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Water-soluble and UV traceable isatoic anhydride-based reagents for bioconjugation

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Instrumentation.

NMR data was collected on either a JEOL ECA-500 or a JEOL ECX-300 NMR spectrometer. GC/MS data was collected on an Agilent 6850 GC/5975C MSD. The UV-visible and fluorescence data was obtained on a Molecular Devices Spectramax M5 plate reader. Routine ESI MS data were collected on a Thermo-Fisher MSQ Plus single quadrupole mass spectrometer. High resolution MS data were collected by dissolving 1 mg in 1 mL acetonitrile then brought to 10 μ M in 50:50 MeOH and H₂O with 0.1% Formic Acid. HRMS was performed using an Orbitrap XL in positive ion mode, through direct injection of 2 μ L using an Accela 1250 LC system, at a solvent flow rate of 200 μ L/min of 50% H₂O with 0.1% formic acid and 50% methanol with 0.1% formic acid. MS1 was collected at a resolution of 60000. Spray voltage of 5 kV, Sheath 12, Aux 10, Sweep 0, Capillary Temp 275°C, Capillary Voltage 4.00 V, Tube Lens 50.00 V.

Synthesis of DMIA (1). To a Schlenk flask under dry N₂ was added 60% wt/wt NaH suspended in oil (1.5 g, 72 mmol). To this was added 30 ml of anhydrous hexanes, the suspension briefly stirred then allowed to settle, and the hexanes removed via cannula. This process was repeated 3 x. To the freshly rinsed NaH was

added anhydrous DMF (30 ml) at room temperature, resulting in a cloudy suspension. To this suspension was added isatoic anhydride (5.1 g, 30 mmol) in one portion. Following this addition, an additional 20 ml of anhydrous DMF was added and the resulting suspension was stirred for 60 min, at which time 2-chloro-N,N-dimethylethylamine HCl (5.19 g, 36 mmol) was added in one portion. The resulting suspension was stirred for 12 h at room temperature under a N₂ atmosphere yielding a clear and colored solution containing the desired product. The resulting solution is concentrated under vacuum at 100 °C resulting in a darkly colored viscous residue. This residue is then dissolved into DCM (150 ml) and extracted 3 x with 100 ml of saturated NaHCO₃ (aq). Finally, the organic layer is rinsed 1 x wash with 100 ml of brine solution. The organic layer is then filtered through a plug of MgSO₄ to both remove the activated carbon and dry the solution of residual water. The resulting solution is then concentrated under vacuum to remove the DCM resulting in a lightly colored solid that is then recrystallized from isopropanol.

1. $C_{12}H_{14}N_{2}O_{3}$ (234.25), ¹H NMR (500 MHz, acetone-d6): δ 2.27 (s, 6H), 2.62 (t, 2H, J = 7.1 Hz), 4.21 (t, 2H, J = 7.1 Hz), 7.35 (m, 1H), 7.50 (d, 1H, J = 8.4 Hz), 7.87 (m, 1H), 8.07 (dd, 1H, J = 7.9, 1.6 Hz) ppm, ¹³C NMR (500 MHz, acetone-d6): δ 43.6, 46.0, 56.5, 112.8, 115.5, 124.4, 130.8, 138.0, 142.8, 148.6, 159.7 ppm, MS (EI): Calc. 234.10; Found 234.1 (M⁺, < 1%), 58.1 (100%) m/z; HRMS (ESI): Calc. 235.1083; Found 235.1077 (MH⁺) m/z.



¹H NMR spectrum of **1** in acetone-d6

General Procedure Synthesis of 2a-2e: To a 3 ml screw capped vial containing compound **1** (100 mg, 0.43 mmol) was added 1 ml of reagent grade acetone (unless otherwise noted) and the solution warmed to completely dissolve the reagent. To this solution was added the corresponding alkyl halide electrophile (0.47 mmol) and the solution let sit over 18 h at 40 °C (unless otherwise noted). The solution was then stored at -20 °C overnight followed by filtration of the product. When no solid formed ESI mass spectrometry of the reaction mix was used to confirm product formation.



