz), 118.6 (q, J = 320.0 Hz), 108.9, 72.8, 40.9 (app t, J = 22.3 Hz), 35.1, 30.5, 27.2, 26.6, 22.6, 21.8, 14.6. ^{19}F NMR (376 MHz, $CDCl_3$): δ −74.52 (s, 3F), −93.80 (d, 1F, J = 269.8 Hz), −104.75 (dm, 1F, J = 278.0 Hz). HRMS (FAB): calcd for $C_{16}H_{21}O_6F_5S$ [M + H]$^+$, 437.1057; found, 437.1050.

Compound 14. In a round-bottom flask, vinyl triflate **13** (407 mg, 0.932 mmol) was dissolved in toluene (20 mL) and concentrated under reduced pressure to remove trace moisture. This procedure was repeated four times. The material was then dissolved in THF (20 mL), and the solution was cooled to −20 °C with stirring. In a separate flame-dried round-bottom flask, a 0.20 M solution of LDA was made by adding *n*-butyllithium (1.12 mL of a 2.5 M solution in hexanes, 2.80 mmol) dropwise to a solution of diisopropylamine (475 μL, 3.36 mmol) in THF (12.4 mL) at −78 °C. A portion of the LDA solution (5.6 mL, 1.12 mmol) was added dropwise via syringe pump to the first mixture over 1 h. The reaction mixture was then brought to rt over 30 min, and the reaction was quenched with deactivated silica gel; the mixture was then concentrated under reduced pressure and purified by flash chromatography (20:1 to 15:1 hexanes/EtOAc with 1% Et_3N over deactivated silica gel) to yield a white solid (159 mg, 59%). R_f = 0.73 (2:1 hexanes/EtOAc); mp 80.1−86.0 °C. ^{1}H NMR (500 MHz, $CDCl_3$): δ 3.90 (s, 6H), 2.54 (app dq, 1H, J = 23.5, 9.1 Hz), 2.40−2.25 (m, 2H), 2.25−2.11 (m, 2H), 2.10−2.02 (m, 2H), 1.81−1.71 (m, 1H), 1.65 (dd, 1H, J = 14.6, 10.3 Hz), 1.50 (app quint, 1H, J = 7.5 Hz), 1.33 (m, 1H), 0.81 (s, 3H). ^{13}C NMR (125 MHz, $CDCl_3$): δ 120.0 (t, J = 238.3 Hz), 109.8 (t, J = 11.2 Hz), 85.4 (dd, 47.1, 41.8 Hz), 72.8, 51.9 (t, J = 23.8 Hz), 35.2 (dd, J = 4.5, 1.6 Hz), 32.8 (d, J = 4.8 Hz), 32.7 (d, J = 2.1 Hz), 30.5, 29.9, 28.2, 20.5, 14.8. ^{19}F NMR (376 MHz, CD_3CN): δ −94.82 (d, 1F, J = 258.7 Hz), −101.81 (ddt, 1F, J = 259.3, 24.1, 7.2 Hz). HRMS (FAB): calcd for $C_{15}H_{20}O_3F_2$ [M + H]$^+$, 287.1459; found, 287.1461.

