Supplementary Information

Deuteration of terminal alkynes realizes simultaneous live cell Raman imaging of similar alkyne-tagged biomolecules

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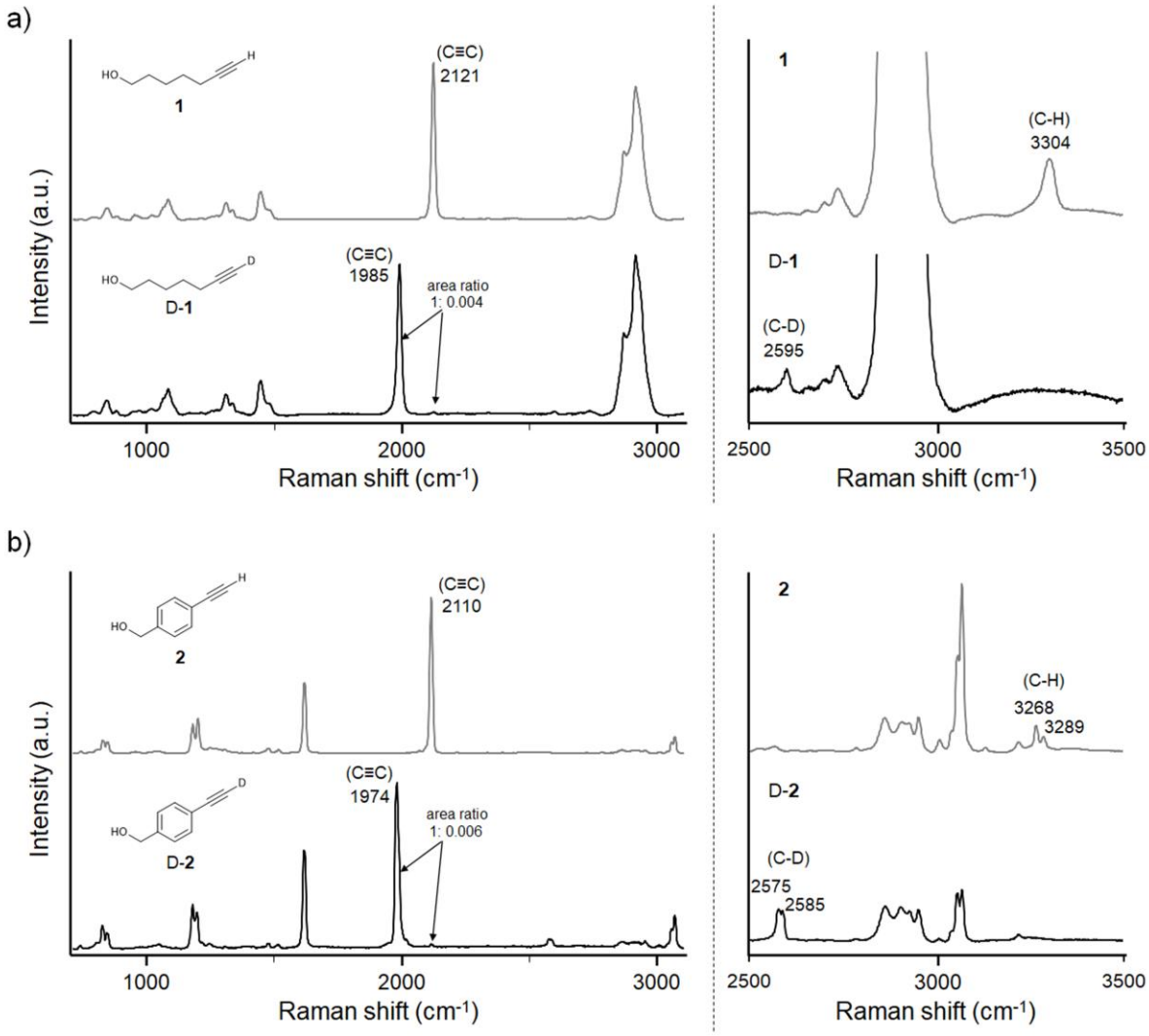


Figure S1 Comparison of Raman spectra between H-alkynes and D-alkynes. Left: Raman spectra collected from 710 to 3100 cm^{-1}. Right: Raman spectra obtained from 2500 to 3500 cm^{-1}. Raman spectra are shown without background using a modified polyfit technique.[1] a) Raman spectra of **1** and D-**1**. The ratio of the peak areas at 1985 cm^{-1} for deuterated C≡C and 2121 cm^{-1} for protonated C≡C was 1:0.004 in Raman spectra of D-**1**. b) Raman spectra of **2** and D-**2**. The ratio of the peak areas at 1874 cm^{-1} for deuterated C≡C and 2110 cm^{-1} for protonated C≡C was 1:0.006 in Raman spectra of D-**2**.

