

Site-Selective Azide Incorporation into Endogenous RNase A *via* a “Chemistry” Approach

Xi Chen^{a,b}, Lars Henschke^a, Qianzhen Wu^a, Kasturi Muthoosamy^b Boris Neumann^c, and Tanja Weil^{a,b,*}

^a Institute of Organic Chemistry III/Macromolecular Chemistry and Biological Chemistry, University of Ulm, Albert-Einstein Allee 11, 89069 Ulm, Germany. Tel. (+)49-731-5022870, Fax. (+)49-731-50-22883, E-mail: tanjaweil@uni-ulm.de

^b Department of Chemistry, National University of Singapore, 3 Science Drive 3, 117543 Singapore

^c Proteome Factory AG, Magnusstr. 11, Haus 2, 1. OG, 12489 Berlin, Germany

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1. THE AUTO-SYRINGE PUMP SETUP IN KINETIC CONJUGATION STEP

The auto-syringe pump setup consists eight key components in total: i) A slender 1 mL syringe where the bioconjugation reagent solution is hold; such a syringe is readily available in most of chemistry as well as biology laboratory; 2) an auto-syringe pump for automatic injection of bioconjugation reagents; 3) a lift which adjusts the height of the auto-syringe pump; 4) a fine needle (Φ 0.4 mm) which is buckled to \angle 90° and is connected to the slender syringe and inserted into the Falcon tube; 5) an ion stand to fix the Falcon tube; 6) a Falcon tube which contains the protein solution in PBS buffer (pH 7.2); the Falcon tube is capped but is made with a tiny hole thorough the cap that just allows the fine needle to go inside; 7) a stirrer bar equipped inside the Falcon tube with an appropriate size of ~2 cm; and 8) an electronic stirrer. The whole setup is stand in a cold room (+4 °C) so that the bioconjugation reaction can be performed in a reduced temperature. Herein, a fine needle (e.g. Φ 0.4 mm) is preferred because it produces smaller drops of the bioconjugation reagent solution. Therefore, other needles with larger diameters (e.g. Φ 0.6 mm, 0.8 mm etc.) are not recommended.

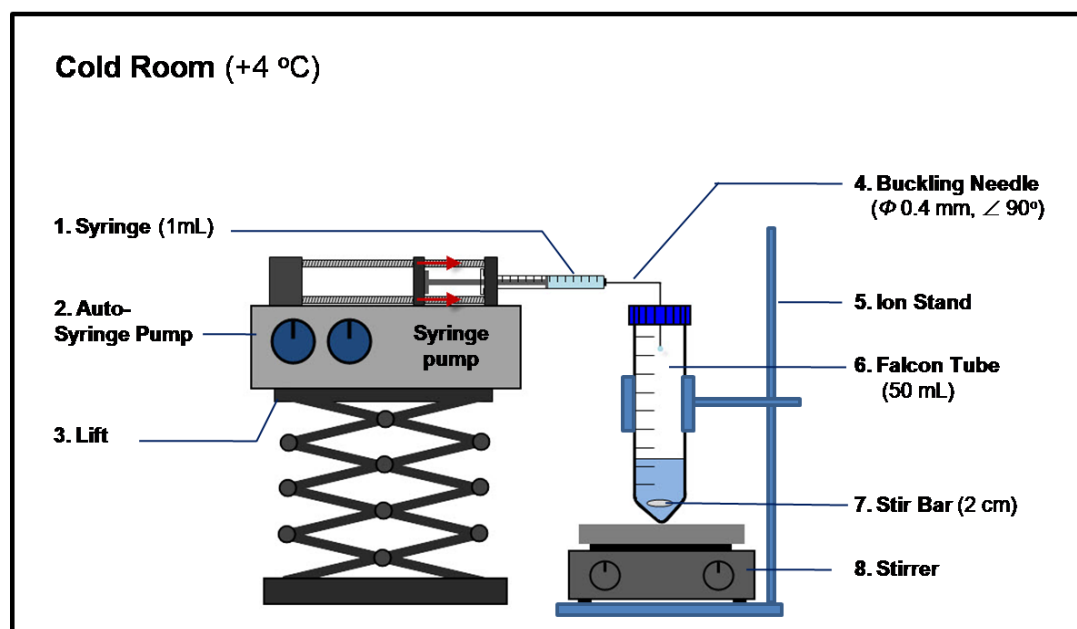


Figure S1. The auto-syringe setup used in the optimized “kinetic protein labeling” (KPL) for the preparation of azido-(K1)RNase A (**13**).

2. INTERPRETATION OF THE NMR SPECTRA OF BIOTIN-TEO-AZIDO-COOH (10)

The carboxylate part of **10** (Figure 3a, highlighted area) displays two sets of signals in both the proton and the carbon NMR spectra, which is attributed to the C-N bond rotation of the tertiary amide bond in molecule **10** (Figure S2a), which complicates the interpretation of the ^1H -NMR and ^{13}C -NMR spectra. However, a combination of 2D-NMR and DEPT 135 allows assigning all signals in the ^1H -NMR and carbon NMR spectra. In the ^1H -NMR of **10** (Figure S2b), the protons ^oH , ^pH , ^qH , ^rH and ^nH all show two sets of signals whereas the remaining protons give only one set of signals. Similarly, in the DEPT 135 spectrum of **10** (Figure S2c), the secondary carbon atoms ^pC , ^oC , ^nC , ^qC and ^rC provide two sets of signals. The tertiary carbon atoms ^gC , ^fC and ^hC refer to the typical carbon signals of the biotin moiety.

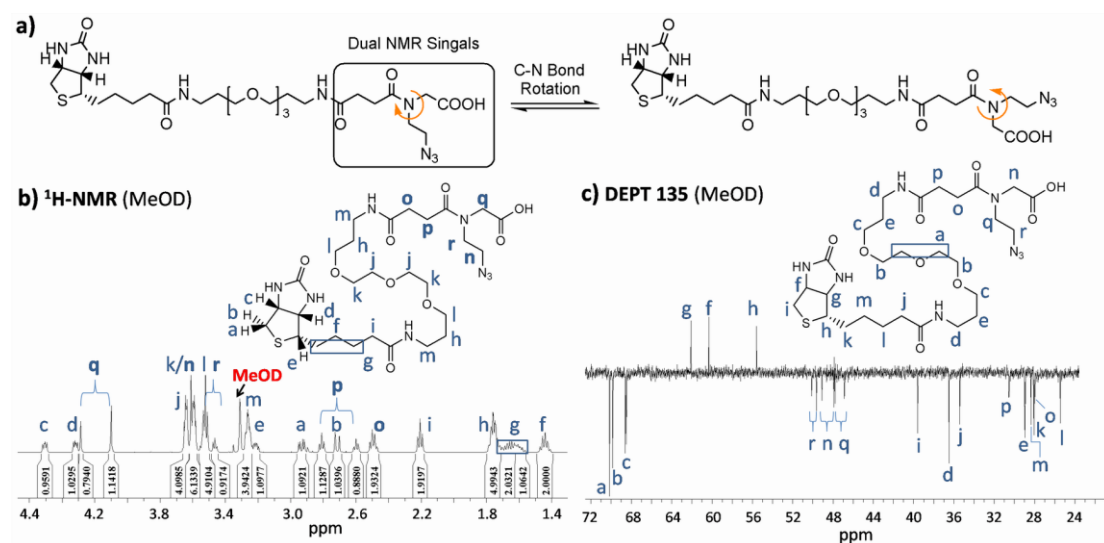
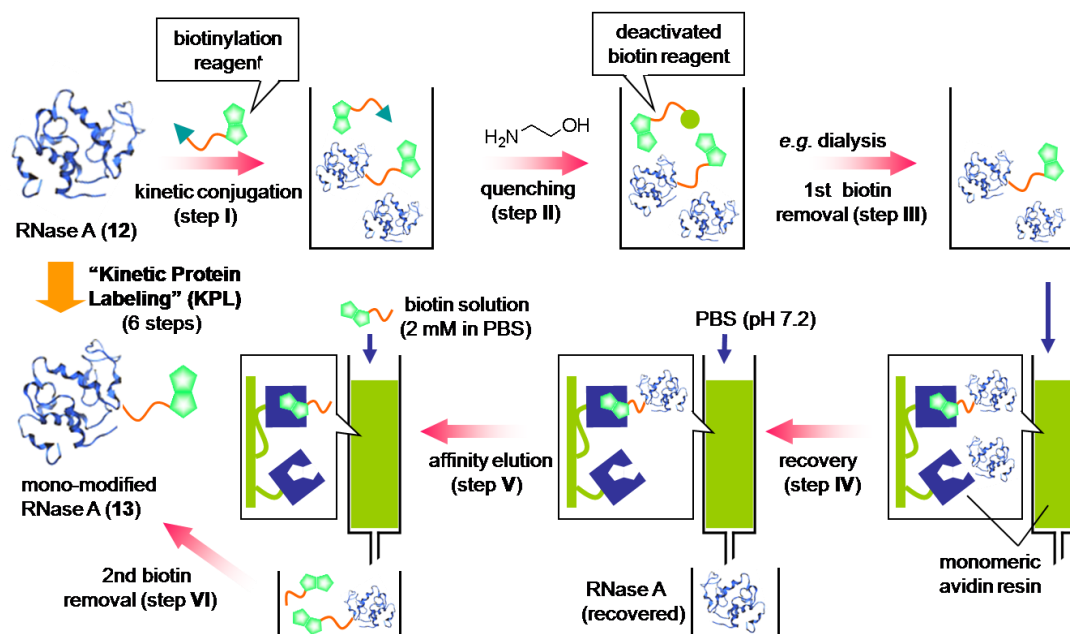


Figure S2. Isomerisation of **11** due to the C-N bond rotation; b) ^1H -NMR spectrum of **11** in MeOD; c) DEPT 135 spectrum of **11** in MeOD.

3. THE SIX KEY STEPS IN KPL

First, the protein solution is treated with a bioconjugation reagent yielding the mono-biotinylated protein as well as the unmodified protein, which is used in excess (**step I: kinetic conjugation**). The next key step comprises the quenching step (step II) in which the remaining, unreacted biotinylation reagent is completely deactivated. This step is

essential since any trace of active NHS bioconjugation reagent will inevitably cause multi-modification of the mono-biotinylated protein after affinity separation in step V. Subsequently, any biotin-containing small molecules are removed either by dialysis or gel filtration in the 1st biotin-removal step (**step III**) since they could block the monomeric avidin resin, which significantly reduces the binding capacity of the column. As result, a pure protein mixture in water is obtained and all small molecule impurities have been separated. Afterwards, the protein solution is loaded onto the monomeric avidin resin column for affinity separation. The monomeric avidin reversibly binds biotinylated molecules under mild conditions so that all the washing and elution steps are performed in PBS buffer at physiological pH at room temperature (RT) or lower temperature, which is particularly attractive for the purification of proteins. After loading the protein mixture, the unreacted native protein which is not bound to the monomeric avidin resin is separated and can be easily collected by elution with PBS buffer (**step IV: recovery step**). This step is particularly attractive for precious protein samples. Subsequently, the mono-biotinylated protein is obtained after elution with 2 mM (+)-biotin solution in PBS buffer (**step V: affinity elution step**). Finally, the obtained protein solution is again dialyzed or subjected to gel filtration to remove the remaining free (+)-biotin molecules in the solution (**step VI: 2nd biotin-removal step**). All six key steps involved in “kinetic protein labeling” are summarized in Scheme S1, which represents a standard procedure for achieving site-selective modification of endogenous proteins and peptides. The entire procedure is fast and can be accomplished within two days (using gel filtration for biotin removal) or four to five days (if dialysis is applied for biotin removal).



Scheme S1. Schematic overview of the six key steps involved in “kinetic protein labeling” for the site-selective introduction of a single azido group into endogenous RNase A.

4. DETAILED RNASE A FUNCTIONAL ASSAY PROCEDURES

The general scheme of RNase A functional assay for the determination of RNase A enzymatic activity is shown in Figure S2a. Briefly, Baker’s yeast ribonucleic acid (yRNA) forms a fluorescent complex in the presence of SYBR Safe DNA gel stain dye, and RNase A quickly degrades yRNA and destroys the fluorescent complex leading to a fluorescence decay. The initial speed of the fluorescence decrease is proportional to the enzymatic activity.

Therefore, the efficiencies of azido-(K1)RNase A, recovered RNase A and RNase A in Tris-EDTA buffer (10 mM/1 mM, pH 8.0) to hydrolyze yRNA-SYBR dye fluorescent complex that cause the fluorescence decay were assayed. A blank control using Tris-EDTA buffer (10 mM/1 mM, pH 8.0) in the absence of any RNase A was also assayed at the same time to determine the auto-hydrolysis of yRNA in buffer. The initial relative fluorescence unit (RFU) decrease of native RNase A (slope $k_1 = 15$ RFU/s), recovered native RNase A (slope $k_2 = 15$ RFU/s) and azido-(K1)RNase A (slope $k_3 = 4.3$ RFU/s) after subtraction of the blank control value were plotted against time, giving the diagram Figure S2b. Therefore, the retained activity of azido-(K1)RNase A compared to

native RNase A is $k_3/k_1 = 29\%$. Recovered RNase A reveals an almost full retaining of the enzymatic activity.