Compound 3. To a scintillation vial under an air atmosphere were added cyclooctyne orthoester **14** (90.4 mg, 0.316 mmol), MeOH (4.5 mL), water (450 μL), and PPTS (159 mg, 0.632 mmol). The reaction mixture was stirred at rt for 24 h, after which the reaction was quenched with saturated $NaHCO_3$ (2 mL) and the mixture concentrated under reduced pressure. The crude product was diluted with brine (8 mL) and extracted with EtOAc (3 × 10 mL). The combined organic layers were washed with brine (10 mL) and a HCl/brine solution (1:1 1 M HCl/brine, 2 × 10 mL), dried over $MgSO_4$, and filtered through a glass frit. The filtrate was concentrated under reduced pressure to yield a white solid (106.8 mg). A portion of this material (57.8 mg) was transferred to a round-bottom flask, where it was dissolved in dioxane (1 mL) and water (200 μL). To this was added LiOH (91 mg, 3.8 mmol), and the reaction mixture was stirred at rt for 3 h under an air atmosphere, after which the reaction was quenched with 1 M HCl (5 mL). The reaction mixture was further diluted with brine (3 mL) and extracted with EtOAc (4 × 10 mL). The combined organic layers were washed (1:1 1 M HCl/brine, 10 mL), dried over $MgSO_4$, and filtered through a glass frit. The filtrate was concentrated and purified by flash chromatography (20:1 hexanes/EtOAc with 2% AcOH) to give a white solid (33 mg, 96% over two steps). R_f = 0.66 (1:1 hexanes/EtOAc with 1% AcOH); mp 87.4−88.9 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ 11.90−10.60 (br s, 1H), 2.84−2.69 (m, 2H), 2.46−2.28 (m, 3H), 2.21−2.05 (m, 2H), 1.90−1.74 (m, 2H), 1.67 (m, 1H), 1.41 (m, 1H). ^{13}C NMR (125 MHz, $CDCl_3$): δ 177.8, 119.1 (t, J = 238.6 Hz), 110.7 (t, J = 11.2 Hz), 84.7 (dd, J = 47.0, 41.6 Hz), 52.6 (t, J = 24.4 Hz), 33.7 (app d, J = 4.4 Hz), 32.9 (d, J = 4.6 Hz), 32.7 (d, J = 2.0 Hz), 27.9, 20.5. ^{19}F NMR (376 MHz, $CDCl_3$) δ −94.64 (d, 1F, J = 260.0 Hz),

−100.82 (ddt, 1F, J = 260.2, 21.1, 6.8 Hz). HRMS (ESI$^-$): calcd for $C_{10}H_{11}O_2F_2$ $[M]^-$, 201.0722; found, 201.0729.

Compound 15b. To a flame-dried round-bottom flask were added cyclooctyne **2** (10.5 mg, 0.0377 mmol), CH_2Cl_2 (0.5 mL), and diisopropylethylamine (16.4 μL, 0.0943 mmol), and the mixture was cooled to 0 °C with stirring. Pentafluorophenyl trifluoroacetate (7.1 μL, 0.042 mmol) was then added, and after 10 min, the system was warmed to rt. The reaction mixture was stirred an additional 1.5 h, concentrated, filtered through a plug of silica gel eluting with hexanes, and then concentrated to yield a white solid. This material was transferred to a new round-bottom flask and dissolved in anhydrous DMF (0.5 mL). Biotinylated amine **16**[25] (12.0 mg, 0.0268 mmol) was then added, followed by diisopropylethylamine (7.0 μL, 0.040 mmol). The reaction mixture was stirred overnight, concentrated, and purified twice by flash chromatography (1% Et_3N in CH_2Cl_2, then 5% MeOH in CH_2Cl_2) to yield a clear oil (9.7 mg, 36% over two steps). R_f = 0.65 (9:1 CH_2Cl_2/MeOH). ^{1}H NMR (400 MHz, CD_3OD): δ 8.41 (m, 1H), 7.93 (m, 1H), 7.77 (d, 2H, J = 8.2 Hz), 7.32 (d, 2H, J = 8.2 Hz), 4.48 (dd, 1H, J = 7.8, 4.7 Hz), 4.29 (dd, 1H, J = 7.9, 4.5 Hz), 3.68−3.53 (m, 10H), 3.48 (q, 4H, J = 6.0 Hz), 3.28−3.16 (m, 4H), 3.10 (d, 1H, J = 10.8 Hz), 2.92 (dd, 1H, J = 12.7, 5.0 Hz), 2.70 (d, 1H, J = 12.7 Hz), 2.63−2.47 (m, 2H), 2.44−2.27 (m, 2H), 2.19 (t, 2H, J = 7.4 Hz), 2.08−1.99 (m, 1H), 1.98−1.79 (m, 4H), 1.79−1.69 (m, 4H), 1.69−1.55 (m, 3H), 1.51 (dd, 1H, J = 15.9, 8.1 Hz), 1.47−1.37 (m, 2H), 1.31 (t, 1H, J = 7.3 Hz), 1.23−1.12 (m, 1H). ^{13}C NMR (125 MHz, CD_3OD): δ 176.1, 170.1, 166.3, 145.2, 134.0, 130.5, 128.7, 121.0 (dd, J = 238.9, 236.0 Hz), 111.2 (t, J = 11.2 Hz), 86.0 (dd, J = 46.8, 41.8 Hz), 71.7, 71.7, 71.4, 71.4, 70.4, 70.1, 63.5, 61.8, 59.5 (t, J = 24.3 Hz), 57.2, 41.2, 38.9, 38.0, 37.0, 35.2 (d, J = 4.0 Hz), 33.8 (d, J = 1.5 Hz), 32.0 (d, J = 4.6 Hz), 30.6 (d, J = 3.8 Hz), 30.0, 29.7, 29.1, 27.0, 20.8, 9.4. ^{19}F NMR (376 MHz, CD_3OD): δ −95.40 (d, 1H, J = 259.9 Hz), −102.76 (ddt, 1H, J = 259.9, 20.1, 6.8 Hz). HRMS (FAB): calcd for $C_{36}H_{52}N_4O_6F_2S$ $[M + Li]^+$, 713.3736; found, 713.3736.