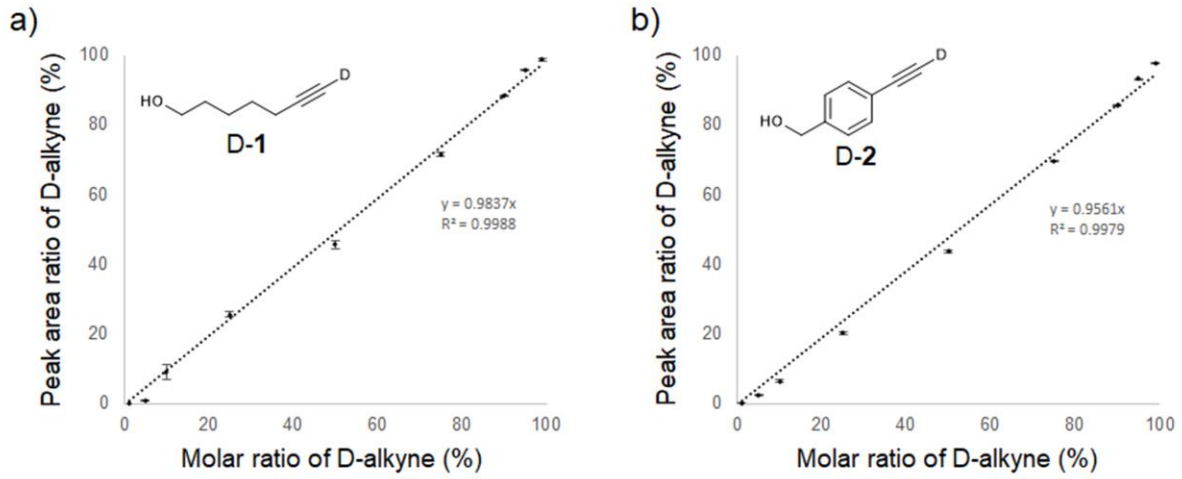


Figure S2 Correlation curves obtained from the molar ratio vs. Raman peak area ratio of a) D-**1**/**1** (D-**1** at 1977 cm^{-1} and **1** at 2116 cm-1) and b) D-**2**/**2** (D-**2** at 1970 cm^{-1} and **2** at 2109 cm^{-1}). The Raman peak area ratios of the D-alkynes were obtained as percentages from mixtures of various molar ratios of the H/D-alkynes (100 mM in total) in DMSO. Data are presented as mean ± SD.

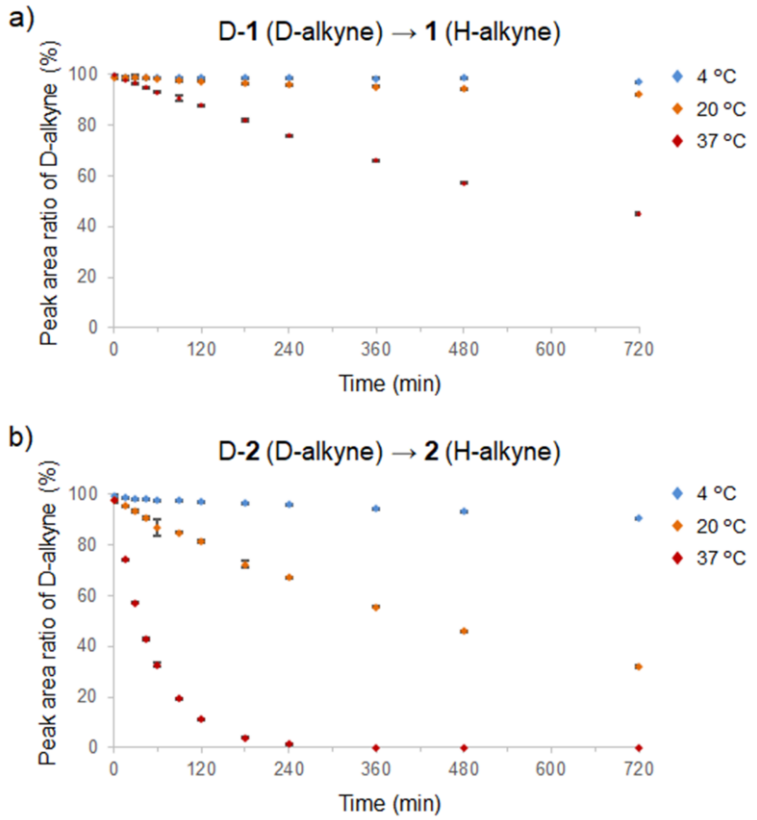


Figure S3 Real-time analysis of D/H exchange from the D-alkynes to the H-alkynes in pH 7.0 phosphate buffer at different temperatures (4, 20, and 37 °C) for a) D-**1** and b) D-**2**. Data are presented as mean ± SD.