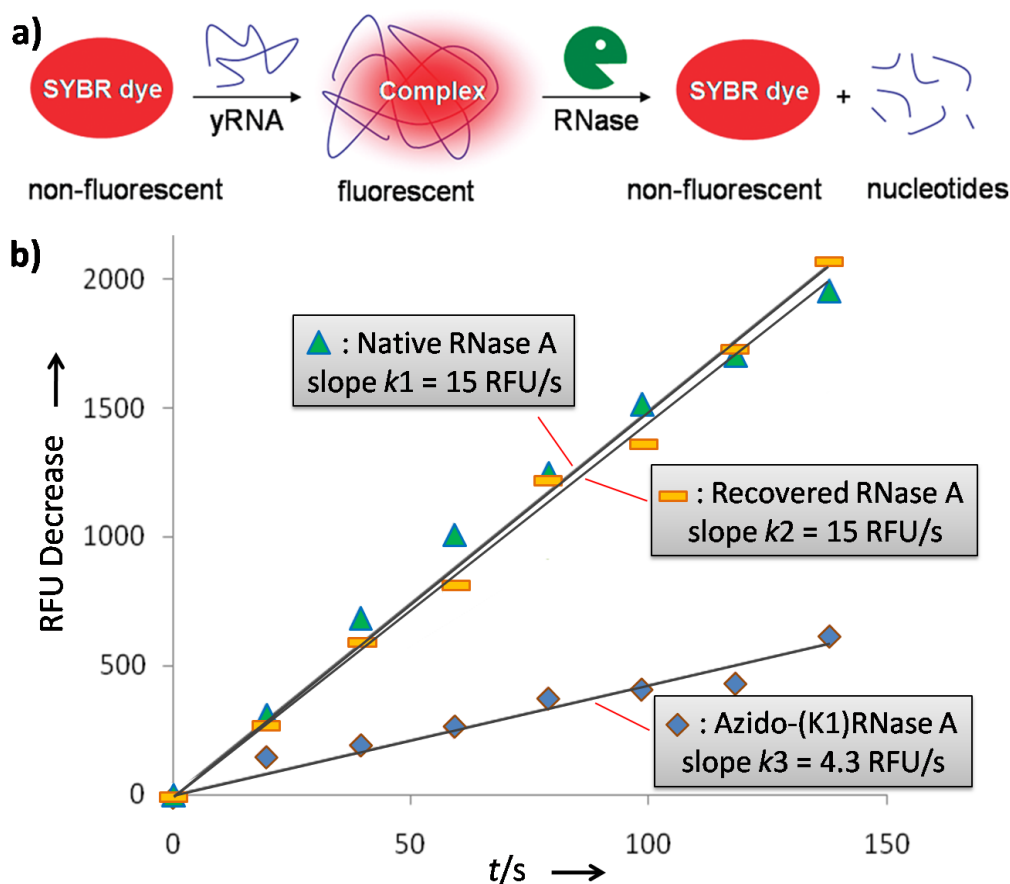


Figure S3. a) The general scheme of RNase A functional assay; b) the determined enzymatic activities of azido-(K1)RNase A (dark blue rhombic dots), recovered RNase A (orange rectangular dots) as well as native RNase A (green triangular dots).

Procedures of RNase A functional assay:

Tris-EDTA buffer solution was freshly prepared from Tris buffer (10 mM) and EDTA (1 mM) and was adjusted to pH 8.0. 10 μL of SYBR dye (diluted to 1 \times concentration with Tris-EDTA buffer) was charged into 12 wells in one line in a sterilized 384-well plate. Then 40 μL of immediately prepared yRNA Tris-EDTA buffer solution (10 $\mu\text{g mL}^{-1}$, keep in $-20\text{ }^{\circ}\text{C}$ and thaw on ice before use) was added to each charged well quickly and mixed. **Then, the 384-well plate was equipped into the holder of BioTek Synergy 4 microplate reader.** Afterwards, three sets of 50 μL of blank Tris-EDTA buffer, native RNaseA (25 ng mL^{-1} in Tris-EDTA buffer), recovered native RNase A (25 ng mL^{-1} in Tris-EDTA buffer) and

azido-(K1)RNase A (25 ng mL^{-1} in Tris-EDTA buffer) were added to 4 charged wells ($3 \times 4 = 12$ wells in total) by a 12-station multi-channel pipette at one time and mixed. Immediately, the BioTek Synergy 4 microplate reader was started and the kinetics of the fluorescence change was determined (excited at 485 nm; emitting at 535 nm). Parameters: one second of shaking with an interval of 13 seconds and monitored for seven minutes. The Relative Fluorescence Unit (RFU) of native RNase A, recovered RNase A, azido-(K1)RNase A and the blank control along time were recorded.

Important Precautions: A fast operation of the whole procedure is essential for the success of this functional assay since the yRNA-SYBR dye fluorescent complex is unstable and should react with active RNase as fast as possible in the assay before it becomes decomposed. Besides, SYBR dye is light sensitive and therefore the assay should be performed in a light-excluded environment. Furthermore, since the wells of 384-well plate are quite small and cautions should be used to avoid any bubbles formation during the process of addition and mixing reagents. Otherwise, the formation of any bubbles in the small well will render the fluorescence readout not reliable.

5. HRMS (ESI) SPECTRA OF K1 MODIFIED KETAAKF (1-8)

The double charged (Figure S3a, m/z 760.403 $[M+2H]^{2+}$) and triple charged (Figure S3b, m/z 507.271 $[M+3H]^{3+}$) HRMS (ESI) spectra of modified KETAAKF (1-8) (calcd. W.M. $1518.790 \text{ g mol}^{-1}$) prove that the labeling occurred at either K1 or K7 in the peptide fragment KETAAKF (1-8) of mono-azido RNase A.

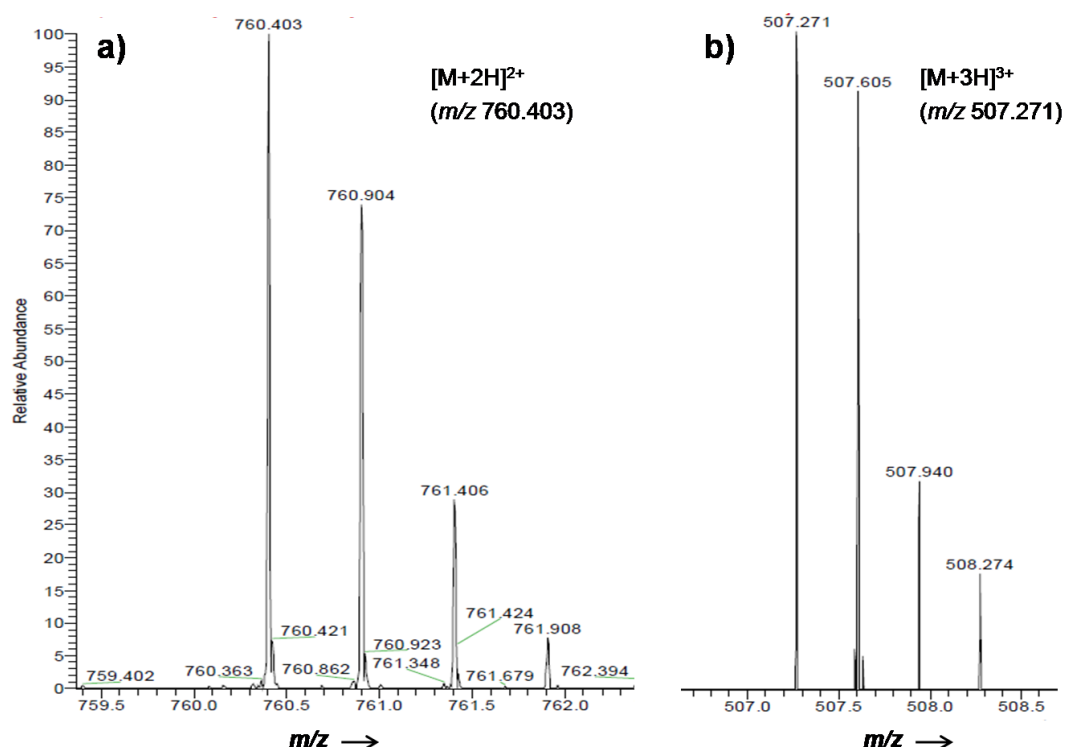


Figure S4. The HRMS (ESI) spectra of K1 modified KETAAKF (1-8). a): $[M+2H]^{2+}$; b): $[M+3H]^{3+}$.

6. INTERPRETATION OF THE THREE FALSE POSITIVE SIGNALS IN FIGURE 4C AND 4D

The peak at t_R 13.05 min in Figure 5c, the peaks at t_R 28.28 min and t_R 29.73 min in Figure 5d represent false positive signals because their identified charges are not in accordance with the charges that they are recorded in their XIC chromatographs. Regarding the t_R 13.05 min peak in Figure 5c, the corresponding chromatograph was recorded as mono-charged at m/z = 865.473-865.481 $[M+H]^+$; however, the HRMS (ESI) spectrum of the t_R 13.05 min peak reveals double charged at m/z 865.874 $[M+2H]^{2+}$ (Figure S4a) which does not match. Similarly, The peaks at t_R 28.28 min and t_R 29.73 min in Figure 5d are recorded as double charged species at m/z 433.241-433.245 $[M+2H]^{2+}$; unfortunately, the HRMS (ESI) mass spectra of the two peaks reveal mono-charged at m/z 432.238 $[M+H]^+$ which are not correct as well (Figure S4b and 4c). Therefore, the three XIC peaks in Figure 5c and 5d are all false positive signals and hence, the unmodified KETAAKF (1-8) peptide fragment is identified to be not existed.

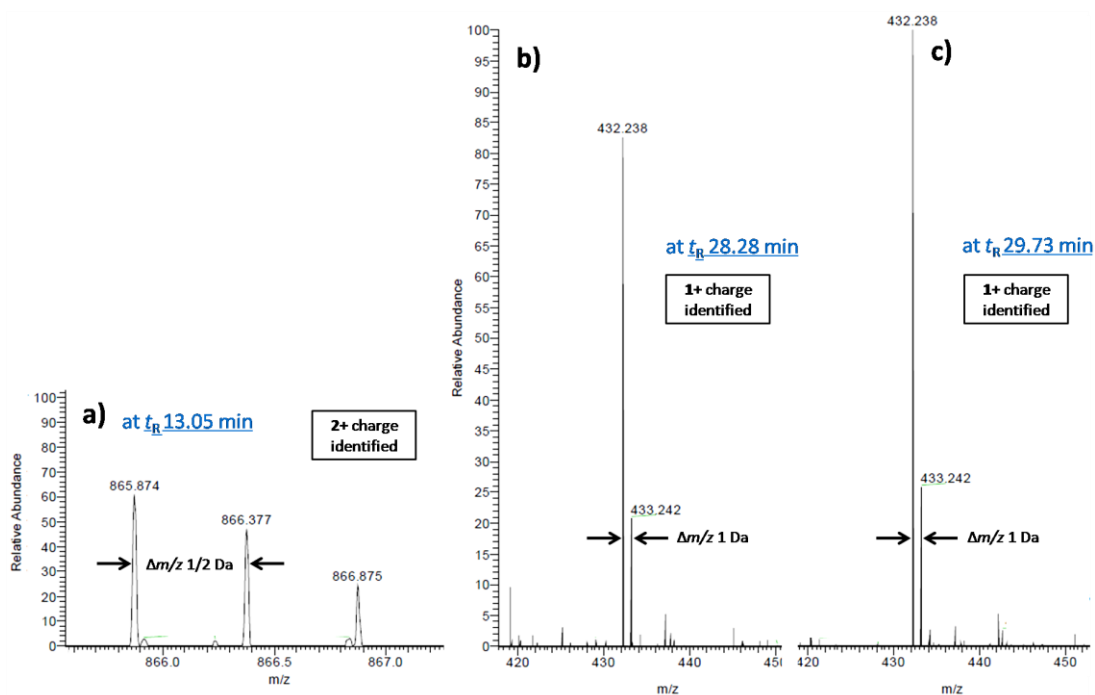


Figure S4. The HRMS (ESI) mass spectra of the XIC peak at t_R 13.05 min in Figure 5c, the peaks at t_R 28.28 min and t_R 29.73 min in Figure 5d reveal all unmatched charges.

7. THE ARGON COLUMN CHROMATOGRAPHY SETUP

Due to the high sensitivity to oxygen of phosphine (III) species, every single step has to be conducted under the exclusion of oxygen. In particular, the workup and column chromatography procedures of phosphine-TEO-COOtBu (**19**) and rho-TEO-phosphine (**21**) have to be performed under argon. Regarding the phosphine species, 2-diphenylphosphino-1-methylterephthalate (**18**) and phosphine-TEO-COOH (**20**), the argon atmospheric procedures are not compulsory and no obvious oxidation of the phosphine (III) had been observed if normal purification procedures were conducted quickly. This is probably due to the presence of acidic groups (e.g. -COOH or CF₃COOH) in **18** and **20** that stabilize the slightly basic phosphine (III) atom.

An argon atmospheric workup procedure can be achieved *via* standard Schenk techniques, similar to the way to exclude oxygen in conducting oxygen-free organic reactions. Extraction and washing steps can be performed in Schenk flasks or tubes and syringes can be used to transfer organic or aqueous layers. All the workup reagents including Na_2CO_3 (sat.), brine and EtOAc should be degassed *via* e.g. ultrasonication first and then filled into Schenk flasks, exchanged with argon and connected to Schenk line before use.

The argon atmospheric column chromatography setup is depicted in Figure S5. The argon column setup is connected to two outlets of a Schenk line. The first argon junction on top of the silica gel column is fixed. It is connected as soon as the sample is loaded and the degassed solvent is poured inside and flushed with argon. Besides, a multiple adapter is equipped, with one top neck connected to the column's outlet. The other top neck is linked to the second argon outlet to make sure that the drops coming out are not exposed to air. The third neck is inserted

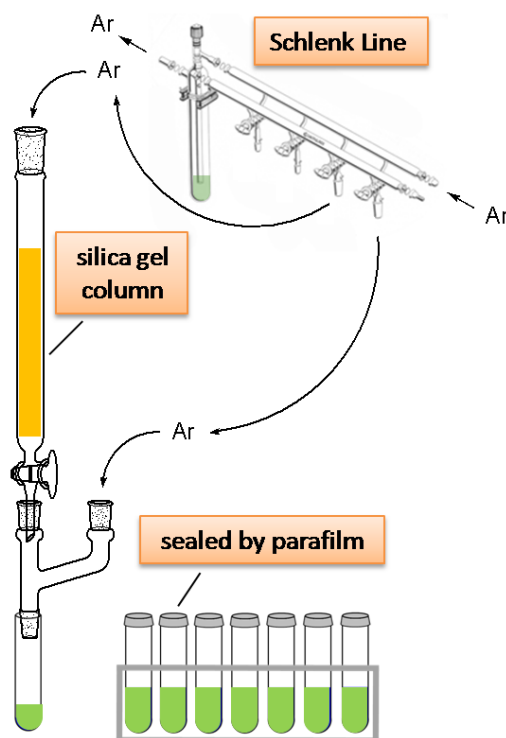


Figure S5. The argon atmospheric silica gel column chromatography setup.

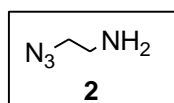
into a glass tube which collects the eluent. Every glass joint of the setup is sealed tightly with parafilm. One advantageous effect of this setup is that the collecting tubes are all filled with argon during the process, since argon is heavier than air. All the test tubes are sealed with parafilms as soon as they are detached from the column.

8. ORGANIC SYNTHESIS

General.