Compound 15c. To a flame-dried round-bottom flask were added cyclooctyne **3** (26.3 mg, 0.130 mmol), CH_2Cl_2 (2.0 mL), and diisopropylethylamine (57.0 μL, 0.325 mmol), and the mixture was cooled to 0 °C with stirring. Pentafluorophenyl trifluoroacetate (25.0 μL, 0.143 mmol) was added, and after 10 min, the system was warmed to rt. The reaction mixture was stirred for an additional 1 h, concentrated, and filtered through a plug of silica gel using 1% EtOAc in hexanes as the eluent to yield a white solid (41.4 mg). A portion of this material (6.5 mg, 0.018 mmol) was then transferred to a flame-dried conical flask and dissolved in anhydrous DMF (0.5 mL). Biotinylated amine **16**[25] (7.9 mg, 0.018 mmol) was added, followed by diisopropylethylamine (5.0 μL, 0.027 mmol). The reaction mixture was stirred for 4 h, concentrated, and purified twice by flash chromatography (0−10% MeOH in CH_2Cl_2) to yield a clear oil. R_f = 0.37 (9:1 CH_2Cl_2/MeOH). The oil was further purified by reversed-phase HPLC (gradient of 5−70% CH_3CN in H_2O over 40 min, eluting at ∼27 min) to yield a clear oil (6.4 mg, 50% over two steps). ^{1}H NMR (400 MHz, CD_3OD): δ 4.49 (dd, 1H, J = 7.9, 4.3 Hz), 4.30 (dd, 1H, J = 7.9, 4.5 Hz), 3.68−3.55 (m, 8H), 3.52 (dt, 4H, J = 6.2, 1.4 Hz), 3.27 (m, 4H), 3.21 (m, 1H), 2.93 (dd, 1H, J = 12.8, 5.0 Hz), 2.82−2.66 (m, 2H), 2.49 (dd, 1H, J = 14.7, 3.9 Hz), 2.46−2.28 (m, 2H), 2.20 (t, 2H, J = 7.4 Hz), 2.18−2.00 (m, 3H), 1.85−1.70 (m, 7H), 1.70−1.52 (m, 4H), 1.50−1.33 (m, 3H). ^{13}C NMR (125 MHz, CD_3OD): δ 176.1, 173.8, 166.3, 120.9 (app t, J = 237.4 Hz), 111.8 (app t, J = 11.0 Hz), 85.7 (dd, J = 46.6, 42.0 Hz), 71.7, 71.7, 71.4, 71.4, 70.1, 70.0, 63.5, 61.8, 57.2, 54.6 (t, J = 24.3 Hz), 41.2, 38.1, 38.0, 37.0, 36.3 (d, J = 4.0 Hz), 33.9 (d, J = 1.9 Hz), 33.5 (d, J = 4.5 Hz), 30.6, 30.0, 29.7, 29.0, 27.1, 20.8, 14.6. ^{19}F NMR (376 MHz, CD_3OD): δ −95.57 (d, 1F, J = 259.0 Hz), −102.08 (ddt, 1F, J = 259.4, 20.9, 6.8 Hz). HRMS (FAB): calcd for $C_{30}H_{48}N_4O_6F_2S$ $[M + H]^+$, 631.3341; found, 631.3324.