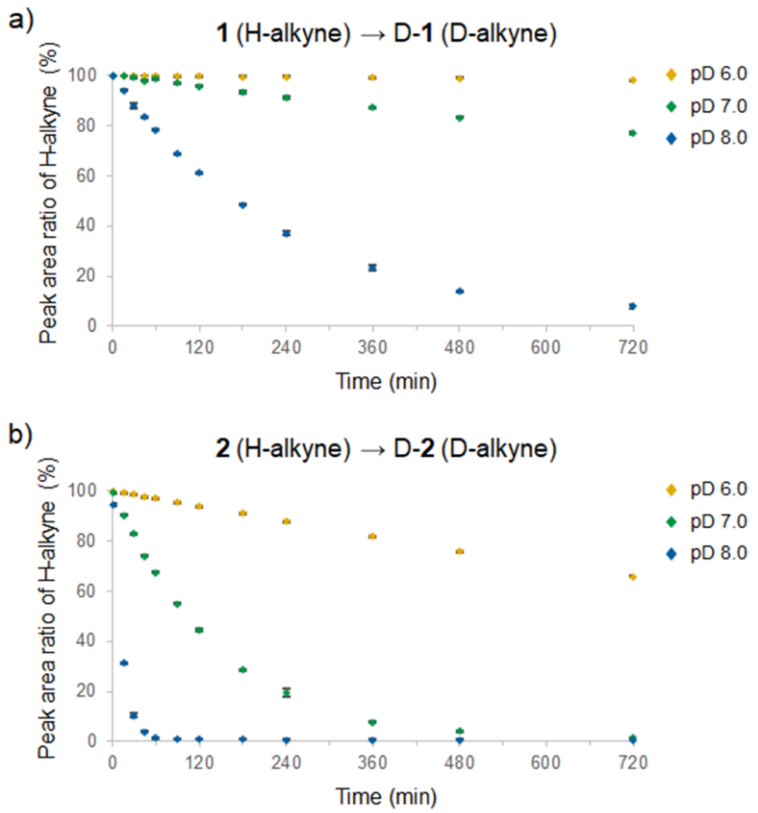
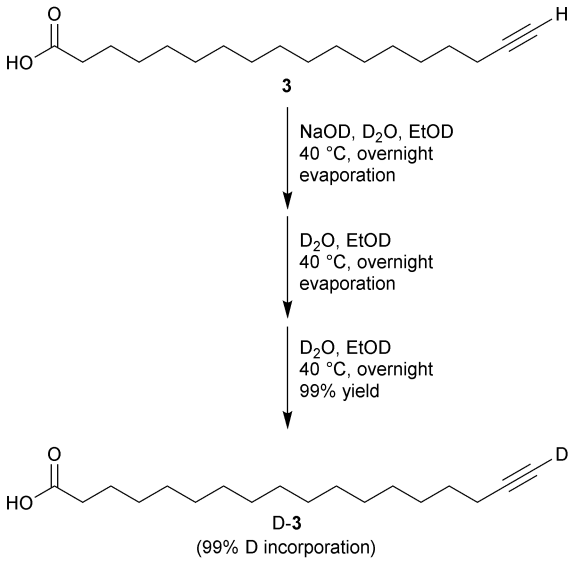


Figure S4 Real-time analysis of H/D exchange from the H-alkynes to the D-alkynes at different pD (6.0, 7.0 and 8.0) in deuterated phosphate buffer at 37 °C for a) **1** and b) **2**. Data are presented as mean ± SD.



Scheme S1 Synthesis of D-labeled octadecynoic acid (D-**3**).