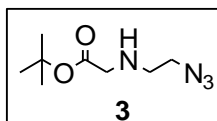
All chemical reagents were purchased from Sigma-Aldrich, Merck or Regent and were used without further purifications unless otherwise mentioned. Anhydrous DMF was dried over freshly activated 4 Å molecular sieves. ^1H -NMR, ^{13}C -NMR, DEPT 135, ^1H - ^1H COSY and HMQC spectra were recorded with Bruker AC 300, AMX 500 or DRX500 NMR spectrometers operating at 400 MHz for ^1H and at 75.48MHz for ^{13}C NMR at 25 °C. Chemical shifts were reported in ppm (δ scale) relative to the solvent signal (CDCl_3 : δH 7.26, δC 77.0; DMSO-d_6 : δH 2.50, δC 39.5; MeOD : δH 3.31, δC 49.0), and coupling constant (J) values were reported in hertz (Hz). High-resolution mass spectra (HRMS) were recorded on a Finnigan MAT95XL-T mass spectrometer by direct infusion of the solution of each compound using electrospray ionization (ESI) in positive mode. All HRMS analysis in this report used $\text{CF}_3\text{COO}^-\text{Na}^+$ solution as the external calibration. Normal ESIMS was determined using Finnigan LCQ quadrupole ion trap mass spectrometer. LC-ESIMS analysis was achieved via Shimadzu LC-20AD/SPD-20A/SIL-20AC/LCMS-2010EV instrument equipped with a C-18 column using $\text{MeCN}/\text{H}_2\text{O}$ as eluent.

Synthesis of Biotin-TEO-Azido-NHS (11)



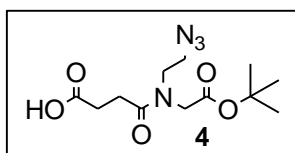
2-Azidoethylamine (2). NaN_3 (1.80 g, 27.7 mmol) was added to a solution of 2-bromoethylamine hydrobromide (**1**) (2.5 g, 13.1 mmol) in H_2O (15 mL) and the resultant solution was heated to 75 °C and stirred overnight. After cooling down to RT, NaOH (488 mg, 12.2 mmol) was added to the reaction mixture. The resultant mixture was extracted with DCM three times (25 mL \times 3). The combined DCM layer was washed with brine (25 mL \times 1), dried over anhydrous Na_2SO_4 , filtered and removed of DCM at 25 °C under 370 mmbar via a rotavap. A light yellow liquid (0.85 g) with slight fluorescence was obtained as the desired product (**2**) in a yield of 60 %. According to ^1H -NMR assay, this liquid contained 80 % of the product (**2**) and 20 % of DCM solvent; ^1H -NMR (CDCl_3 , 300MHz): δ 3.36 (t, $J=5.4\text{Hz}$, 2H, $-\text{CH}_2\text{N}_3$), 2.88 (t,

$J=5.4\text{Hz}$, 2H, $-\text{CH}_2\text{NH}_2$), 1.28 (s, 2H, $-\text{NH}_2$); **MS** (ESI): $\text{C}_2\text{H}_7\text{N}_4^+$ calcd. 87.1, found 87.1. NMR data was in accordance with those published previously¹.



***N*-(2-Azidoethyl)glycine *tert*-butyl ester (3).** K_2CO_3 (491 mg, 3.56 mmol) was added into a 100-mL thoroughly dried Schlenk flask and the flask was further heated for 10 min under high vacuum in order to

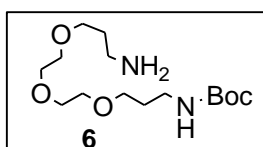
remove any trace of water in K_2CO_3 . DMF (11.9 mL) and 2-azidoethylamine (**3**) (613 mg, 7.12 mmol) were added under Ar. *tert*-Butyl bromoacetate (695 mg, 3.56 mmol) was added dropwise to the stirring suspension and the resultant reaction mixture was stirred at RT for two days. The reaction mixture was partitioned in EtOAc/DI- H_2O (3: 1, V/V) and the organic layer was washed with brine, concentrated and evacuated to remove DMF. Finally, the residue was purified *via* silica gel chromatography (Hexane: EtOAc: NH_3 (20 w %) 2:1:2 %) to afford 488 mg clear viscous liquid as the product (**37**) in a yield of 68 %; **¹H-NMR** (CDCl_3 , 500 MHz): δ 3.41 (t, $J=5.70\text{Hz}$, 2H), 3.31 (s, 2H), 2.80 (t, $J=5.70\text{Hz}$, 2H), 1.73 (s, 1H, NH) 1.46 (s, 9H); **¹³C-NMR** (CDCl_3 , 500 MHz): δ 171.44, 81.36, 51.55, 51.41, 48.10, 28.08; **MS** (ESI): $\text{C}_8\text{H}_{17}\text{N}_4\text{O}_2^+$ calcd. 201.1, found 200.9; **EA**: $\text{C}_8\text{H}_{16}\text{N}_4\text{O}_2$ calcd. C 47.99 %, H 8.05 %, N 27.98 %, found C 47.51 % H 7.55 % N 27.60 %.



***N*-(3-Carboxypropanoyl)-*N*-(2-azidoethyl)glycine *tert*-butyl ester (4).** Succinic anhydride (360 mg, 3.6 mmol) and DMF (9 mL) were added into a dry 25 mL-Schlenk flask under Ar. *N*-(2-Azidoethyl)glycine *tert*-butyl ester (360 mg, 1.8 mmol) was injected dropwise to the stirring solution and the reaction mixture was stirred at

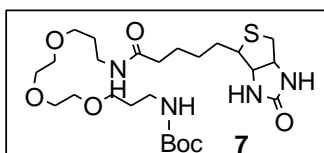
RT overnight. DMF was removed under high vacuum with stirring and the residue was dissolved in EtOAc, washed with DI- H_2O (5 x), brine, dried over anhydrous Na_2SO_4 , concentrated and purified *via* silica gel chromatography (MeOH: CHCl_3 1:5, R_f 0.45, stained by ninhydrin) to yield 437 mg light yellow semisolid as the product (**4**) in a yield of 81 %; **¹H-NMR** ($\text{DMSO}-d_6$ 500MHz): δ 12.03 (br, s, 1H), 4.18 (s, 0.46 x 2H), 3.92 (s, 0.54 x 2H), 3.55 (t, 0.54 x 2H, $J=5.1\text{Hz}$), 3.45 (t, $J=6.0\text{Hz}$, 0.46 x 2H), 3.52 (t, $J=5.1\text{Hz}$, 0.54 x 2H), 3.39 (t, $J=6\text{Hz}$, 0.46 x 2H), 2.63 (t, $J=6.6\text{Hz}$, 0.54 x 2H), 2.43 (t, $J=6.9\text{Hz}$, 0.46 x 2H),

2.42 (m, 2H), 1.44 (s, 0.46 × 9H), 1.39 (s, 0.54 × 9H) (signals from the same proton are underlined); ¹³C-NMR (DMSO-d⁶, 500MHz): δ173.68, 173.65, 172.09, 171.58, 168.79, 168.32, 81.49, 80.55, 50.47, 49.17, 48.51, 48.41, 47.21, 46.02, 28.94, 28.89, 27.67, 27.59, 27.42, 27.19 (signals from the same carbon are underlined); DEPT135 (DMSO-d⁶, 500MHz): δ (-) 50.47, 49.17, 48.51, 48.40, 47.21, 46.02, 28.94, 28.89, 27.42, 27.19; (+) 27.67, 27.59; HRMS(ESI): C₁₂H₂₀N₄NaO₅⁺ calcd. 323.1236, found 323.1233.



N-Boc-4,7,10-trioxa-1,13-tridecanediamine (6).² A solution of 4,7,10-trioxa-1,13-tridecanediamine (7.50 g, 34.1 mmol) in 1,4-dioxane (100 mL) in RBF immersed in a RT water bath was

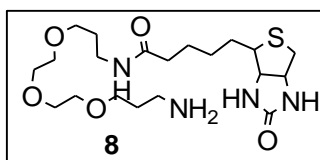
treated with Boc anhydride (3.70 g, 16.9 mmol) and the reaction mixture was stirred at RT overnight. The solvent was removed under reduced pressure and the residue was purified by silica gel chromatography (CHCl₃: MeOH: NH₃ (20 w %) 6:1:0.21, R_f = 0.4, stained by I₂) to afford 1.86 g light yellowish oil as the product (6) in a yield of 34%. ¹H-NMR (CDCl₃, 500 MHz): δ 5.10 (s, br, 1H), 3.62~3.65 (m, 4H), 3.52~3.60 (m, 8H), 3.22 (m, 2H), 2.80 (t, J=6.8Hz, 2H), 1.70~1.78 (m, 4H), 1.43 (s, 9H); MS(ESI): C₁₅H₃₂N₂NaO₅⁺ calcd. 343.2, found 343.2.



Biotin-TEO-NHBoc (7).² Into a dry 25-mL Schlenk flask, (+)-biotin (1.06 g, 4.35 mmol) and EDC·HCl (1.11 g, 5.8 mmol) were combined. Dry DMF (8.5 mL) was added and

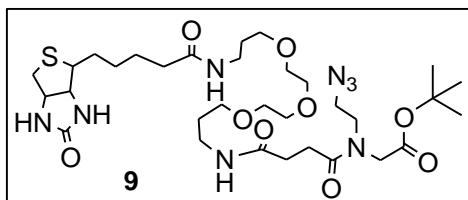
the resultant suspension was stirred in an ice-water bath for 10 min. A solution of N-Boc-4,7,10-trioxa-1,13-tridecanediamine (0.928 g, 2.9 mmol) in dry DMF (6 mL) was added to the suspension and the ice-water bath was removed. The reaction mixture was stirred at RT for two days. Then, the reaction solution was partitioned in CHCl₃/H₂O and the CHCl₃ layer was separated. The aqueous layer was extracted four additional times with CHCl₃ and all organic layers were combined and washed with saturated Na₂CO₃. The Na₂CO₃ layer was anti-extracted with CHCl₃ and CHCl₃ layers were combined again, washed with brine, dried over anhydrous Na₂SO₄, filtered and concentrated. DMF was removed under high vacuum at 35 °C with stirring and the resultant residue was purified

by silica gel flash chromatography (CHCl₃:MeOH 6:1, R_f = 0.5, stained by I₂) to yield 1.15 g off-white solid as the product (**7**) in a yield of 73 %; **¹H-NMR** (CDCl₃, 500 MHz): δ 6.61 (s, br, 1H), 6.41 (s, br, 1H), 5.67 (s, br, 1H), 5.03 (s, br, 1H), 4.48 (m, 1H), 4.29 (m, 1H), 3.60~3.64 (m, 4H), 3.53~3.60 (m, 6H), 3.51 (t, *J*=5.7Hz, 2H), 3.32 (q, *J*=6.1Hz, 2H), 3.19 (m, 2H), 3.13 (m, 1H), 2.88 (dd, *J*₁=12.6Hz, *J*₂=4.4Hz, 1H), 2.72 (d, *J*=12.6Hz, 1H), 2.17 (t, *J*=7.6Hz, 2H), 1.70~1.78 (m, 5H), 1.60~1.70 (m, 3H), 1.42 (m, 2H), 1.417 (s, 9H); **¹³C-NMR** (CDCl₃, 500 MHz): δ 173.08, 163.88, 70.48, 70.44, 70.15, 70.00, 69.91, 69.46, 61.78, 60.15, 55.60, 40.48, 38.43, 37.69, 36.00, 29.68, 28.93, 28.42, 28.22, 28.09, 25.65; **DEPT 135** (CDCl₃, 500 MHz): δ(+) 61.78, 60.15, 55.61, 28.42; δ(-) 70.48, 70.44, 70.15, 70.00, 69.90, 69.44, 40.48, 38.43, 37.67, 36.00, 29.68, 28.93, 28.22, 28.09, 25.65; **HRMS** (ESI): C₂₅H₄₆N₄O₇SN⁺ calcd. 569.2979, found 569.2987.



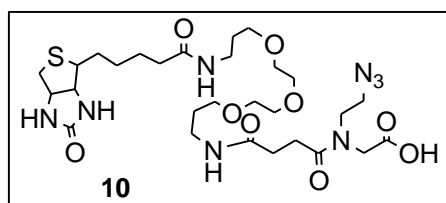
Biotin-TEO-NH₂ (8).² Dry DCM (15 mL) was added into a 100-mL RBF equipped with a stir bar containing biotin-TEO-NHBoc (**7**) (921 mg, 1.69 mmol) under argon to form a homogenous solution. The RBF was cooled in an

ice-water bath and TFA (7.5 mL) was added under a flush of argon. The reaction solution was stirred for 12 min and DCM and TFA were removed under high vacuum overnight. The residue was purified by silica gel flash chromatography (CHCl₃: MeOH: NH₃ (20 w %) 1: 0.25: 0.1, R_f = 0.35, stained by ninhydrin) to afford 974 mg light yellow sticky solid as the product (**8**) in a quantitative yield; **¹H-NMR** (DMSO-d₆, 500 MHz): δ 7.75 (t, *J*=5.68Hz, 1H), 6.41 (s, 1H), 6.35 (s, 1H), 4.30 (m, 1H), 4.13 (m, 1H), 3.51 (m, 4H), 3.47 (m, 4H), 3.43 (t, *J*=6.3Hz, 2H), 3.38 (t, *J*=6.3Hz, 2H), 3.20 (s, br, 2H), 3.10 (m, 1H), 3.07 (m 1H), 2.82 (dd, *J*₁=12.0Hz, *J*₂=5.1Hz, 1H), 2.62 (t, *J*=6.9Hz, 2H), 2.57 (d, *J*=12.0Hz, 1H), 2.04 (t, *J*=7.6Hz, 2H), 1.60 (m, 5H), 1.49 (m 3H), 1.30 (m, 2H); **MS**(ESI): C₂₀H₃₉N₄O₅S⁺ calcd. 447.2, found 447.2. NMR data was in accordance with those published previously³



Biotin-TEO-azido-COOtBu (9). A solution of *N*-(3-carboxylpropanoyl)-*N*-(2-azidoethyl)glycine