Kinetic Evaluation of [3 + 2] Cycloaddition of 2 and 3 with Benzyl Azide. Cyclooctyne **2** or **3** was mixed at a 1:1 molar ratio (∼15 mM) with benzyl azide in either (a) CD_3CN or (b) a 7:3 mixture of CD_3CN and 25 mM potassium phosphate in D_2O (pH 7), and the reaction was monitored by ^{1}H NMR. The kinetic data were derived by following the change in integration of resonances corresponding to the benzylic protons in benzyl azide (δ ∼4.4) compared to those of the benzylic protons in the triazole products (δ ∼5.5−5.7). The second-order rate constants for the reaction were determined by plotting 1/[azide] versus time and subsequently using analysis by linear regression. The second-order rate constant k ($M^{-1}s^{-1}$) corresponds to the determined slope.

Cell Culture. Jurkat or Chinese hamster ovary (CHO) cells were maintained in a 5% CO_2, water-saturated atmosphere and grown in RPMI-1640 (Jurkat) or F12 (CHO) media supplemented with 10% FBS, penicillin (100 units/mL), and streptomycin (0.1 mg/mL). Cell densities were maintained between 1×10^5 and 1.6×10^6 cells/mL.

Cell-Surface Labeling of Azide-Bearing Glycans with Biotinylated Conjugates. Jurkat cells were incubated for 3 days in media containing 25 μM Ac_4ManNAz or no sugar. The cells were distributed into a 96-well V-bottom tissue culture plate and washed three times by sequential concentration by centrifugation (2500g, 3 min, 4 °C) and resuspension in 200 μL of labeling buffer [PBS (pH 7.4) containing 1% FBS]. The cells were then incubated at rt with 100 μL of 0−10 μM **15a−c** in labeling buffer for 0−60 min, with dilutions made from a 2.5 mM stock solution in 7:3 PBS/DMF. After incubation, cells were washed three times and resuspended in 100 μL of FITC-labeled avidin (1:200 dilution in labeling buffer of a 1 mg/mL stock solution). After a 15 min incubation in the dark at 4 °C, the cells were washed once and then incubated with FITC-labeled avidin for an additional 15 min at 4 °C. The cells were washed three times and then diluted to a volume of 400 μL for flow cytometry analysis. Annexin V-PE staining was performed according to instructions from the manufacturer immediately prior to flow cytometry analysis.

Live-Cell Imaging of Azide-Labeled Glycans. CHO cells were incubated for 3 days in media containing 25 μM Ac_4ManNAz or no sugar in an eight-well LabTek II chambered coverglass (Nunc). The cells were washed three times by sequential gentle aspiration of the media and addition of 500 μL of media. The cells were then treated with 100 μL of a 10 μM solution of **15b** or **15c** (1:250 dilution in media from a 2.5 mM stock solution in 7:3 PBS/DMF) for 60 min at rt. The cells were washed three times, stained with FITC-labeled avidin (100 μL of a 1:200 dilution in media from a 1 mg/mL stock solution) for 10 min at rt, washed three times, treated with Hoechst 33342 dye (100 μL of a 1:1000 dilution in media from a 1 mg/mL DMSO stock solution) for 2 min at rt to stain the nuclei, washed twice, and imaged by epifluorescence microscopy.