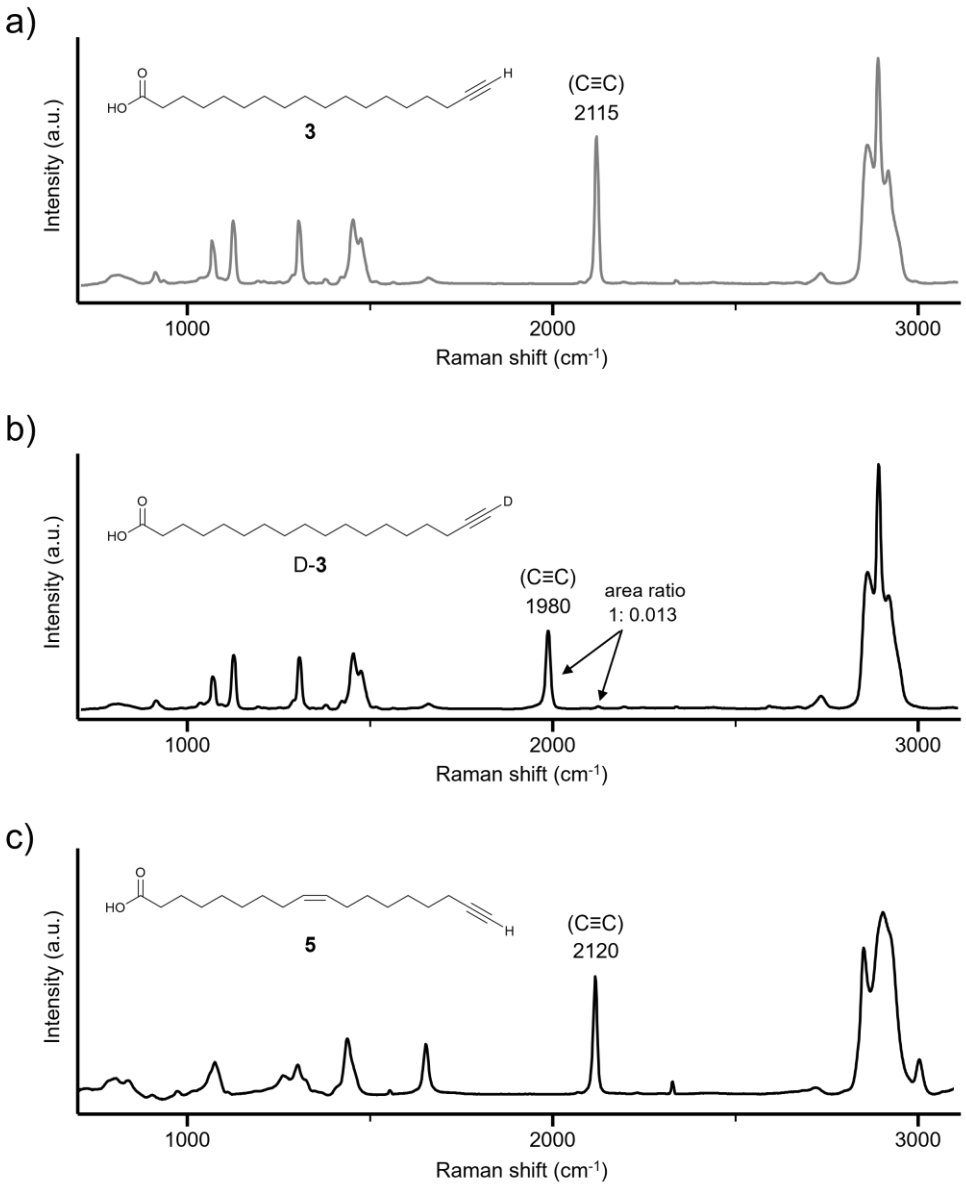


Figure S5 Raman spectra of the alkyne-containing C18 fatty acids of a) **3**, b) D-**3** (alkyne peak area of D-**3**:**3** = 1:0.013), and c) **5**.

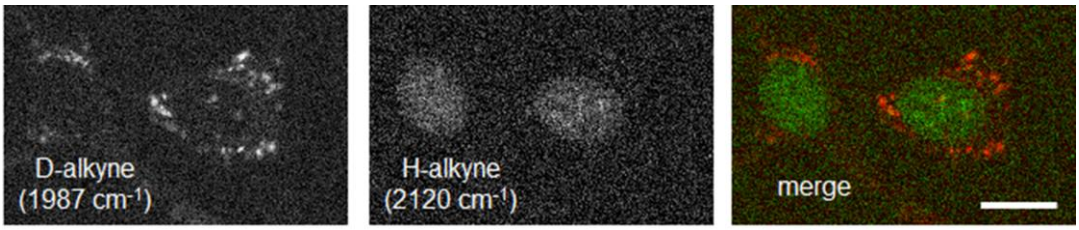


Figure S6 Raman images of H-alkyne and D-alkyne signals in Hela cells treated with D-**3** and EdU. HeLa cells were treated with 100 μM D-**3** for 6 h after 22 hour cultivation with 40 μM EdU. Images were obtained from the Raman intensity of the D-alkyne detected at 1987 cm^{-1} and H-alkyne detected at 2120 cm^{-1}. Two-color Raman images are the overlapped Raman images of the D-alkyne (red) and H-alkyne (green). Scale bars are 10 μm.