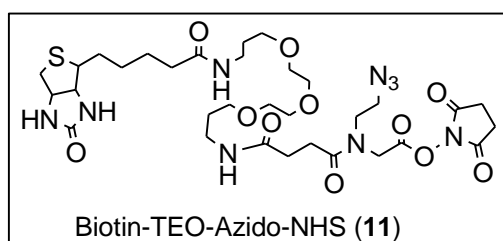
tert-butyl ester (**4**, 218 mg, 0.726 mmol) in DCM was added into a 25 mL Schlenk tube equipped with a stir bar. DCM was removed under high vacuum and EDC·HCl (166 mg, 0.864 mmol) and DMF (1.73 mL) were added. The resultant reaction solution was stirred at 0 °C for 10 min. Subsequently, a solution of biotin-TEO-NH₂ (**8**, 308 mg, 0.691 mmol) in dry DMF (1.73 mL) was added and the ice-water bath was removed. The reaction tube was wrapped with aluminum foil to exclude light and the reaction solution was stirred at RT for two days. DMF was removed under high vacuum with stirring and the residue was partitioned in CHCl₃/ sat. NaHCO₃ (25 mL/25 mL). The organic layer was separated and the aqueous phase was extracted several times by CHCl₃. Organic layers were combined, washed with brine, dried over anhydrous MgSO₄, filtered, concentrated and purified *via* silica gel chromatography (CHCl₃: MeOH 5:1, R_f = 0.5, stained by I₂) to afford 349 mg yellowish solid as the product in a yield of 69 %; **¹H-NMR** (CDCl₃, 500 MHz): δ 6.84 (m, 1H), 6.77 (t, *J*=5.38Hz, 0.5 H), 6.74 (t, *J*=5.4Hz, 0.5H), 6.15 (s, 1H), 5.48 (d, *J*=6.0Hz, 1H), 4.49 (m, 1H), 4.30 (m, 1H), 4.07 (s, 0.5 × 2H), 3.97 (m, 0.5 × 2H), 3.63 (m, 4H), 3.62~50 (m, 10H), 3.48~46 (m, 2H) 3.32 (m, 4H), 3.14 (dd, *J*₁=12.6Hz, 1H), 2.87 (d, *J*=12.6Hz, 1H), 2.55~51 (m, 2H), 2.17 (t, *J*=7.05Hz, 4H), 1.76 (m, 5H), 1.66 (m, 3H), 1.47 (m, 2H), 1.47 (s, 0.5 × 9H), 1.44 (s, 0.5 × 9H); (signals from the same proton are underlined) **¹³C-NMR** (CDCl₃, 500 MHz): δ 173.24, 172.73, 173.09, 173.08, 172.06, 172.03, 168.35, 168.33, 163.64, 163.55, 82.77, 81.87(-C(CH₃)₃), 70.45, 70.41, 70.03, 69.96, 69.52, 69.48, 61.83, 60.11, 55.59, 51.83, 50.13, 49.82, 49.42, 48.11, 47.46, 40.50, 37.73, 37.39, 35.89, 31.15, 31.13, 29.05, 28.89, 28.46, 28.34, 28.17, 28.06, 28.04, 28.01 (-C(CH₃)₃), 25.59; **DEPT135** (CDCl₃, 500 MHz): δ(+) 61.83, 60.11, 25.59, 28.01; δ(-) 70.46, 70.42, 70.03, 69.96, 69.51, 69.48, 63.83, 60.12, 55.60, 50.83, 50.13, 49.82, 49.43, 48.11, 47.46, 40.50, 37.72, 37.39, 35.89, 31.35, 31.13, 29.05, 28.89, 28.46, 28.34, 28.17, 28.06, 28.04, 28.01 -C(CH₃)₃, 25.59; (signals from the same carbon are underlined) **HRMS** (ESI): C₃₂H₅₆N₈NaO₉S⁺ calcd. 751.3783, found 751.3797.



Biotin-TEO-azido-COOH (10).

Biotin-TEO-azido-COOtBu (**9**, 307 mg, 0.421

mmol) and anhydrous DCM (6 mL) were added into a thoroughly dried 25-mL RBF equipped with a stir bar. A clear solution was formed and the RBF was cooled in an ice-water bath. The solution was degassed *via* vacuum/Ar three times and TFA (3 mL) was injected. The ice-water bath was removed and the RBF was wrapped with aluminum foil. The reaction solution was stirred at RT for 6 h. DCM and TFA were removed under high vacuum overnight and the residue was partitioned in CHCl₃/MQ-H₂O (10 mL/5 mL). The organic layer was drained out and the aqueous layer was washed two additional times by CHCl₃. Water was removed under reduced pressure *via* a rotavap and the residue was further dried in *vacuo* to afford 262 mg sticky solid as the product in a yield of 93 %. **¹H-NMR** (MeOD, 500 MHz): δ4.51 (m, 1H), 4.32 (m, 1H), 3.64 (m, 4H), 3.61~5.9 (m, 6H), 3.52 (t, *J*=6.13Hz, 4H), 3.54 (t, *J*=6.35Hz, 0.5H), 3.46 (t, *J*=5.95Hz, 0.5H), 3.26(m, 4H), 3.21 (m, 1H), 2.93 (dd, *J*₁=12.75Hz, *J*₂=4.95Hz, 1H), 2.81 (t, *J*=6.95Hz, 0.5H), 2.60 (t, *J*=6.90Hz, 0.5H), 2.71 (d, *J*=12.70Hz, 1H), 2.49 (q, *J*=7.48Hz, 2H), 2.21 (t, *J*=7.35Hz, 2H), 1.76 (m, 5H), 1.64 (m, 3H), 1.44 (p, *J*=7.69Hz, 2H); (signals from the same proton are underlined) **¹³C-NMR** (MeOD, 500 MHz): δ176.00, 174.90, 175.27, 174.69, 172.61, 172.58, 159.64, 71.54, 71.22, 69.97, 69.89, 63.47, 61.72, 56.99, 51.51, 51.05, 50.49, 49.33, 49.22, 48.26, 41.02, 37.90, 36.84, 31.97, 31.93, 30.40, 30.35, 29.78, 29.50, 29.39, 26.88; **DEPT135** (MeOD, 500MHz): δ(+)63.47, 61.72, 56.97; δ(-) 71.54, 71.24, 71.22, 69.97, 69.89, 51.04, 49.33, 49.08, 47.93, 47.82, 46.85, 39.62, 36.50, 35.43, 30.57, 30.52, 29.00, 28.95, 28.38, 28.10, 27.99, 25.47; (signals from the same carbon are underlined) **HRMS** (ESI): C₂₈H₄₈O₉N₈NaS⁺ calcd. 695.3157, found: 695.3159; **LCMS**: 97.2 % purity (214 nm).

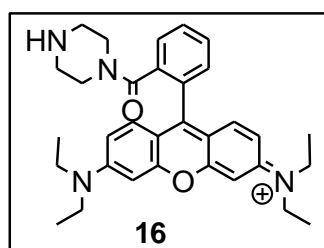


Biotin-TEO-azido-NHS (11). A solution of biotin-TEO-azido-COOH (**10**) in anhydrous DMF (0.2 M) (60 μL, 12 μmol, 1.2 equiv) was mixed with a solution of EDC·HCl/DMF (0.4 M) (25 μL, 10 μmol, 1.0 equiv) and a NHS

solution in DMF (0.4 M) (30 μL, 12 μmol, 1.2 equiv) under Ar. The resultant clear solution was stood overnight, yielding an *in-situ* generated biotin-TEO-azido-NHS (**11**) solution in

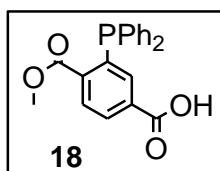
anhydrous DMF at the concentration of 0.1 M. This solution was directly used for subsequent preparation of azido-(K1)-RNase A (**13**) *via* kinetically controlled labeling; **HRMS**(ESI): C₃₂H₅₂N₉O₁₁S⁺ calcd. 770.3502, found 770.3507. In KPL approach, the NHS ester can be *in situ* prepared by NHS/EDC and there is no need to isolate the active NHS ester *via* flash column chromatography.

Synthesis of Rho-TEO-Phosphine (21)

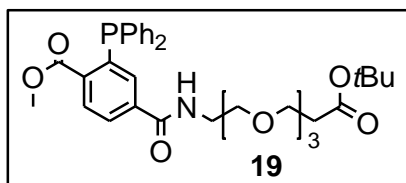


Rhodamine B piperazine amide (16). This is a new synthetic procedure toward the synthesis of rhodamine B piperazine amide⁴ involving the intermediate rhodamine B acyl chloride. Rhodamine B (**14**) (479 mg, 1 mmol) was placed into a 2 necked 25 mL-RBF equipped with a stir bar

under Ar and SOCl₂ (5 ml) was added. The resultant reaction mixture was cooled in a water bath and stirred at RT overnight. Excess of SOCl₂ was removed under high vacuum. Anhydrous DCM (5 mL) was injected and piperazine (430 mg, 5 mmol) was added. The reaction mixture was cooled in a water-bath and stirred overnight. The reaction mixture was removed of DCM under high vacuum and the residue was partitioned in EtOAc/sat. NaHCO₃. The aqueous layer was separated and extracted three times by DCM. Organic layers were combined, washed with 1M HCl/brine (4:1 v/v), brine, dried over anhydrous MgSO₄, filtered, removed of DCM under reduced pressure and dried in *vacuo* to afford the 172 mg dark violet powder as rhodamine B piperazine amide (**16**) in a yield of 31 %; ¹H-NMR (MeOD, 500 MHz): δ 7.76~7.79 (m, 3H), 7.52~54 (m, 1H), 7.27 (d, J=9.4Hz, 2H), 7.10 (dd, J₁=9.4Hz, J₂=2.5Hz, 2H), 6.98 (d, J=2.5Hz, 2H), 2.68~73 (m, 12H), 3.12 (t, J=4.7Hz, 4H), 1.32 (t, J=7.3Hz, 12H); ¹³C-NMR (MeOD): δ 169.53, 159.29, 157.31, 156.76, 135.64, 133.07, 132.48, 131.91, 131.63, 131.42, 128.87, 115.49, 114.89, 97.41, 46.92, 12.85; DEPT135 (MeOD, 500MHz): δ (+) 133.07, 131.91, 131.63, 131.42, 128.86, 115.49, 9.40, 12.85; (-) 46.92; **LCMS**(ESI): C₃₂H₃₉N₄O₂⁺ calcd. 511.31, found 511.25. NMR data was in accordance with those published previously²⁻⁴



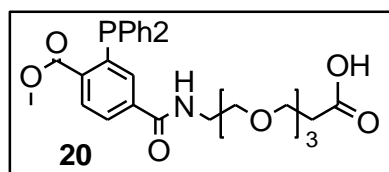
2-Diphenylphosphino-1-methylterephthalate (18).⁵ K_2CO_3 (220 mg, 1.60 mmol) was added in to a 2 neck 25 mL-Schlenk tube equipped with a stir bar and then dried under high vacuum with heat for 10 min. 1-Methyl-2-iodoterephthalate (**17**, 90 w %) (247 mg→ 222 mg, 0.726 mmol), $Pd(OAc)_2$ (8.1 mg, 2.5 mol %) and anhydrous MeCN (8 mL) were added under Ar. The volume of MeCN cannot be reduced because otherwise the reaction solution will become a viscous suspension. $HPPH_2$ (270 mg, 1.45 mmol) was injected under Ar and the reaction mixture was allowed to stir at 85 °C for 24 hours. MeCN and DIEA were removed under high vacuum and the residue was partitioned in EtOAc/ H_2O and was further acidified by dropwise addition of 1 M HCl (~2.5 mL) to pH ~3. The organic layer was separated and the aqueous phase was extracted once by EtOAc. All ester layers were combined, washed with brine, dried over anhydrous Na_2SO_4 , filtered, concentrated and purified *via* silica gel chromatography (MeOH: $CHCl_3$ 1:7, R_f 0.4, visualized under UV) to yield 227 mg yellow solid as the product in a yield of 86 %. **¹H-NMR** (MeOD, 500MHz): δ 8.04 (s, 2H), 7.65 (d, $J=3.8$ Hz, 1H), 7.35~37 (m, 6H), 7.24~28 (m, 4H), 3.69 (s, 3H); **DEPT** 135 (MeOD, 500MHz): δ 135.98, 134.78, 134.61, 131.17, 131.15, 130.17, 129.81, 129.46, 129.40, 52.32; **³¹P-NMR** ($CDCl_3$): δ -5.29; **MS**(ESI): $C_{21}H_{16}O_4P^-$ calcd. 363.08, found 363.10. NMR data was in accordance with those published previously⁶.



Phosphine-TEO-COOtBu (19).

1-Diphenylphosphino-2-methylterephthalate (**18**, 95 mg, 0.26 mmol) and HBTU (117 mg, 0.31 mmol) were added into a 10 mL-Schlenk tube equipped with a stir bar under Ar. DMF (1.3 mL), *tert*-butyl 12-amino-4,7,10-trioxadecanoate (80 w %) (100 mg→ 80 mg, 0.29 mmol) and DIEA (90 mg, 0.7 mmol) were injected. The resultant solution was stirred at RT under Ar for 2 days. The following workup and column chromatography procedures were performed under Ar in order to minimize the oxidation of this phosphine intermediate (19). DMF and DIEA were removed under high vacuum and the residue was partitioned in EtOAc/ Na_2CO_3 . The organic layer was separated and

the aqueous phase was extracted several times by EtOAc. Organic layers were combined, dried over anhydrous Na₂SO₄, removed of Na₂SO₄, concentrated and purified *via* silica gel chromatography (pure EtOAc, R_f 0.5, visualized under UV and using ninhydrin stain) to yield 98 mg light yellow sticky solid as the product in a yield of 60 %; **¹H-NMR** (CDCl₃, 400 MHz): δ 8.07 (dd, *J*₁=8.04Hz, *J*₂=3.6Hz, 1H), 7.77 (dd, *J*₁=8.08Hz, *J*₂=1.6Hz, 1H), 7.35~7.27 (m, 1H), 6.46 (t, *J*=4.6Hz, 1H), 3.73 (s, 3H), 3.67 (t, *J*=6.52Hz, 2H), 3.62~3.55 (m, 12H), 2.46 (t, *J*=6.52Hz, 2H), 1.43 (s, 9H); **¹³C-NMR** (CDCl₃, 400 MHz): δ 170.70, 166.65 (d, *J*=9.12Hz), 166.36, 141.40, 137.37, 137.14 (d, *J*=51.85), 136.62 (d, *J*=88.55), 133.87 (d, *J*=103.80), 132.1, 130.78 (d, *J*=11.85), 128.95, 128.90 (d, *J*=36.65), 126.71, 80.51, 70.52, 70.49, 70.34, 70.28, 69.53, 66.87, 58.17, 39.84, 36.21, 28.07; **³¹P-NMR** (CDCl₃): (400 MHz, CDCl₃): δ -3.67; **HRMS**(ESI): C₃₄H₄₂NO₈PNa⁺ calcd. 646.2540, found 646.2538.