Results and Discussion

Synthesis of Second-Generation DIFO Reagent 2. The synthesis of cyclooctyne **2** (Scheme 1) began with 1,3-cyclooctanedione, which was prepared in ∼67% yield as previously described.[26] Difluorination of 1,3-dicarbonyl compounds with Selectfluor has been reported to occur sluggishly under neutral conditions[27] and more rapidly using enamine intermediates[28] or microwave-assisted strategies.[29] We found that simply

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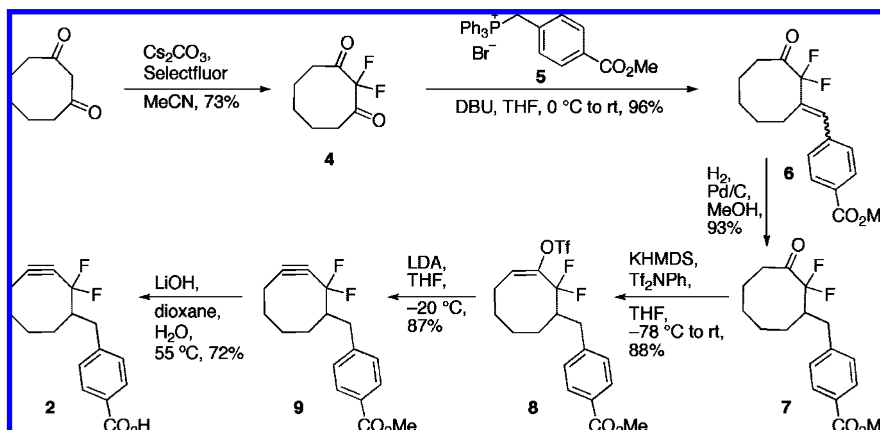
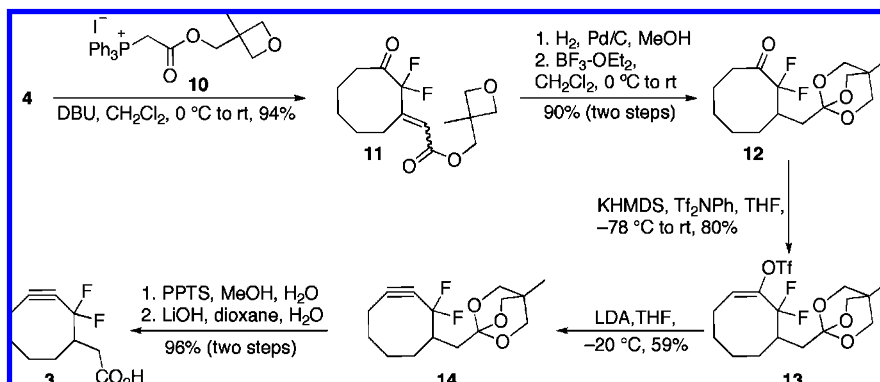
Scheme 1. Synthesis of Second-Generation DIFO Reagent **2**



Scheme 2. Synthesis of Second-Generation DIFO Reagent **3**



treating the diketone with Selectfluor and Cs_2CO_3 at 0 °C produced 2,2-difluoro-1,3-cyclooctanedione (**4**) in 73% yield. In order to install a linker with a protected carboxylic acid, we performed a Wittig reaction using phosphonium salt **5** and DBU. Fortuitously, we observed exclusive formation of monosubstituted product **6** even when excess amounts of **5** and base were used, thus enabling efficient desymmetrization of the diketone. The observed chemoselectivity is likely due to the high electrophilicity of the first ketone equivalent compounded with the low nucleophilicity of the stabilized ylide. Hydrogenation of the olefin to saturated compound **7** and subsequent conversion of the ketone to a vinyl triflate (**8**) proceeded in good yield (82% over two steps). Cyclooctyne **9** was formed by LDA-mediated elimination, and the methyl ester was saponified to yield DIFO reagent **2** in a total of six steps in 36% overall yield from 1,3-cyclooctanedione. This yield is ~25-fold higher than that for the previously reported synthesis of **1**[19] and was achieved in half as many steps.

Synthesis of Second-Generation DIFO Reagent 3. A lesson from previous studies is that the hydrophobicity of DIFO reagents can contribute to nonspecific protein and cell binding. Thus, we also synthesized a second-generation DIFO analogue lacking the nonessential phenyl moiety in the linker. The target, compound **3** (Figure 1), possessed a carboxymethyl group to which probes were later attached. A key protecting-group strategy masked the carboxymethyl group as an oxabicycloortho (OBO) ester in order to avoid acidic protons that would later interfere with vinyl triflate formation and elimination.