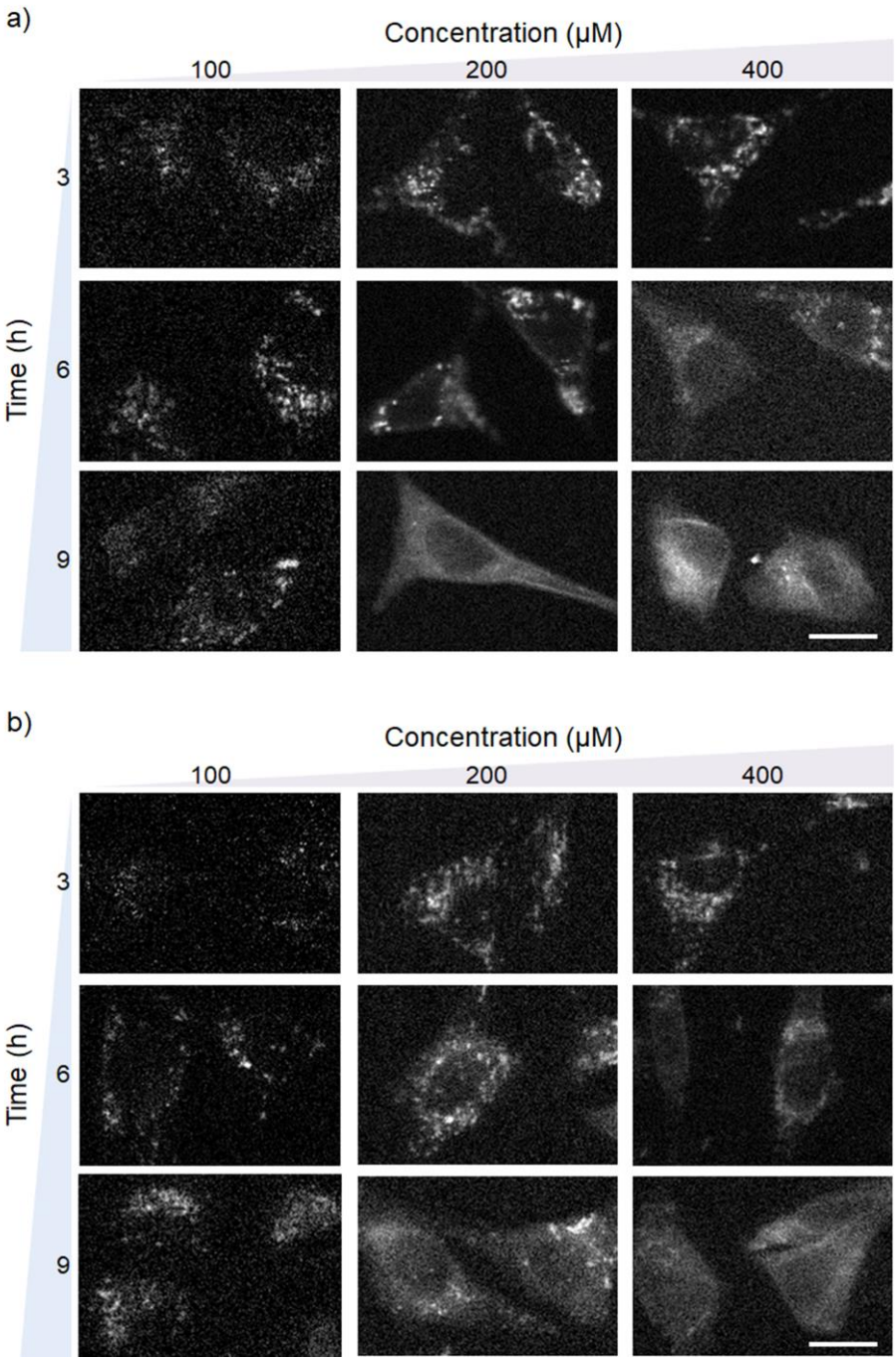


Figure S7 Raman images of H-alkyne or D-alkyne in HeLa cells treated with **3** or D-**3** at different doses (100, 200, or 400 μM) and times (3, 6, or 9 h). Raman images were obtained from the Raman intensity of the a) H-alkyne of **3** detected at 2120 cm^{-1} and b) D-alkyne of D-**3** detected at 1987 cm^{-1}. Scale bars are 10 μm.