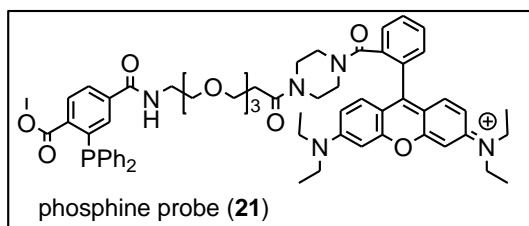


Phosphine-TEO-COOH

(20).

Phosphine-TEO-COOtBu (**19**, 98 mg, 0.158 mmol) was dissolved in DCM (3 mL) under Ar. TFA (degassed) (1.5 mL) was added to this stirring solution at 0 °C. The

ice-water bath was removed and the resultant solution was allowed to stir at RT under Ar for 24 hours. DCM and TFA were removed under high vacuum to yield the light yellow solid as the product in a quantitative yield; **¹H-NMR** (DMSO-d₆, 400 MHz): δ 10.57 (s, br, 1H), 8.59 (t, *J*=5.47Hz, 1H, *NH*), 8.00 (dd, *J*₁=8.04Hz, *J*₂=3.60Hz, 1H), 7.91 (dd, *J*₁=8.0Hz, *J*₂=1.56Hz, 1H), 7.45 (dd, *J*₁=3.88Hz, *J*₂=1.52Hz, 1H), 7.39~7.37 (m, 6H), 7.24~7.19 (m, 4H), 3.65 (s, 3H), 3.58 (t, *J*=6.32Hz, 2H), 3.48~3.44 (m, 10H), 3.32 (q, *J*=5.69Hz, 2H), 2.42 (t, *J*=6.34Hz, 2H); **¹³C-NMR** (DMSO-d₆, 400MHz): δ 172.56, 166.32 (d, *J*=6.84Hz), 165.32, 139.75 (d, *J*=111.4Hz), 137.26, 137.02 (d, *J*=45.32Hz), 136.35 (d, *J*=81.2Hz), 133.48 (d, *J*=83.04Hz), 133.19, 130.02 (d, *J*=10.44Hz), 128.93, 128.66 (d, *J*=28.68Hz), 126.76, 69.69, 69.68, 69.60, 69.57, 68.71, 66.22, 52.07, 34.73; **³¹P-NMR** (DMSO-d₆, 400MHz): δ -5.27; **HRMS**(ESI): C₃₀H₃₄NO₉PNa⁺ calcd. 590.1914, found 590.1910.



Rho-TEO-phosphine (21).

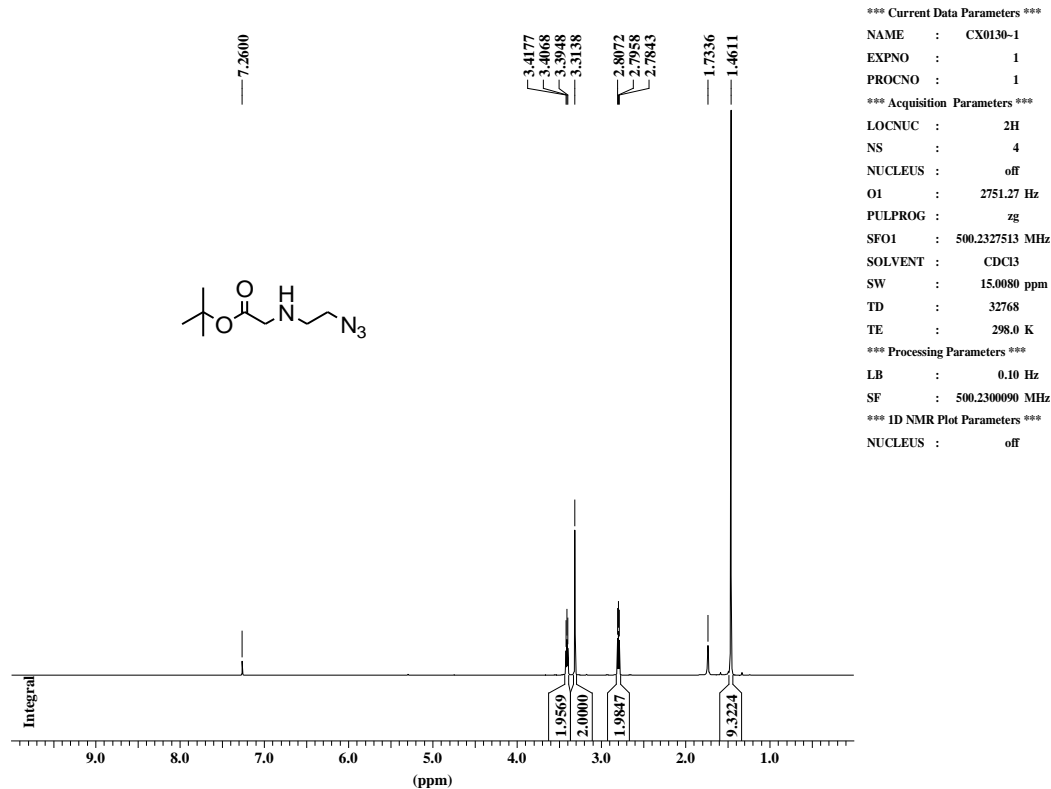
Phosphine-TEO-COOH (**20**, 41.5 mg, 0.073 mmol), rhodamine-piperazine amide (40 mg, 0.073 mmol) and HBTU (30.4 mg, 0.080

mmol) were combined in a Schlenk tube equipped with a stir bar under Ar. DMF (0.73 mL) and DIEA (24.5 mg, 0.190 mmol) were injected. The resultant solution was allowed to stir at RT under Ar for two days. The following workup and purification procedures were performed under Ar using Schlenk techniques in order to minimize the oxidation of the phosphine probe (21). DMF and DIEA were removed under high vacuum and the residue was partitioned in EtOAc/sat. NaHCO₃. The organic layer was separated and the aqueous phase was extracted several times by EtOAc. Organic layers were combined, washed with brine, dried over anhydrous Na₂SO₄ and the organic phase was transferred out, concentrated and purified *via* silica gel chromatography (CHCl₃:MeOH 5:1, R_f 0.5) to yield 36.3 mg dark violet solid as the rhodamine probe (**21**) in a yield of 41 % yield; ¹H-NMR (CDCl₃, 400 MHz): δ 8.05 (m, 1H), 7.79 (d, J=8.48Hz, 1H), 7.62~68 (m, 3H), 7.48~57 (m, 2H), 7.46 (m, 1H), 7.38 (dd, J₁=3.68Hz, J₂=1.60Hz, 1H), 7.31~35 (m, 6H), 7.26~30 (m, 5H), 7.08 (m, 1H), 6.84 (m, 1H), 6.69 (m, 2H), 3.72 (s, 3H), 3.64~53 (m, 2H), 3.45 (br, 6H), 3.35 (br, 2H), 2.65 (t, 2H), 1.31 (t, J=7.08Hz, 12H); ¹³C-NMR (CDCl₃, 400MHz): δ 172.33, 169.07, 166.75, 166.37, 157.74, 155.73, 154.77, 139.84 (d, J=94.04Hz), 137.43, 137.22 (d, J=65Hz), 136.65 (d, J=82.96Hz), 135.21, 133.90 (d, J=83Hz), 133.31, 132.96, 131.83, 131.73, 130.87, 130.79, 129.98 (d, J=30.80Hz), 128.95, 128.62 (d, J=29.12Hz), 128.38, 126.72, 114.69, 114.64, 95.87, 70.54, 70.40, 70.30, 70.09, 69.43, 67.11, 52.15, 46.07, 39.87, 38.61, 33.49, 12.60; ³¹P-NMR(CDCl₃, 400 MHz): δ -3.80 (PAr₃), -144.45 (hep, J=1760Hz) (PF₆⁻); HRMS (ESI): C₆₂H₇₁N₅O₉P⁺ calcd. 1060.4984, found 1060.4999.

8. NMR SPECTRA

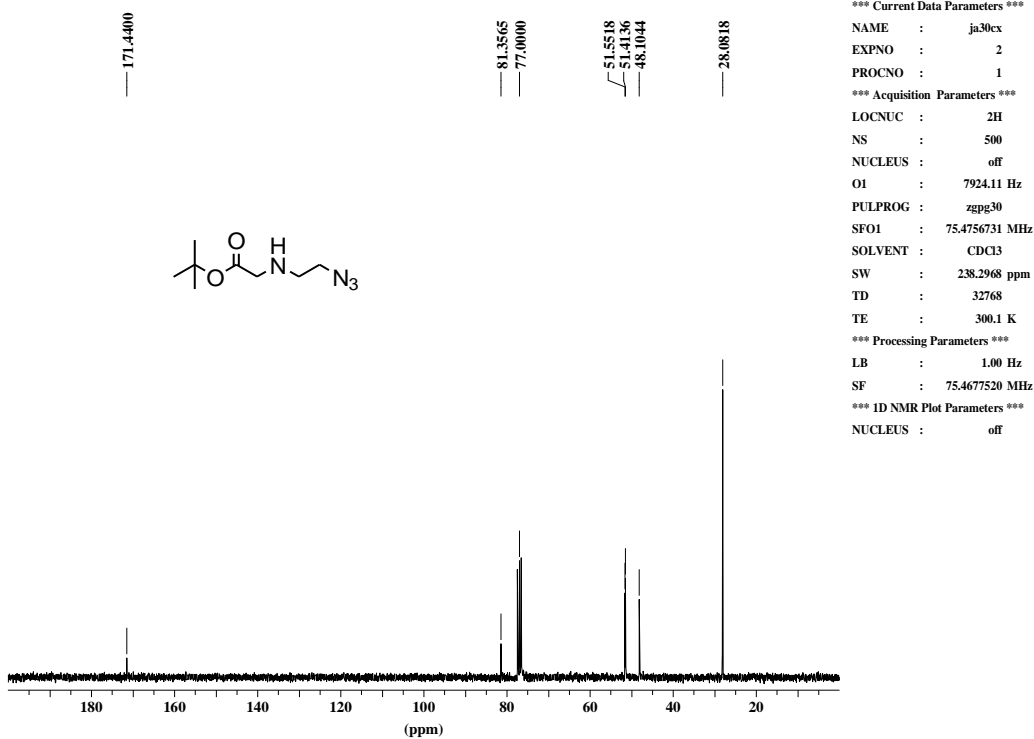
¹H-NMR (CDCl₃, DRX500): *N*-(2-Azidoethyl)glycine *tert*-Butyl Ester

N-(2-Azidoethyl)glycine *tert*-Butyl ester in CDCl₃ DRX500



¹³C-NMR (CDCl₃, AC300): *N*-(2-Azidoethyl)glycine *tert*-Butyl Ester

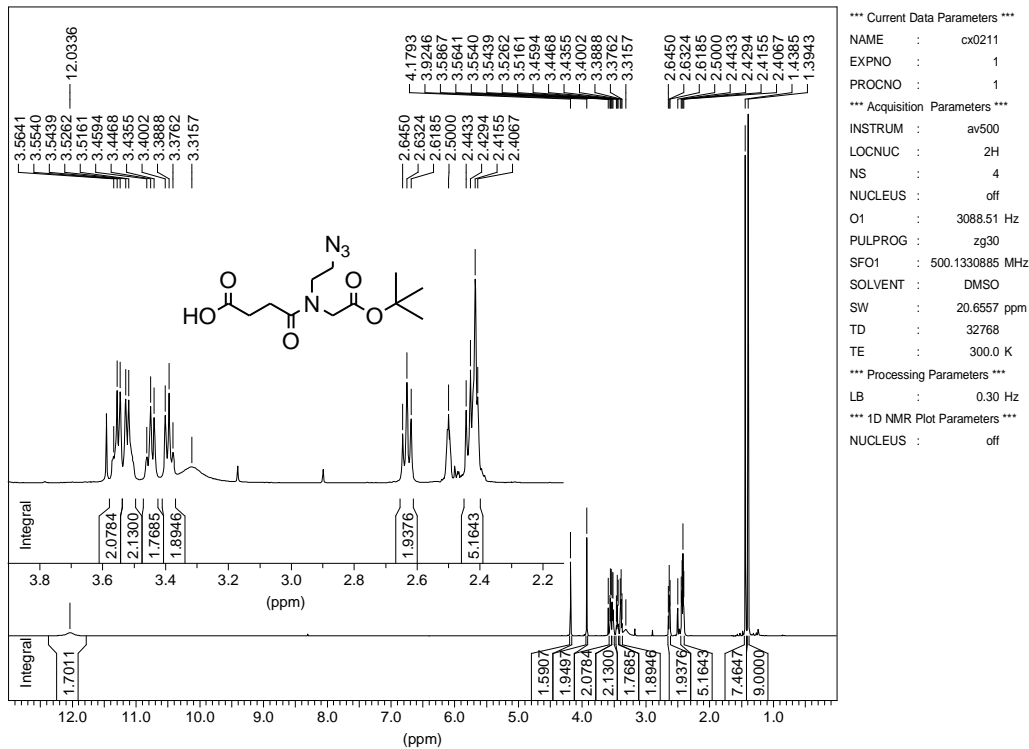
N-Azidoethylglycine tert-Butyl ester in CDCl₃ 13C Standard AC300



¹H-NMR (DMSO-d₆, AMX500):

N-(2-Azidoethyl)-N-Carboxypropanoylglycine tert-Butyl Ester

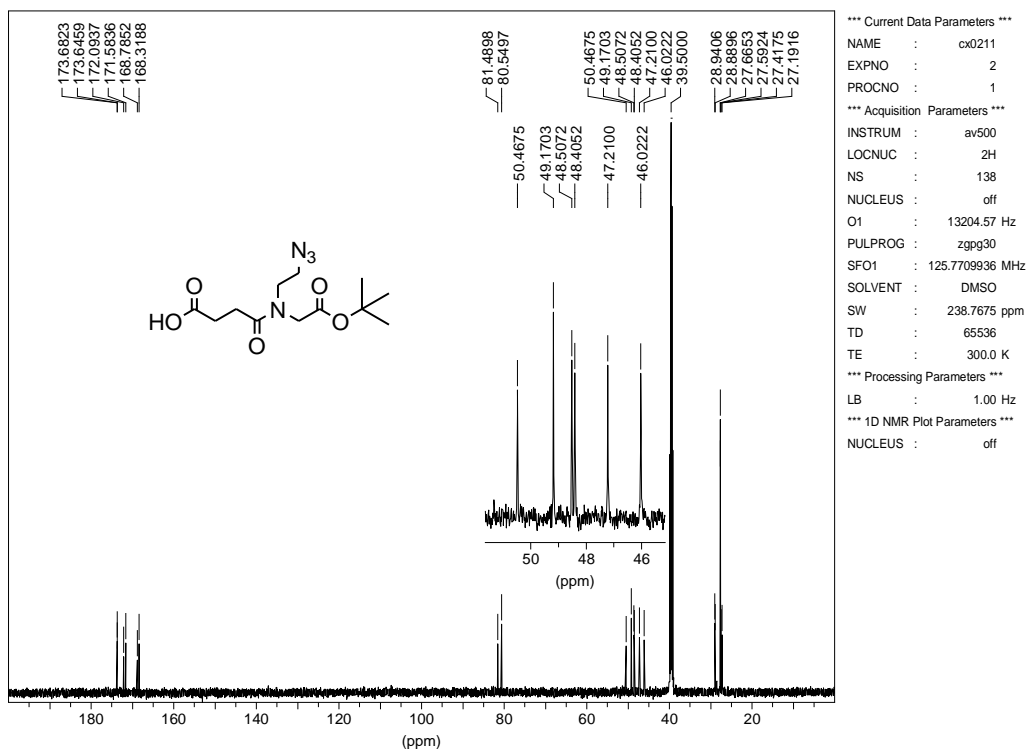
N-Azidoethyl-N'-carboxypropanoylglycine tert-Butyl ester in DMSO 1H AMX500