2a. The general procedure was followed as above using compound **1** (117 mg, 0.50 mmol) and iodomethane (35 μ L, 0.56 mmol) as the electrophile with the following exceptions. Solids started precipitating after 20 mins and the reaction was filtered. The product (130 mg, 69%) was isolated as a colorless solid that

formed readily upon addition of the halide. $C_{13}H_{17}N_2O_3I$ (376.19), ¹H NMR (500 MHz, dimethyl sulfoxide-d6): δ 3.23 (s, 9H), 3.62 (t, 2H, J = 7.8 Hz), 4.49 (t, 2H, J = 7.8 Hz), 7.40 (m, 1H), 7.53 (d, 1H, J = 8.5 Hz), 7.91 (m, 1H), 8.06 (dd, 1H, J = 7.8, 1.6 Hz) ppm, ¹³C NMR (500 MHz, dimethyl sulfoxide-d6): δ 38.0, 52.7, 60.4, 112.1, 114.4, 124.0, 129.8, 137.3, 140.8, 147.6, 158.6 ppm, HRMS (ESI): Calc. 249.1239; Found 249.1233 (M⁺) m/z.



2b. The general procedure was followed as above using an 80% solution of propargyl bromide (54 μ L, 0.5 mmol) as the electrophile with the following exceptions. Product formation was complete within 20 min at room temperature. The resulting product (157 mg, 89%) was isolated as a colorless

solid which formed readily upon addition of the halide and precipitated from acetone. $C_{15}H_{17}N_2O_3Br$ (353.21), ¹H NMR (500 MHz, dimethyl sulfoxide-d6): δ 3.27 (s, 6H), 3.68 (t, 2H, J = 7.8 Hz), 4.16 (t, 1H, J = 2.4 Hz), 4.52 (t, 2H, J = 7.8 Hz), 4.58 (d, 2H, J = 2.3 Hz), 7.39 (t, 1H, J = 7.5 Hz), 7.55 (d, 1H, J = 8.5 Hz), 7.90 (m, 1H), 8.06 (dd, 1H, J = 7.7, 1.6 Hz) ppm, ¹³C NMR (500 MHz, dimethyl sulfoxide-d6): δ 37.8, 50.2, 54.4, 58.3, 72.2, 83.6, 112.1, 114.4, 124.1, 129.8, 137.3, 140.8, 147.7, 158.7 ppm, HRMS (ESI): Calc. 273.1239; Found 273.1232 (M⁺) m/z.



¹H NMR spectrum of **2b** in dimethyl sulfoxide-d6

2c. The general procedure was followed as above using 1-azido-3iodopropane (0.24 g, 1.1 mmol) as the electrophile. The final product (123 mg, 64%) was isolated as a light brown solid which precipitated readily N_{+} , N_{3} from the acetone at -20 °C. C₁₅H₂₀N₅O₃I (445.26), ¹H NMR (500 MHz, dimethyl sulfoxide-d6): δ 2.02 (m, 2H), 3.21 (s, 6H), 3.51 (m, 4H), 3.62 (t, 2H, J = 7.7 Hz), 4.49 (t, 2H, J = 7.7 Hz), 7.40 (m, 1H), 7.55 (d, 1H, J = 8.5 Hz), 7.91 (m, 1H), 8.06 (dd, 1H, J = 7.8, 1.5 Hz) ppm, ¹³C NMR (500 MHz, dimethyl sulfoxide-d6): δ 21.9, 37.7, 47.8, 50.6, 58.4, 61.4, 112.0, 114.5, 124.1, 129.7, 137.3, 140.8, 147.6, 158.6 ppm, HRMS (ESI): Calc. 318.1566; Found 318.1558 (M⁺) m/z.