As shown in Scheme 2, 2,2-difluoro-1,3-cyclooctanedione (**4**) was reacted under basic conditions with phosphonium salt **10**, which bears an oxetane ester, an OBO ester precursor.[30] Again, we observed selective monosubstitution to yield olefin **11** in 85% yield. Hydrogenation of the olefin and conversion of the oxetane ester to the orthoester using $BF_3 \cdot OEt_2$[31] yielded ketone **12** in 90% yield. Formation of vinyl triflate **13** and elimination to form cyclooctyne **14** proceeded in 80% and 59% yields, respectively. Two-step deprotection of the OBO ester to carboxylic acid **3** was accomplished by acidic deprotection using PPTS to form a simple ester and subsequent saponification using LiOH (86% yield).[32] The synthesis of **3** was accomplished with an overall yield of 28% from 1,3-cyclooctanedione.

Kinetic Evaluation of [3 + 2] Cycloaddition of 2 and 3 with Benzyl Azide. With these reagents in hand, we used ^1H NMR to measure the kinetics of the copper-free click reaction with the model compound benzyl azide. For the reactions with **2** and **3** performed in CD_3CN, we obtained second-order rate constants of $(4.2 \pm 0.1) \times 10^{-2}$ and $(5.2 \pm 0.2) \times 10^{-2}$ M^{-1} s^{-1}, respectively; these values are comparable to that for the reaction of **1** with benzyl azide under identical conditions (7.6×10^{-2} $M^{-1}s^{-1}$).[19] To evaluate the kinetics of the [3 + 2] cycloaddition in an aqueous environment, we performed reactions of **2** and **3** with benzyl azide in a 7:3 mixture of CD_3CN and 25 mM potassium phosphate in D_2O (pH 7) and obtained k values of $(9.0 \pm 0.3) \times 10^{-2}$ and $(8.6 \pm 0.9) \times 10^{-2}$ M^{-1}

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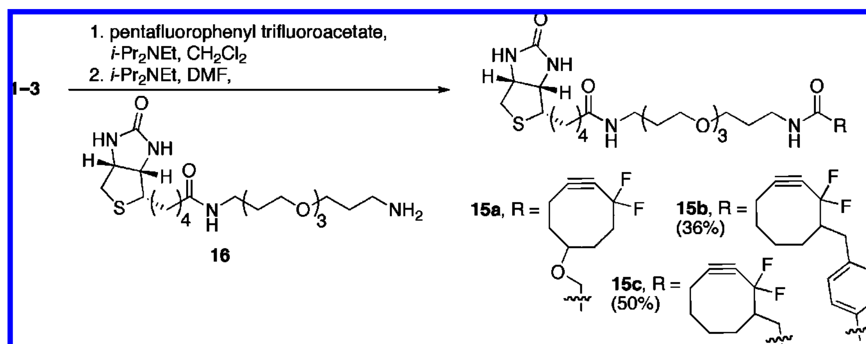
Scheme 3. Synthesis of Biotinylated Derivatives of Difluorinated Cyclooctynes (**15a**−**c**)



s^{-1}, respectively. All three reagents are considerably more reactive than cyclooctynes lacking the difluoromethylene group.[33]

Additionally, we investigated the stabilities of compounds **2** and **3** with respect to biologically relevant nucleophiles. We dissolved each cyclooctyne (20 mM) in a 7:3 mixture of CD_3CN and 25 mM potassium phosphate in D_2O (pH 7) and then added either 2-mercaptoethanol or 2-aminoethanol (20 mM). We observed no reaction after 24 h, as monitored by ^{1}H and ^{19}F NMR, suggesting that compounds **2** and **3** are stable to water, thiols, amines, and alcohols at physiological pH. Finally, compounds **2** and **3** were found to be stable for many months when stored at −20 °C.