¹³C-NMR (DMSO-d₆, AMX500):

N-(2-Azidoethyl)-N-Carboxypropanoylglycine tert-Butyl Ester

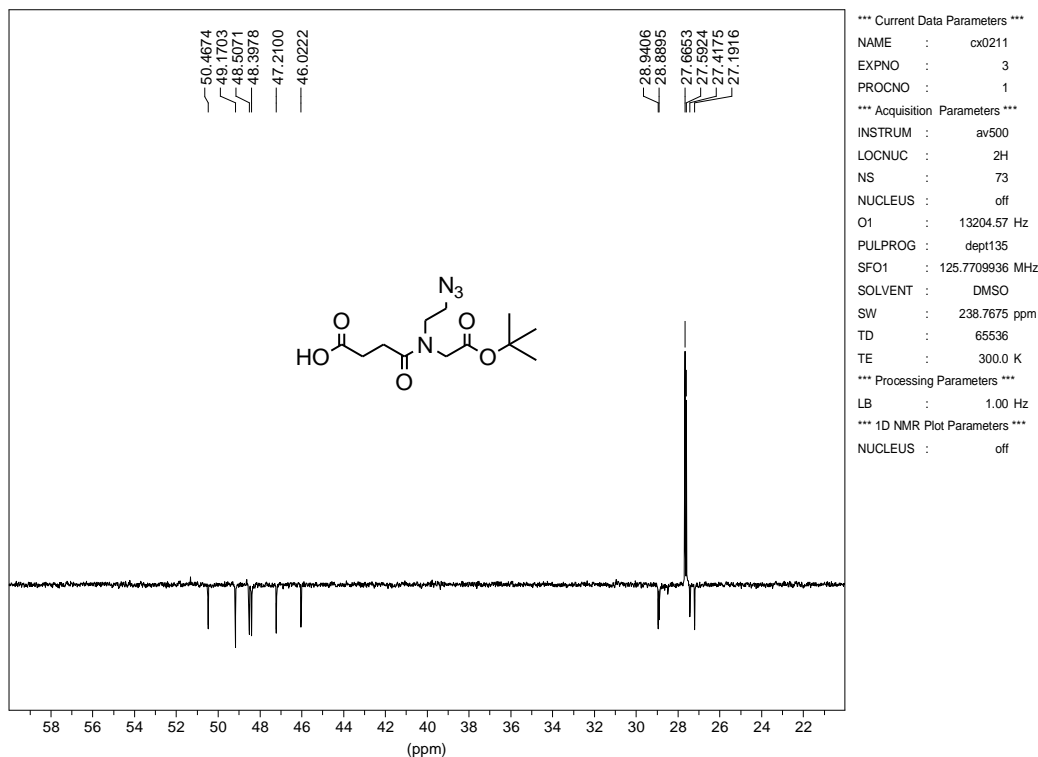
N-Azidoethyl-N'-carboxypropanoylglycine tert-Butyl ester in DMSO 13C AMX500



DEPT 135 (DMSO-d⁶, AMX500):

N-(2-Azidoethyl)-N'-Carboxypropanoylglycine tert-Butyl Ester

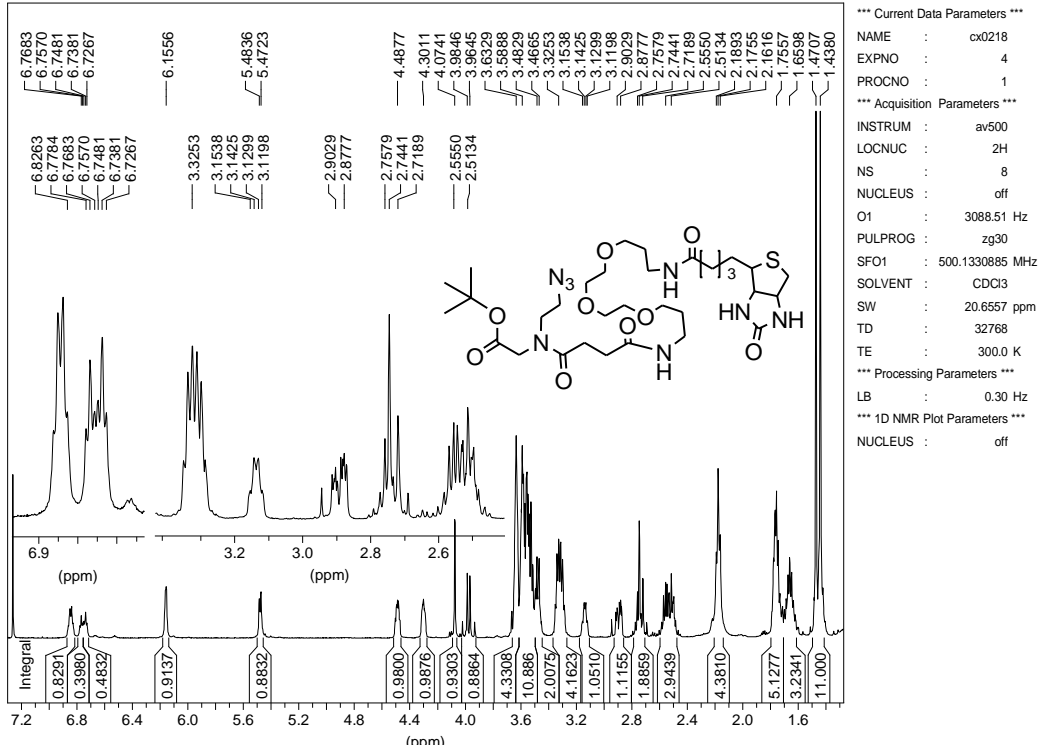
N-Azidoethyl-N'-carboxypropanoylglycine tert-Butyl ester in DMSO DEPT135 AMX500



¹H-NMR (CDCl₃, AMX500):

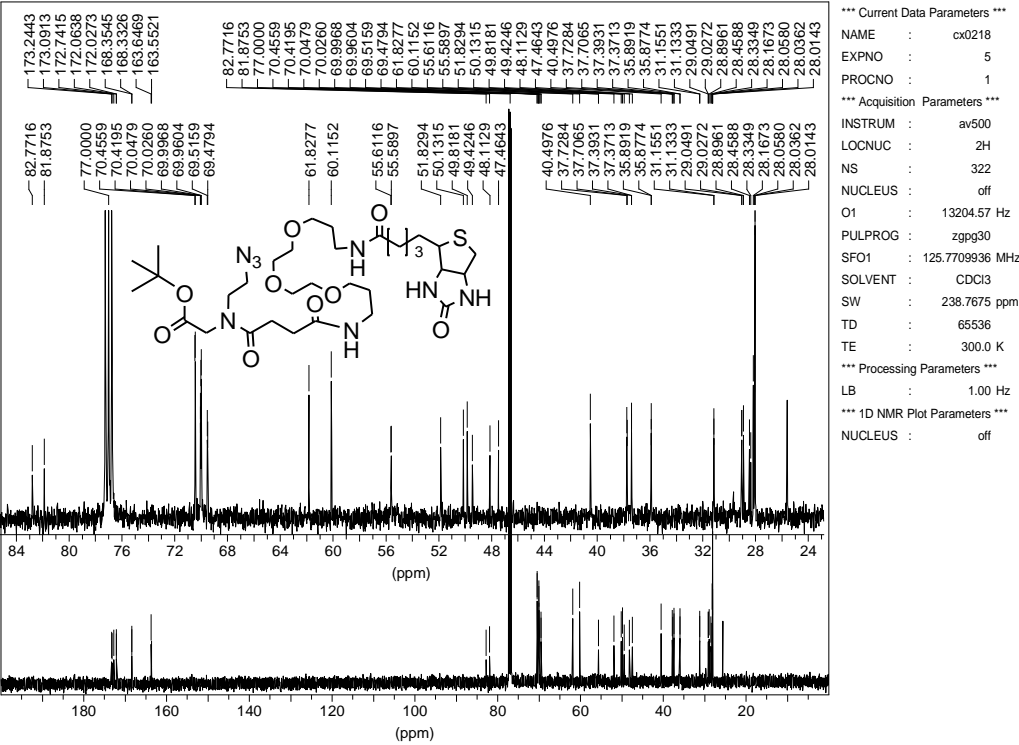
Biotin-TEO-Azido-COOtBu

Biotin-PEG-Ethynyl in CDCl₃ 1H AMX500

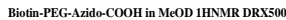


¹³C-NMR (CDCl₃, AMX500):
Biotin-TEO-Azido-COOtBu

Biotin-PEG-Ethynyl in CDCl₃ ¹³C AMX500

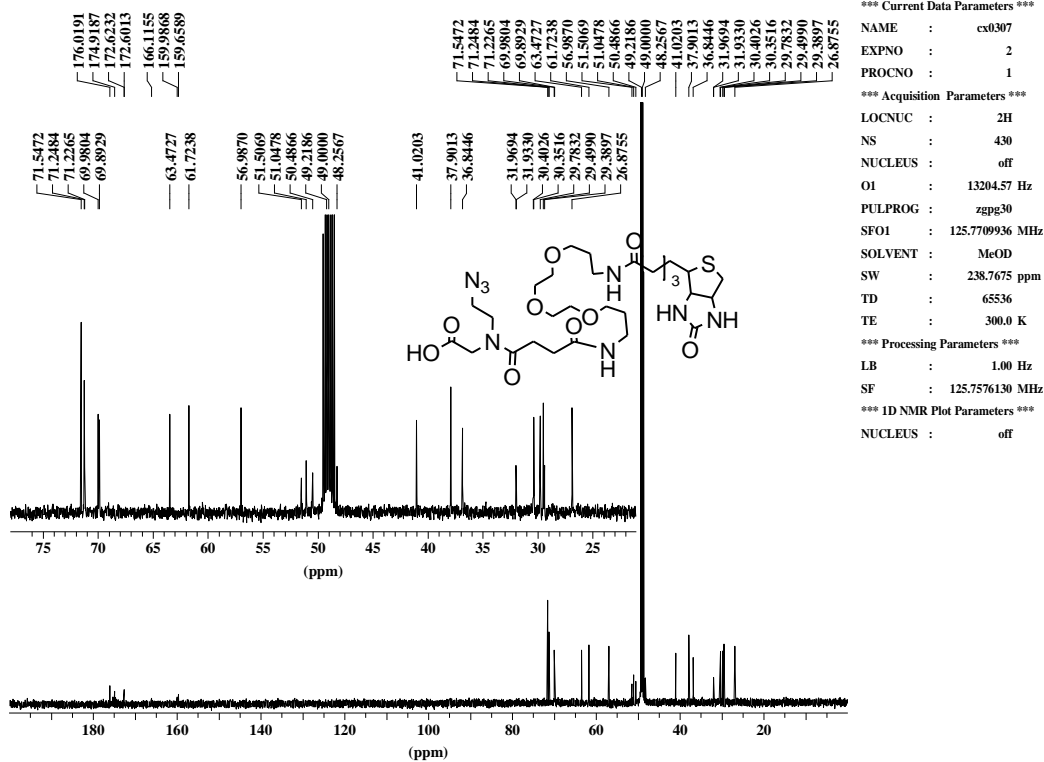


DEPT 135 (CDCl₃, AMX500):
Biotin-TEO-Azido-COOtBu



¹³C-NMR (MeOD DRX500):
Biotin-TEO-Azido-COOH

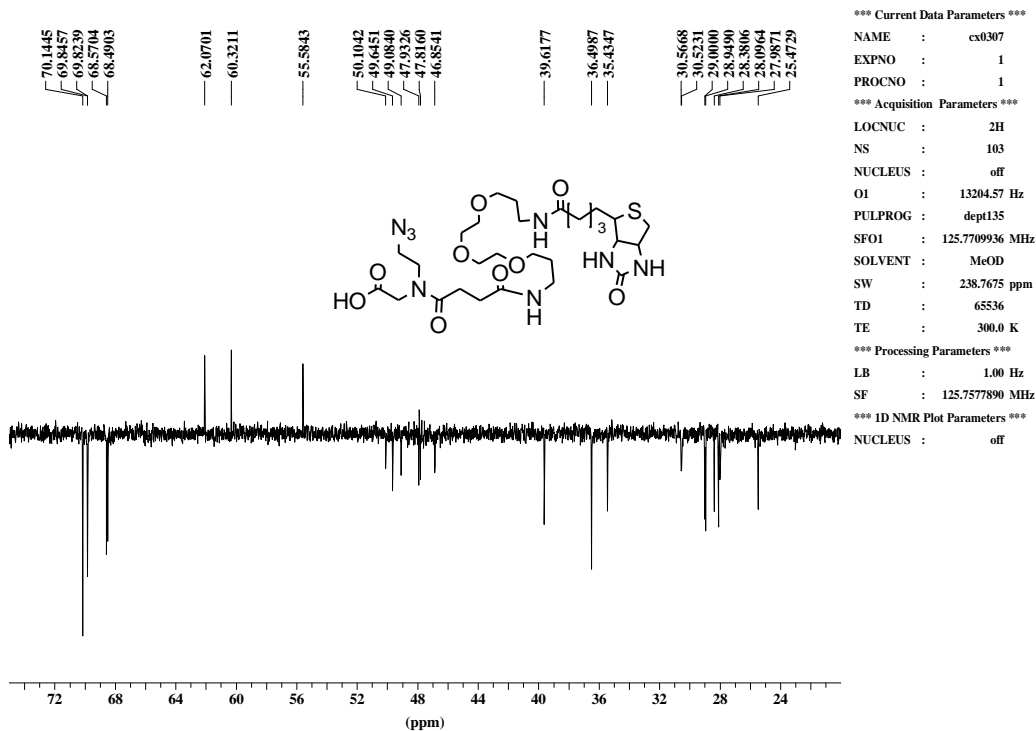
Biotin-PEG-Azido-COOH in MeOD 13C AMX500