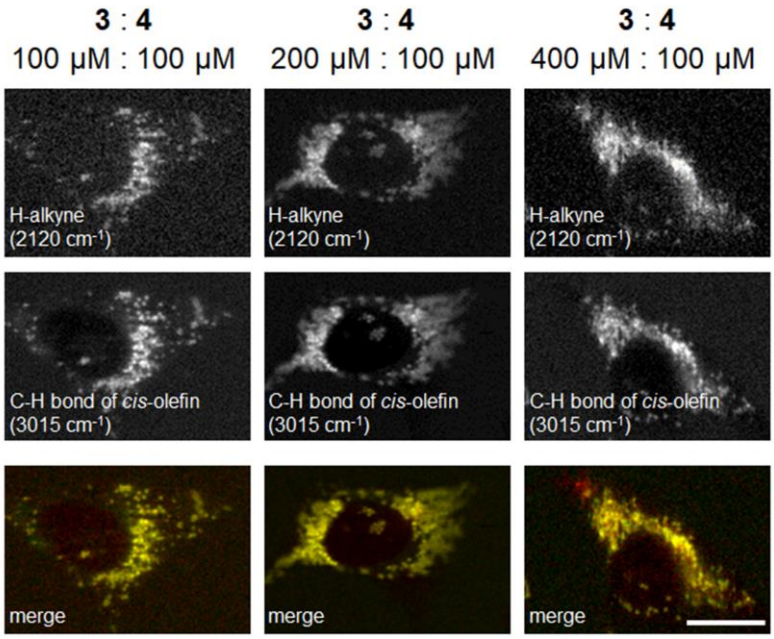


Figure S8 Raman images of the H-alkyne and C-H bonds of *cis*-olefin in HeLa cells treated with **3** and **4** at different dose ratios (1:1, 2:1, or 4:1) for 6 h. Images were obtained from the Raman intensity of the H-alkyne detected at 2120 cm^{-1} and the C-H bonds of *cis*-olefin detected at 3015 cm^{-1}. Two-color Raman images are the overlapped Raman images of the H-alkyne (red) and the C-H bond of *cis*-olefin (green). Scale bars are 10 μm.

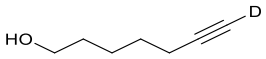
Chemistry

General

^{1}H, ^{2}H, and ^{13}C NMR spectra were recorded using a JNM-ECS 400 spectrometer and JNM-ECA 500 spectrometer (JEOL Inc., Japan). ESI-HRMS was taken on a micrOTOF-QII-$_{RSL}$ (Bruker Daltonics Inc., USA). APCI-HRMS was taken on a Synapt G2 (Waters corp., USA). Column chromatography was performed using silica gel 60 (40-100 μm) purchased from Kanto Chemical Co., Ltd. (Japan). Chemical reagents and solvents were purchased from Tokyo Chemical Industry Co., Ltd. (Japan), FUJIFILM Wako Pure Chemical Corp. (Japan), Nacalai Tesque, Inc. (Japan), and Sigma-Aldrich Japan, Inc. (Japan). 17-octadecynoic acid (**3**) and (9Z)-9-octadecen-17-ynoic acid (**5**) were purchased from Cayman Chemical Co., Inc. (USA), and oleic acid (**4**) was purchased from Tokyo Chemical Industry Co., Ltd. (Japan).

Synthesis of deuterated terminal alkynes

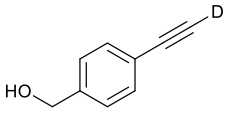
Deuterated 6-heptyn-1-ol (D-**1**)



To a solution of 6-heptyn-1-ol (**1**, 184.5 mg, 1.645 mmol) in dry ethanol-d_1 (6.0 mL) was added D_2O (1.0 mL) and 1 N NaOD (1.0 mL, in D_2O), and then the solution was stirred at room temperature overnight. After stirring overnight, the solvent was evaporated, and ethanol-d_1 (6.0 mL) and D_2O (2.0 mL) were added to the dried residue. The solution was stirred again overnight, and the same operation was repeated twice. The resulting solution was cooled to 0 °C, and then 1 N $KDSO_4$ (1.2 mL, in D_2O) was added. After the solution was concentrated, the residue was purified by column chromatography (EtOAc:hexane = 1:4 to 1:1) to yield the desired alkyne (D-**1**, 96.0 mg, 0.848 mmol, 52%) as a colorless oil.

^{1}H NMR (400 MHz, $CDCl_3$): δ 3.65 (t, *J* = 6.4 Hz, 2H), 2.21 (t, *J* = 6.8 Hz, 2H), 1.63-1.45 (m, 6H), 1.39 (s, 1H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 84.1 (t, *J* = 7.7 Hz, 1C), 68.2 (t, *J* = 38.1 Hz, 1C), 62.9, 32.3, 28.3, 25.0, 18.4. ^{2}H NMR (77 MHz, $CHCl_3$): 1.94 (br s, 1H). HRMS (APCI): Calculated for $C_7H_{11}DO^+$ $[M+H]^+$, 114.1024: found, 114.1028.

Deuterated 4-ethynylbenzyl alcohol (D-**2**)

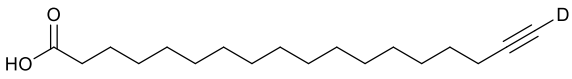


To a solution of 4-ethynylbenzyl alcohol (**2**, 131.2 mg, 0.993 mmol) in dry ethanol-d_1 (7.5 mL) was added D_2O (1.0 mL) and 1 N NaOD (1.5 mL, in D_2O), and then the solution was stirred at room temperature overnight. The solvent was then evaporated, and ethanol-d_1 (7.5 mL) and D_2O (2.5 mL)

were added to the dried residue. The solution was stirred again overnight, and solvent was evaporated; this same procedure was repeated twice more. The final solution was cooled to 0 °C and then 1 N $KDSO_4$ (2mL, in D_2O) was added. After the solution was concentrated, the residue was purified by column chromatography (EtOAc:hexane = 1:4 to 1:1) to yield the desired alkyne (D-**2**, 130.6 mg, 0.981 mmol, 99%) as a white solid.