Copper-Free Click Labeling of Cell-Surface Azido Glycans Using Biotinylated Conjugates. An important application of copper-free click chemistry is the nontoxic and rapid detection of azides within living systems, either for molecular imaging or for subsequent affinity capture. DIFO reagent **1** and its derivatives (e.g., the biotinylated derivative **15a**[19]) have proven useful for bioorthogonal labeling of cell-surface glycans bearing azides, and we set out to evaluate the second-generation DIFO reagents in this context. We first derivatized carboxylic acids **2** and **3** as the biotinylated reagents **15b** and **15c**, respectively, via formation of the corresponding activated pentafluorophenyl esters and subsequent conjugation to an amine-derivatized biotin reagent (Scheme 3).

Jurkat cells were incubated with 25 μM peracetylated *N*-azidoacetylmannosamine (Ac_4ManNAz) for 3 days, resulting in the metabolic labeling of cell-surface glycans with azido sialic acid (SiaNAz) residues (Figure 2A).[34] The cells were washed and labeled with biotinylated reagents **15b** and **15c** either at various concentrations (0−10 μM) for 60 min (Figure 2B) or for various reaction times (0−60 min) at 10 μM (Figure 2C). In all cases, the cells were subsequently stained with FITC-labeled avidin and analyzed by flow cytometry, as described previously.[35] Both of the second-generation DIFO reagents displayed concentration- and time-dependent reaction profiles with cell-surface-associated azides. We were pleased to observe that both reagents displayed very little background fluorescence labeling, though the more hydrophobic **15b** resulted in slightly higher background fluorescence than **15c**.

We then compared the efficacy of cell-surface azide labeling of **15b** and **15c** to the first-generation reagent **15a** in a 60 min reaction at 10 μM (Figure 2D). The observed labeling with **15b** and **15c** was approximately half of that with **15a**, consistent with their relative rate constants. These labeling efficiencies are all significantly higher than those previously reported for mono-