DEPT 135 (MeOD DRX500):

Biotin-TEO-Azido-COOH

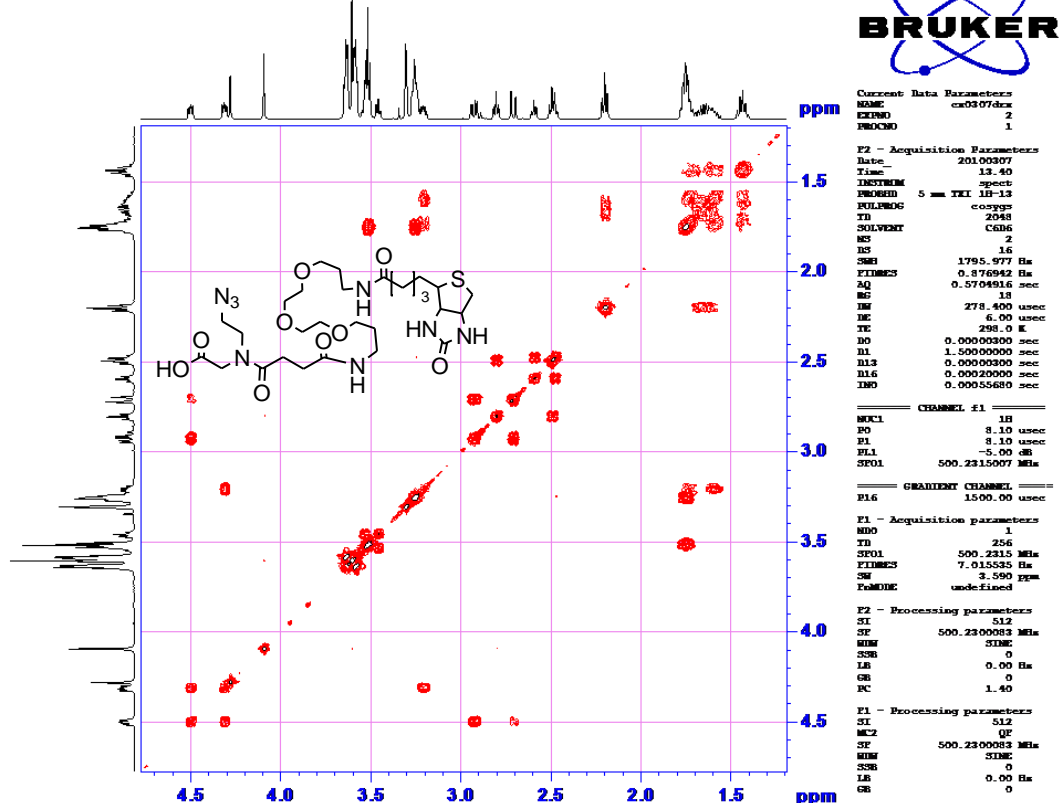
Biotin-PEG-Azido-COOH in MeOD DEPT135 AMX500



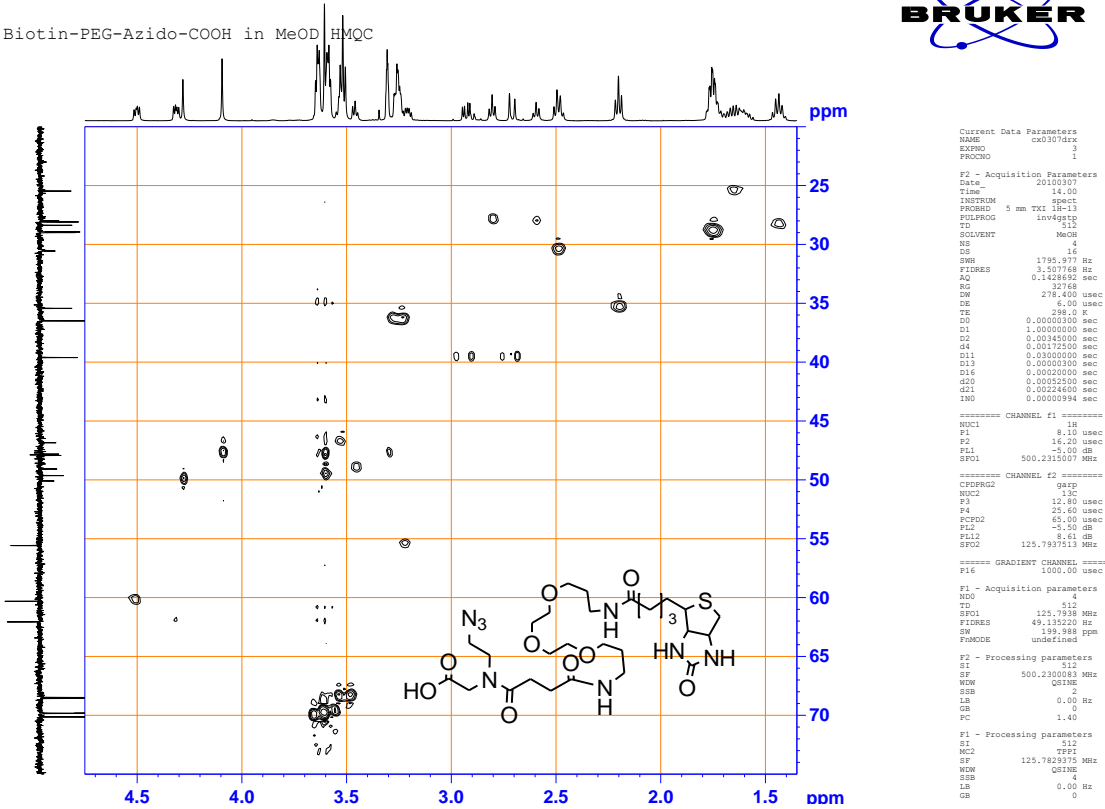
¹H-¹H COSY (MeOD, DRX500):

Biotin-TEO-Azido-COOH

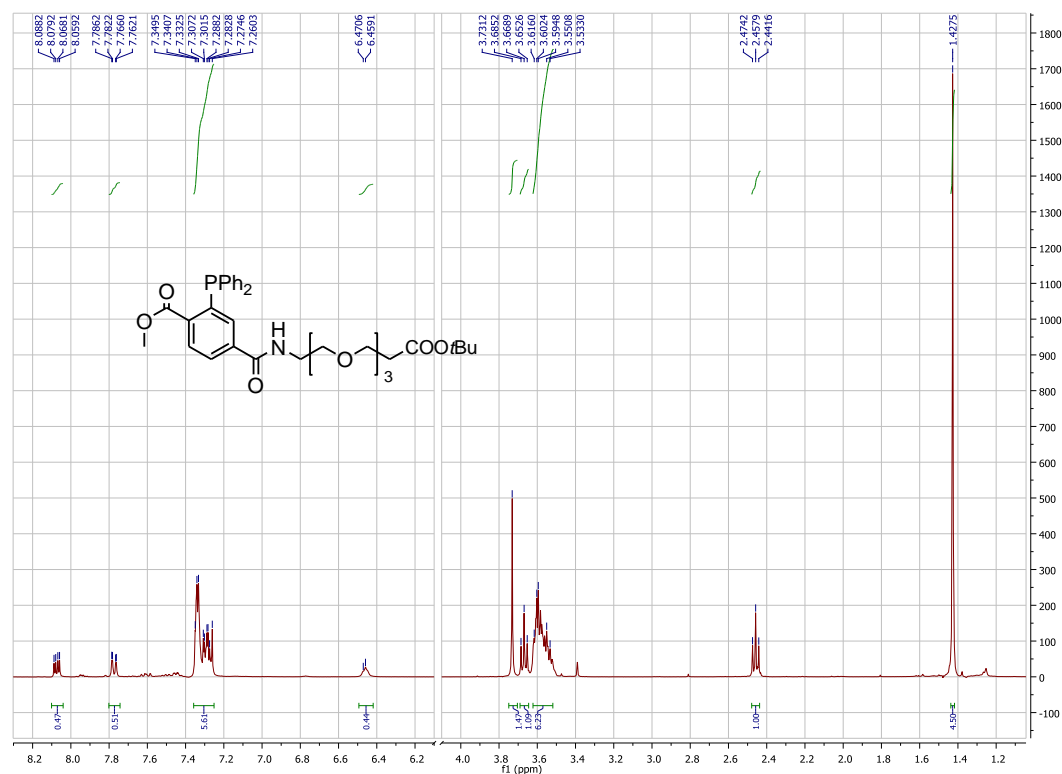
Biotin-PEG-Azido-COOH in MeOD COSY



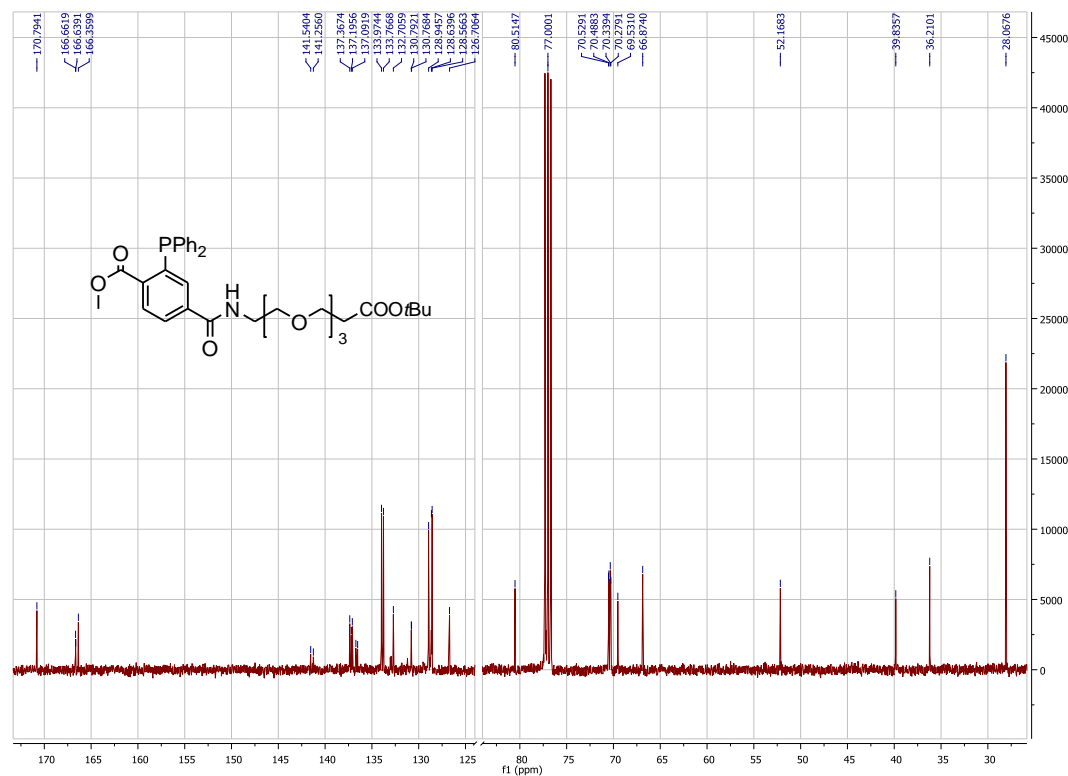
HMQC (MeOD DRX500):
Biotin-TEO-Azido-COOH



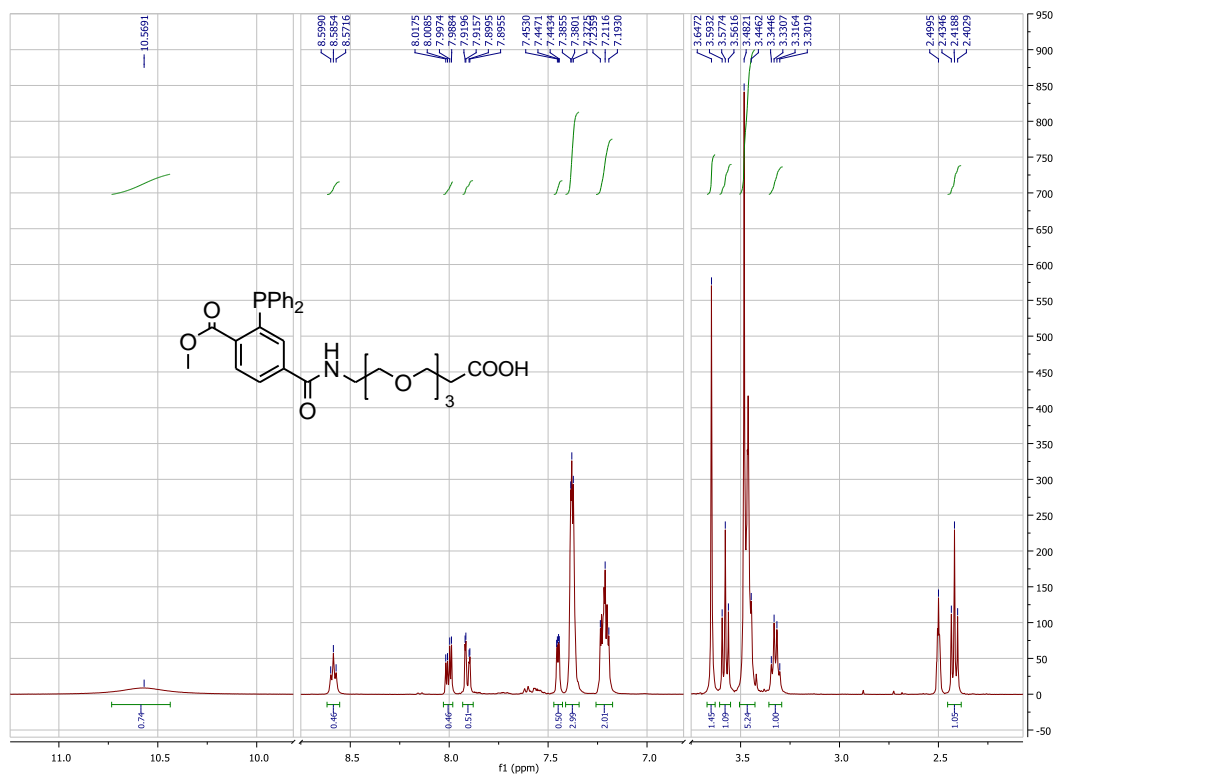
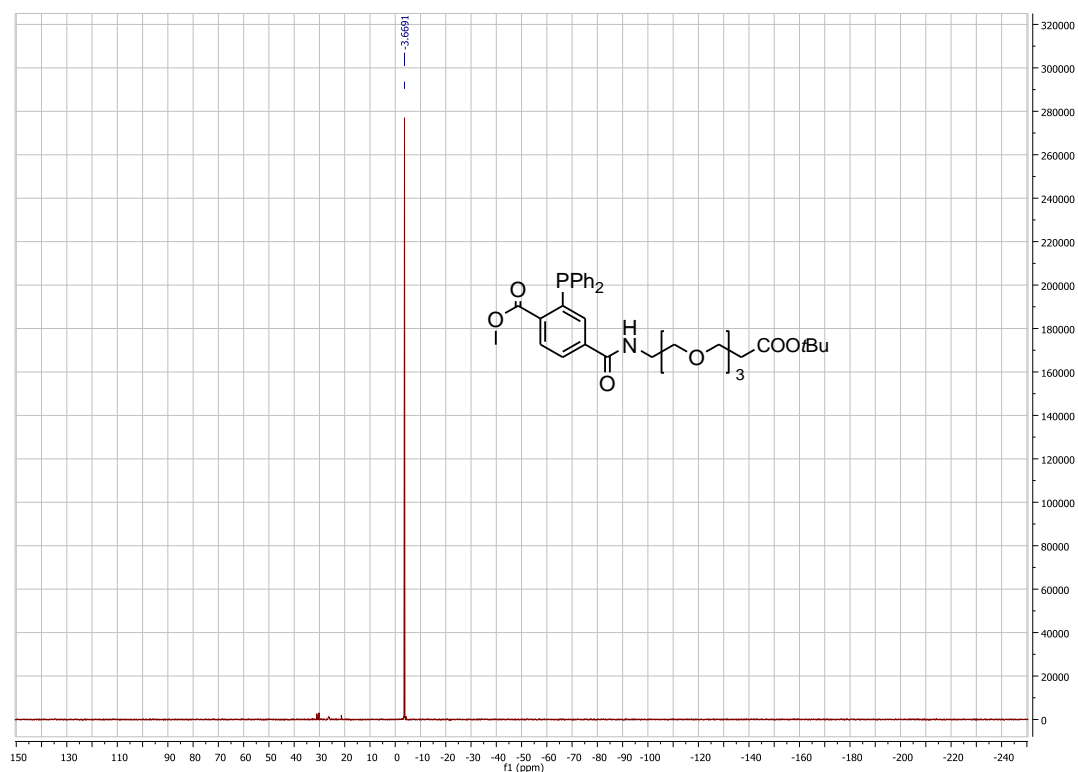
¹H-NMR (CDCl₃, 400MHz):
Phosphine-TEO-COOtBu