^{1}H NMR (400 MHz, methanol-d_4): δ 7.43 (d, *J* = 8.4 Hz, 2H), 7.33 (d, *J* = 8.4 Hz, 2H), 4.60 (s, 2H). ^{13}C NMR (100 MHz, methanol-d_4): δ 143.6, 133.0, 127.8 (2C), 122.5 (2C), 83.9 (t, *J* = 7.7 Hz, 1C), 78.1 (t, *J* = 38.6 Hz, 1C), 64.7. ^{2}H NMR (77 MHz, methanol): 3.39 (br s, 1H). HRMS (ESI): Calculated for $C_9H_6DO^-$ $[M-H]^-$, 132.0565: found, 132.0566.

Octadec-17-ynoic-18-d acid (D-**3**)



To a solution of 17-octadecynoic acid (**3**, 9.8 mg, 35.9 μmol) in dry ethanol-d_1 (1.0 mL) was added D_2O (450 μL) and 1 N NaOD (50 μL, in D_2O), and then the solution was warmed to 40 °C and stirred overnight. After stirring overnight, the solvent was evaporated, and ethanol-d_1 (600 μL) and D_2O (300 μL) were added to the dried residue. The solution was then warmed again at 40 °C, stirred overnight, and the solvent was evaporated; this same operation was repeated twice. The resulting solution was cooled to 0 °C, and then 1 N $KDSO_4$ (100 μL, in D_2O) was added. After the solution was concentrated, the residue was purified by column chromatography (EtOAc:hexane = 1:4) to yield the desired alkyne (D-**3**, 9.8 mg, 0.848 mmol, 99%) as a white solid.

^{1}H NMR (400 MHz, $CDCl_3$): δ 2.35 (t, *J* = 7.4 Hz, 2H), 2.18 (t, *J* = 7.0 Hz, 2H), 1.67-1.59 (m, 2H), 1.56-1.48 (m, 2H), 1.40-1.25 (m, 22H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 179.9, 84.5 (t, ***J*** = 7.3 Hz, 1C), 67.9 (t, ***J*** = 37.6 Hz, 1C), 34.1, 29.8 (3C), 29.8 (2C), 29.8, 29.7, 29.6, 29.4, 29.3, 29.2, 28.9, 28.7, 24.8. ^{2}H NMR (77 MHz, $CHCl_3$): 1.93 (br s, 1H). HRMS (ESI): Calculated for $C_{18}H_{30}DO_2^-$ $[M-H]^-$, 280.2392: found, 280.2390.

Biology

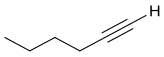
Cell culture

HeLa cells were maintained in Dulbecco's modified Eagle's medium supplemented with 100 U/mL penicillin, 100 μg/mL streptomycin, and 10% heat-inactivated fetal bovine serum (FBS). The cells were grown in a humidified incubator at 37 °C under 5% CO_2/95% air.

Raman experiments

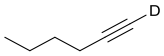
Density functional theory calculation method

DFT calculations were done by using "Gaussian 16" software.[2] The calculations (geometry optimizations and frequency calculations) of 1-hexyne/D-1-hexyne were done in water by using the B3LYP functional and the 6-31G* basis set. Final geometries of structure follow.



(C≡C vibrational frequency: 2120 cm^{-1})

C1	-2.8811452	-0.497967	0.0000102
C2	-1.6923752	0.4684373	-0.0001685
C3	-0.3391783	-0.2517399	0.0001258
C4	0.8507237	0.731886	0.000085
C5	2.1538408	0.0625163	0.0000746
C6	3.2204756	-0.5076774	-0.0001149
H7	-3.8336728	0.0439821	-0.00022
H8	-2.8647596	-1.1456779	-0.8851171
H9	-2.864903	-1.145175	0.8855074
H10	-1.7529609	1.1256167	0.8786768
H11	-1.752832	1.1251327	-0.8793828
H12	-0.2647647	-0.9040426	-0.8797705
H13	-0.2649664	-0.9036813	0.8803099
H14	0.7804759	1.3891677	0.8778825
H15	0.7804158	1.3891707	-0.8777081
H16	4.163919	-1.0072244	-0.0002513



(C≡C vibrational frequency: 1972 cm^{-1})