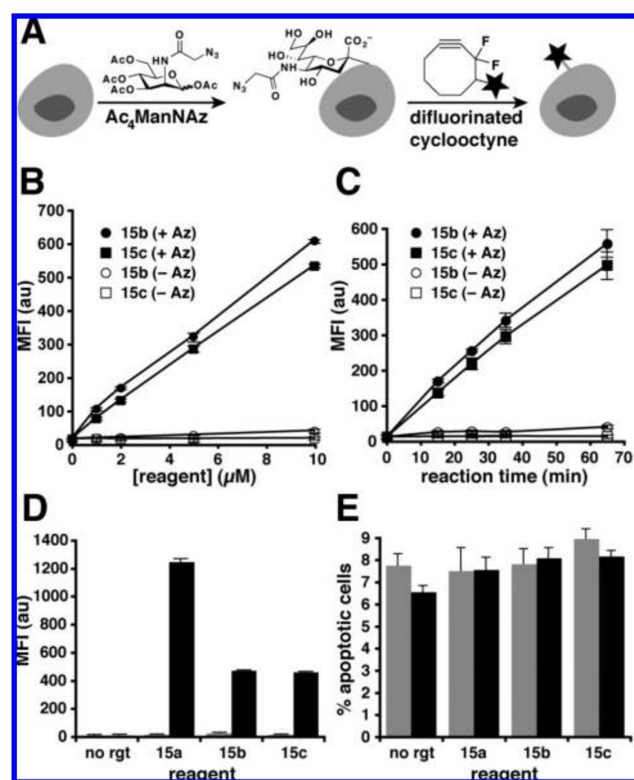


Figure 2. Labeling of cell-surface glycans with azido sugars and DIFO reagents. (A) Bioorthogonal chemical reporter strategy for two-step detection of glycans. Cells were first incubated with Ac_4ManNAz, which is metabolically converted to cell-surface SiaNAz residues, and subsequently reacted with difluorinated cyclooctyne probes for visualization. (B−E) Jurkat cells were metabolically labeled with 25 μM Ac_4ManNAz (+Az) or no sugar (−Az) for 3 days. The cells were labeled with **15b** or **15c** either (B) for 60 min at 0, 1, 2, 5, or 10 μM or (C) for 0, 15, 25, 35, or 65 min at 10 μM. The cells were then stained with FITC-labeled avidin and analyzed by flow cytometry. (D−E) The cells were labeled with **15a**−**c** or no reagent (no rgt) for 60 min at 10 μM and then sequentially stained with FITC-labeled avidin and Annexin V-PE, followed by flow cytometry analysis. Gray bars denote no sugar and black bars 25 μM Ac_4ManNAz. The error bars indicate the standard deviation of three replicate samples. Shown in (D) is the mean fluorescence intensity (MFI) in arbitrary units (au). Shown in (E) is the percentage of cells in each sample belonging to the population that stains highly with Annexin V-PE.

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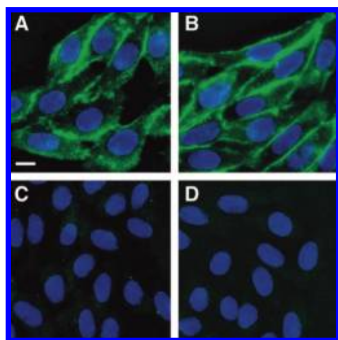

Figure 3. Live-cell imaging of cell-surface glycans using second-generation difluorinated cyclooctynes. CHO cells were metabolically labeled with (A, B) 25 μM $Ac_4ManNAz$ or (C, D) no sugar for 3 days. The cells were labeled for 60 min with 10 μM (A, C) **15b** or (B, D) **15c**, washed, stained with FITC-labeled avidin and Hoechst 33342, and imaged by epifluorescence microscopy. Images were deconvolved using the nearest-neighbor algorithm of Slidebook 4.2 and are shown as maximum intensity *z*-projection fluorescence images over 7.5 μm. Green, FITC; blue, Hoechst 33342. Scale bar: 10 μm.

or nonfluorinated cyclooctyne reagents or triarylphosphines capable of Staudinger ligation.[33]

Finally, we tested the toxicity of probes **15a−c** by incubation of cells labeled as above (60 min, 10 μM) with phycoerythrin-conjugated Annexin V, a marker of apoptosis. As shown in Figure 2E, none of the reagents caused a significant change in the percentage of Annexin V-positive cells, indicating that DIFO reagents **15a−c** are not toxic to Jurkat cells.

Live-Cell Imaging of Membrane-Associated Glycans Using Second-Generation DIFO Reagents. Finally, we applied these novel cyclooctynes to image cell-surface glycans in live CHO cells. The cells were incubated for 3 days with 25 μM $Ac_4ManNAz$ or with no sugar as a negative control and labeled with 10 μM **15b** or **15c** for 60 min. Subsequently, the cells were treated with FITC-labeled avidin and Hoechst 33342, a live-cell nuclear stain, and imaged by epifluorescence microscopy. We observed clear azide-dependent labeling at the cell surface in the $Ac_4ManNAz$-treated cells and minimal background fluorescence in the negative control for both **15b** and **15c** (Figure 3), indicating that these second-generation DIFO reagents can be utilized for live-cell imaging applications.

Conclusion

We have developed synthetically tractable second-generation difluorinated cyclooctyne reagents for copper-free click chemistry. The synthesis of these reagents involves a novel method for one-step difluorination of 1,3-diketones, a selective Wittig reaction to install the linkers, and the use of an orthoester as a cyclooctyne-compatible protecting group. Furthermore, these reagents can be prepared efficiently, with overall yields that are more than 20-fold higher than that of the first-generation reagent. Critically, we determined that these novel DIFO reagents are nontoxic to cells and can selectively tag azide-labeled biomolecules in living systems with efficiencies approaching that of the parent compound. We envision that these reagents will enable widespread use of copper-free click chemistry in the context of in vivo imaging and ex vivo labeling of biomolecules and in the generation of novel biocompatible materials.[36]

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Supporting Information Available: ^{1}H and ^{13}C NMR spectra for all of the new compounds, kinetic data, and flow cytometry plots. This material is available free of charge via the Internet at http://pubs.acs.org.

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