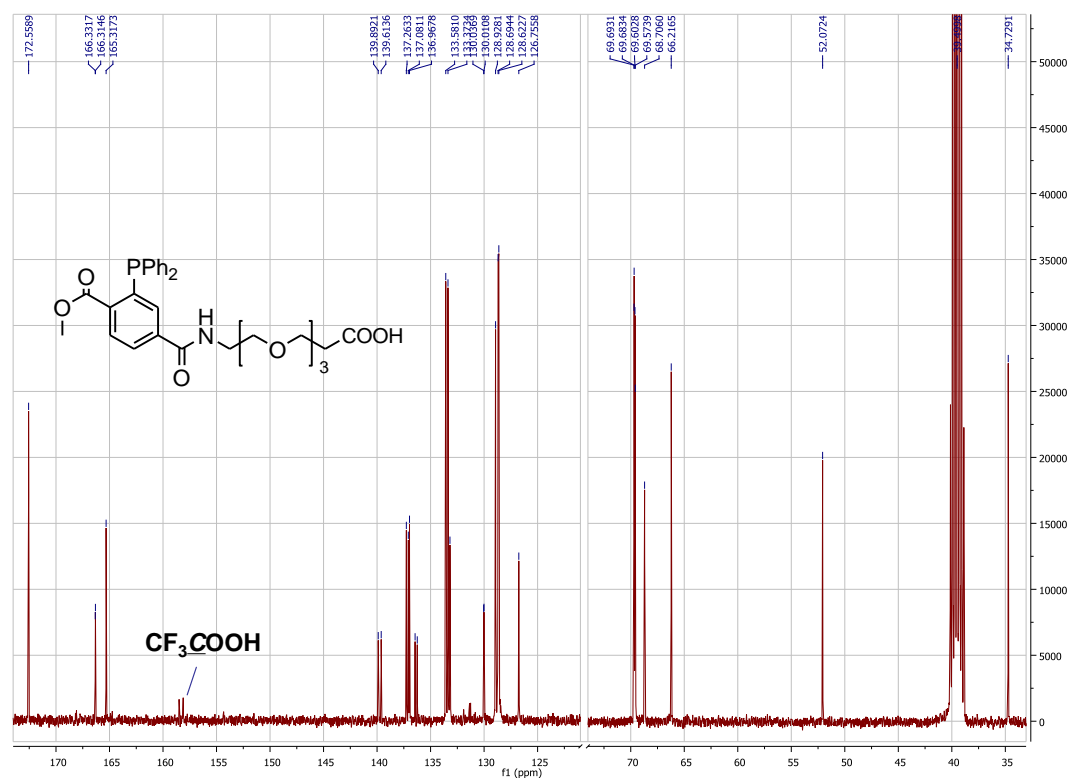


¹³C-NMR (CDCl₃, 400MHz):
Phosphine-TEO-COOtBu

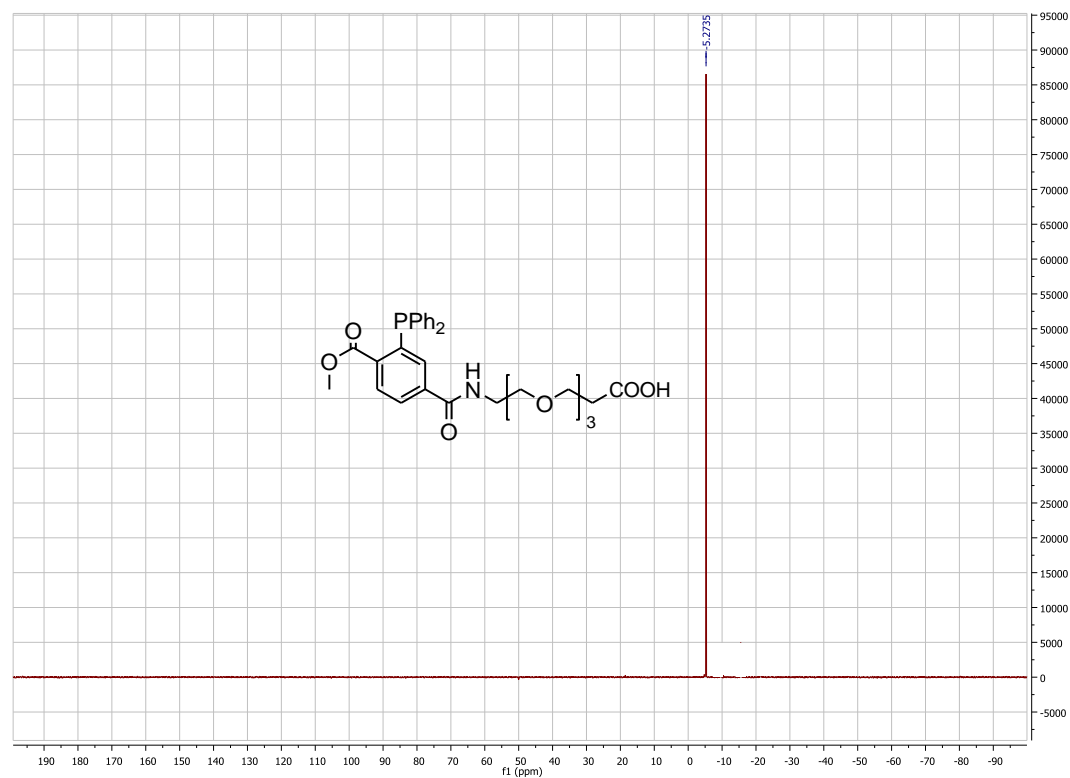


³¹P-NMR (CDCl₃, 400MHz):
Phosphine-TEO-COOtBu

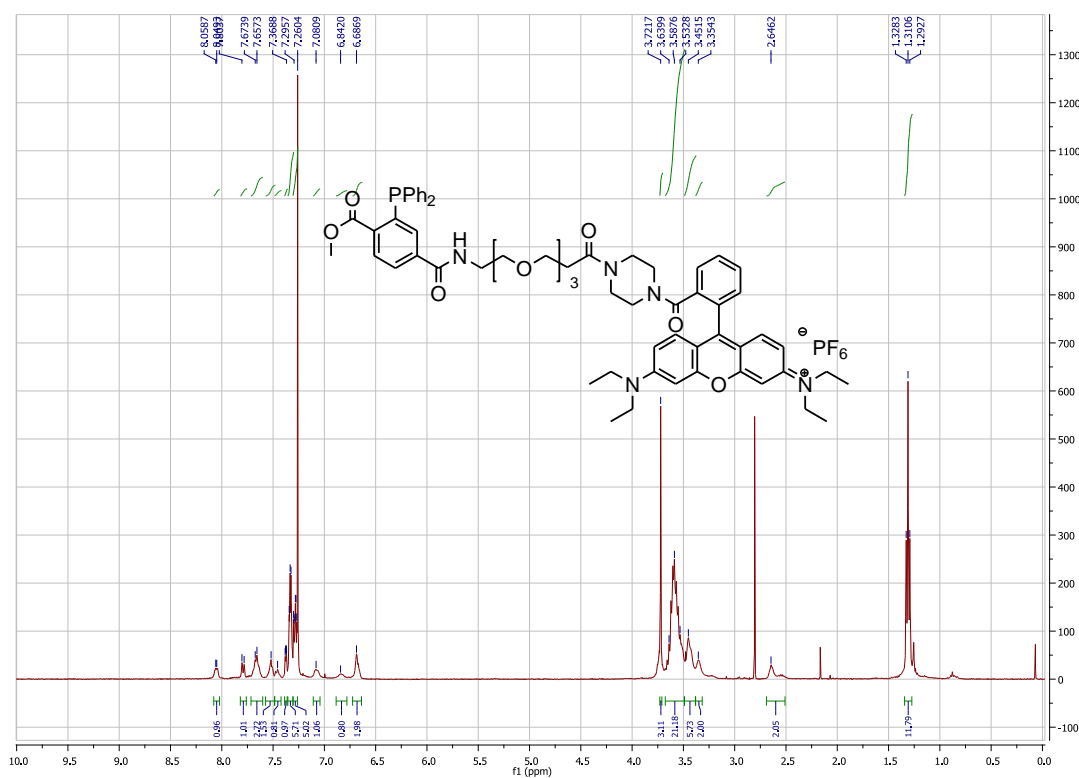




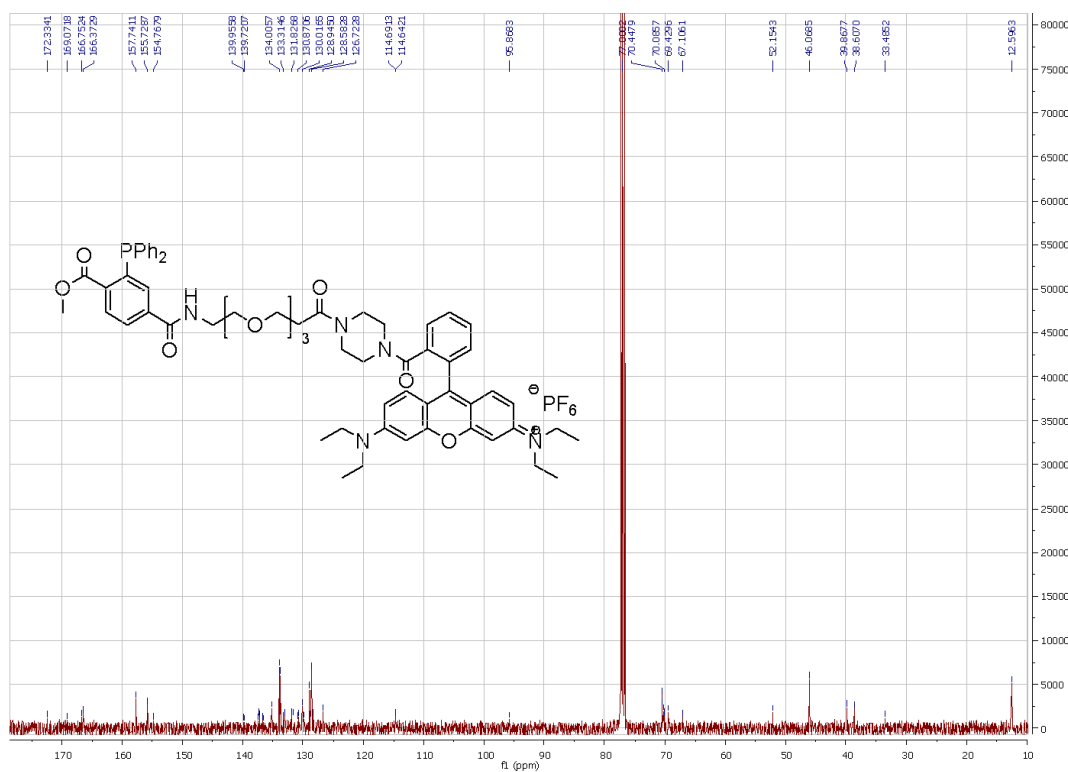
³¹P-NMR (DMSO-d₆, 400MHz):
Phosphine-TEO-COOH



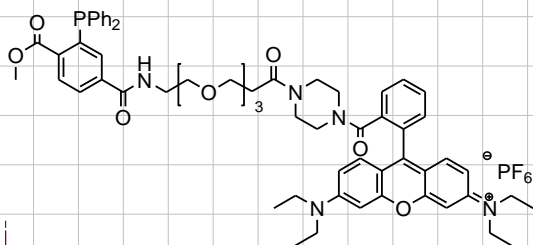
¹H-NMR (CDCl₃, 400 MHz):
Rho-TEO-Phosphine



¹³C-NMR (CDCl₃, 400 MHz):
Rho-TEO-Phosphine



³¹P-NMR (CDCl₃, 400 MHz):
Rho-TEO-Phosphine



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