¹H NMR spectrum of **2c** in dimethyl sulfoxide-d6

2d. The general procedure was followed as above using anhydrous THF as the solvent (1 mL) and allyl bromide (41 μ L, 0.47 mmol) as the electrophile. The final product (128 mg, 84%) was isolated as a colorless solid which precipitated readily from the acetone. C₁₅H₁₉N₂O₃Br (355.23), ¹H NMR (500 MHz, dimethyl

sulfoxide-d6): δ 3.20 (s, 6H), 3.60 (t, 2H, J = 7.8 Hz), 4.18 (d, 2H, J = 7.3 Hz), 4.53 (t, 2H, J = 7.8 Hz), 5.70 (m, 2H), 6.10 (m, 1H), 7.39 (m, 1H), 7.59 (d, 1H, J = 8.5 Hz), 7.9 (m, 1H), 8.05 (dd, 1H, J = 7.8, 1.6 Hz) ppm, ¹³C NMR (500 MHz, dimethyl sulfoxide-d6): δ 37.7, 49.9, 58.3, 66.0, 112.0, 114.5, 124.0, 125.6, 128.2, 129.7, 137.3, 140.8, 147.6, 158.6 ppm. HRMS (ESI): Calc. 275.1395; Found 275.1389 (M⁺) m/z.

2e. The general procedure was followed as above using compound **1** (117 mg, 0.50 mmol) and 1-(bromomethyl)-vinylbenzene (0.10 mg, 0.51 mmol) as the electrophile with the following exceptions. A small amount of

butylated hydroxytoluene was added to the solution. The product (115 mg, 53%) was isolated as a colorless solid which formed readily upon addition of the halide. $C_{21}H_{23}N_2O_3Br$ (431.32), ¹H NMR (500 MHz, dimethyl sulfoxide-d6): δ 3.19 (s, 6H), 3.69 (t, 2H, J = 7.7 Hz), 4.62 (t, 2H, J = 7.7 Hz), 4.78 (s, 2H), 5.37 (d, 1H, J = 11.0 Hz), 5.94 (d, 1H, J = 17.6 Hz), 6.79 (dd, 1H, J = 17.6, 11.0 Hz), 7.39 (m, 1H), 7.62 (m, 5H), 7.89 (m, 1H), 8.04 (dd, 1H, J = 7.8, 1.2 Hz) ppm, ¹³C NMR (500 MHz, dimethyl sulfoxide-d6): δ 38.1, 49.4, 58.9, 66.9, 112.0, 114.7, 116.3, 124.1, 126.5, 127.1, 129.7, 133.5, 135.8, 137.5, 139.0, 140.8, 147.7, 158.7 ppm, HRMS (ESI): Calc. 351.1709; Found 351.1700 (M⁺) m/z.

Reaction with D₂O at pH 8.4. In an NMR tube containing compound **2b** (2.6 mg, 7.4 µmol) was dissolved in sodium bicarbonate D₂O buffer (500 µL) pH 8.4 at the desired concentration (25 mM NaHCO₃). The hydrolysis reaction was monitored at 25 °C by ¹H NMR on a JEOL ECA 500 MHz NMR. The reaction was tracked by the disappearance of the reagent peak at 8.1 ppm and by the growth of the product peak at 7.7 ppm. Scans were taken every 20 min with the initial scan being taken at 7 min. The signals at 8.1 ppm and 7.7 ppm are equivalent at 107 minutes, $t_{V_2} \approx 1.8$ h.



Figure S1: Kinetics trace of the hydrolysis of **2b** at 25 $^{\circ}$ C in 25 mM bicarbonate buffer at pH 8.4 tracked by ¹H NMR

Reaction of 2b with *n*-butylamine followed by NMR. A solution of *n*-butylamine was injected (60 μ L of a 0.42 M *n*-butylamine in D₂O) into the solution of 2b at 25 °C. Sixteen individual spectra were collected every 5 seconds immediately upon injection. The first spectrum after injection (at 5s) clearly shows that 2b is gone, see figure below.



Figure S2 – A ¹H NMR spectrum (the aromatic region; 6.5 ppm to 8.2 ppm) of **2b** in D₂O (0.9 mg **2b** in 390 μ L D₂O) before injection of *n*-butylamine. B ¹H NMR spectrum 5 seconds after injection of *n*-butylamine (5.5 mM **2b**; 55 mM *n*-butylamine).

Bioconjugation

Labeling of BSA by with 2b.



A stock solution of BSA (40 mg/ mL) (0.60 mM) was prepared in 25 mM bicarbonate buffer (pH 8.40). 300 μ L of the solution (0.18 μ mol) was transferred to an Eppendorf tube. To this was added 40 μ L of **2b** (15mM, 0.6 μ mol) and reacted for 1 h, RT. To this was added 5 μ L NH₄OH. Princeton Separations CS-800 Pro Spin GPC columns were used to purify 300 μ L of the solution. 20 μ L of purified solution was diluted with 480 μ L 25 mM bicarbonate buffer (pH 8.40). This solution was placed in a 500 μ L quartz cuvette in a Molecular Devices Spectramax M5 plate reader and absorbance was read (250-450 nm). The degree of labelling (DOL) of **3** was determined to be 3.0.

Reagents efficiency and speed.



Chart S1. The labeling of BSA with **2b**. Degree of labeling (DOL) vs time.

Taking advantage of the solubility of BSA we can demonstrate the very high efficiency that is possible with these reagents and the speed of these reagents. The efficiency increases with the increasing concentration of nucleophile (or conversely decreases with decreasing concentration of nucleophile) for this 2nd order reaction that is in competition with hydrolysis reaction.

BSA (0.54 mM) in 10 mM NaHCO₃ is stirred while a solution of **2b** in neutral water (5.18 equivalents) is quickly introduced with stirring. Aliquots were taken and quenched with ammonium hydroxide and cleaned up with a GPC spin column and the degree of labeling was determined using the absorption at 330 nm and the extinction coefficient according the formula on page 18 below. The reaction reached completion near the 1 h time point (88% efficiency; DOL=4.54). The initial time point gives an idea of the speed of reaction (DOL= 3.65 at 2 min).

Although a direct comparison to NHS esters was not carried out. These are second order reactions with a 1-2 hour reaction time being typical for labeling with NHS esters, comparable to the reaction with these isatoic anhydride based reagents. These new reagents are certainly more hydrolytically stable than the typical NHS ester. The half-life of an NHS ester is typically 4-5 h at neutral pH at 0° C¹ while at neutral pH the half-life of **2b** is 43 h at 4°C and 16 h at RT.

¹*Thermo Fisher Scientific*, www.thermofisher.com/us/en/home/life-science/proteinbiology/protein-biology-learning-center/protein-biology-resource-library/pierce-proteinmethods/amine-reactive-crosslinker-chemistry.html.

CuAAC reaction for biomolecules.



The procedure of Finn et. al^2 was used without modification. Briefly, the bioconjugate **3** (25 µL, 34 mg/ml) with a DOL of 3.0 was diluted in 407.5 µL of 100 mM PBS buffer (pH 7.0) in a 1.5 ml Eppendorf tube. To this was added 10 µL of the 3-azido-7-hydroxy-coumarin (20 mM). THPTA ligand (50 mM, 5 µL) was premixed with CuSO₄ (20 mM, 2.5 µL) and added to the solution. Aminoguanidine hydrochloride (100 mM, 25 µL) was added to the solution followed by freshly made sodium ascorbate (100 mM, 25 µL). The solution was vortexed and 150 µL transferred to an opaque walled 96 well plate. The reaction was monitored for evolution of signal at 470 nm, indicating the formation of **4**, using the excitation wavelength of 404 nm.

² V. Hong, S. I. Presolski, C. Ma and M. G. Finn, *Angew. Chem. Int. Ed. Engl.*, 2009, **48**, 9879-9883.



Figure S3: Evolution of fluorescence at 470 nm upon formation of the bioconjugate, **4**. Measured at λ_{ex} 404 nm, λ_{em} 470 nm.



2c': Ring opening of 2c with *n*-butylamine. 2c was transferred to a 1.5 mL Eppendorf tube (5.0 mg, 0.01 mmol), to this was added butylamine (20 μ L, 0.2 mmol) and vortexed until all salts were dissolved into solution.

The solution was then diluted to 15 mM with DI H_2O and excess butylamine is removed under reduced vacuum. The solution is then used directly without further purification. m/z (ESI): Calc. 347.26; Found (M+) 347.23.



The procedure of Finn et. al.² was used with minor modification. Briefly, bioconjugate **3** (25 μ L, 34 mg/ml) with a DOL of 3.0 was diluted in 385.5 μ L of 100 mM PBS buffer (pH 7.0). To this was added 32 μ L of the butylamine ring-opened azide from **2c**, above. THPTA ligand (50 mM, 5 μ L) was premixed with CuSO₄ (20 mM, 2.5 μ L) and added to the solution. Aminoguanidine hydrochloride (100 mM, 25 μ L) was added to the solution followed by freshly made sodium ascorbate (100 mM, 25 μ L). The solution was vortexed and incubated at 25 °C for 24 h. The reaction was stopped by passing 100 μ L of the reaction mixture through a Princeton Separations CS-800 Pro Spin GPC column. 20 μ L of purified solution was diluted with 480 μ L 25 mM

² V. Hong, S. I. Presolski, C. Ma and M. G. Finn, *Angew. Chem. Int. Ed. Engl.*, 2009, **48**, 9879-9883.

bicarbonate buffer (pH 8.40). This solution was placed in a 500 μ L quartz cuvette and the absorbance was recorded (250-450 nm).

Degree of labeling (DOL) was determined using Equation 1 below:

$$DOL = \frac{A_{330}\varepsilon_{prot}}{(A_{280} - A_{330}C_{280})\varepsilon_{reagent}}$$

Equation S1: Degree of labeling formula

Abbreviation	Definition	
A330	Absorbance of the sample at 330 nm	
Eprot	Molar extinction coefficient (in M ⁻¹ cm ⁻¹) of pure protein at 280 nm	
A ₂₈₀	Absorbance of the sample at 280 nm	
C ₂₈₀	C ₂₈₀ Correction factor of reagents contribution to absorbance at 280 nm	
Ereagent	Molar extinction coefficient (in M ⁻¹ cm ⁻¹) of the reagent at 330 nm	

Table S1: Degree of labeling variables

Abbreviation	Value
A330	0.1707
Eprot	43,824 M ⁻¹ cm ⁻¹
A ₂₈₀	0.7215
C ₂₈₀	0.3184
Ereagent	3750 M ⁻¹ cm ⁻¹

Table S2: Data for bioconjugate **3** for determination of DOL

$$DOL = \frac{0.1707 * 43824 M^{-1} cm^{-1}}{(0.7215 - 0.1707 * 0.3184) * 3750 M^{-1} cm^{-1}} = 2.99$$

Equation S2: Degree of labeling calculation for bioconjugate 3

Abbreviation	Definition
A ₃₃₀	0.2069
€ _{prot}	43,824 M ⁻¹ cm ⁻¹
A ₂₈₀	0.5086
C ₂₈₀	0.3037
Ereagent	$3680 \text{ M}^{-1} \text{ cm}^{-1}$

Table S3: Data for the bioconjugate **5** after the CUAAC reaction at 35 °C for determination of DOL

For the calculation of DOL for bioconjugate 5, $\varepsilon_{reagent}$ represents an average of the extinction coefficient of the two reagents, this was done since the value of the individual extinction coefficients fall within the uncertainty.

$$DOL = \frac{0.2069 * 43824 M^{-1} cm^{-1}}{(0.5086 - 0.2069 * 0.3037) * 3680 M^{-1} cm^{-1}} = 5.54$$

Equation S3: Degree of labeling calculation for bioconjugate **5**

Degree of Cargo Loading (DOCL) in 5

$$DOCL = 5.54 - 2.99 = 2.55$$

Equation S4: Calculation of the amount of cargo incorporation onto 3 to form 5

Derivatizatio
$$effeciency = \frac{5.54 - 2.99}{2.99} * 100\% = 85\%$$

Equation S5: Efficiency of CuAAC reaction to yield 5.