C1	-2.8811435	-0.4979682	0.0000099
C2	-1.6923748	0.4684372	-0.0001666
C3	-0.3391778	-0.2517395	0.0001247
C4	0.8507232	0.7318875	0.0000843
C5	2.1538404	0.0625183	0.0000728
C6	3.2204734	-0.5076795	-0.0001132
H7	-3.8336705	0.0439811	-0.0002171
H8	-2.8647589	-1.1456759	-0.8851196
H9	-2.8649002	-1.1451793	0.8855048
H10	-1.7529606	1.1256142	0.8786804
H11	-1.7528328	1.1251351	-0.8793791
H12	-0.2647644	-0.9040403	-0.879773
H13	-0.2649642	-0.9036826	0.8803074
H14	0.780475	1.389169	0.8778818
H15	0.7804154	1.3891719	-0.8777088
D16	4.1639156	-1.0072291	-0.0002491

Raman spectra of alkynes (Figure S1 and S5)

Raman spectra were obtained using a RAMAN-11 slit-scanning Raman microscope (Nanophoton Corp., Japan) at 532 nm excitation. The alkynes were placed on a quartz substrate during the measurements. The laser output was focused on each sample by a 60×/1.27 numerical aperture (NA) water immersion objective lens (CFI Plan Apo IR 60X WI, NIKON Corp., Japan). The slit width of the spectrograph was 50 μm. The exposure time for each line was 10 s. The light intensity at the sample plane was 1.5 mW/μm^2. Raman spectra of alkynes were obtained from an average intensity of 900 (X × Y = 30 × 30 or 60 × 15) pixels.

Molar ratio vs. Raman peak area ratio of H-alkyne and D-alkyne (Figure S2)

Solutions containing several proportions of H-alkyne and D-alkyne were obtained by blending 100 mM H-alkynes in DMSO and 100 mM D-alkynes in DMSO. Raman spectra of the solutions were measured using a 532 nm line laser. The exposure time for each line was 10 s, and the light intensity at the sample plane was 3.0 mW/μm^2. The obtained spectra were subtracted from the background of DMSO. The peak areas of the D-alkyne and H-alkyne were computed using "Raman Viewer" software equipped on the Raman microscope. Finally, a correlation curve was calculated from the Raman peak area ratio between H-alkyne and D-alkyne, and the molar ratio of H-alkyne and D-alkyne in the solutions.

Analysis of the H/D exchange rate (Figure 2, S3 and S4)

A 1 M DMSO stock solution of alkynes was dissolved in 25 mM phosphate buffer (pH 6.0, 7.0, or 8.0) or 25mM deuterated phosphate buffer (pD 6.0, 7.0, or 8.0). The sample solutions (20 mM) were maintained at 4-37 °C in a block incubator (BI-516S, Astec Co., Ltd., Japan). Raman spectra of the sample solutions were measured using a 532 nm line laser. The exposure time for each line was 10 s, and the light intensity at the sample plane was 3.0 mW/μm^2. The obtained spectra were subtracted from the background of the buffers containing 2% DMSO. The peak areas of D-alkyne and H-alkyne were computed using "Raman Viewer" software equipped on the Raman microscope. Finally, the conversion ratio was calculated from the Raman peak area ratio between the D-alkyne and H-alkyne at different reaction times.

Raman imaging experiments of HeLa cells treated with C18 fatty acids (Figure 4, 5, S7 and S8)

HeLa cells (2.4 mL, 5.0 × 10^4 cells/mL) were placed on a sterilized Φ25 mm quartz substrate in a TPP tissue culture dish (No. 93040; TPP Techno Plastic Products AG, Switzerland). After overnight incubation, the fatty acid stock solutions (50 mM in EtOH) were added and incubated for different durations (3, 6, 9, 12, or 24 h). The medium was replaced with Tyrode's buffer (150 mM NaCl, 10 mM glucose, 10 mM HEPES, 4 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, and 4 mM NaOH) after washing with

phosphate buffered saline (PBS) (-). A quartz substrate was used to observe the Raman spectra with a RAMAN-11 slit-scanning Raman microscope at 532 nm excitation. The laser output was focused onto the sample using a 60×/1/.27 NA water immersion objective lens. The slit width of the spectrograph was 50 μm. The exposure time for each line was 10 s. The light intensity at the sample plane was calculated as 3.0 $mW/\mu m^2$.

To obtain the Raman images, the Raman spectral data set was further processed using the singular value decomposition (SVD) technique for noise reduction.[3] We selected several spectral regions (705-3105 cm^{-1} for the C-H bond of *cis*-olefin, 1900-2050 cm^{-1} for the C≡C bond of D-alkyne, and 2050-2200 cm^{-1} for the C≡C bond of H-alkyne) in the calculation procedure for SVD to avoid artifacts in the constructed images. A modified polyfit technique was then used at each pixel to determine the autofluorescence baseline signal, which was subtracted from the original Raman spectrum.[1] Finally, the Raman images were reconstructed using each vibrational band of interest. All data processing was performed using "Raman Viewer" image processing software (Nanophoton Corp., Japan).

References

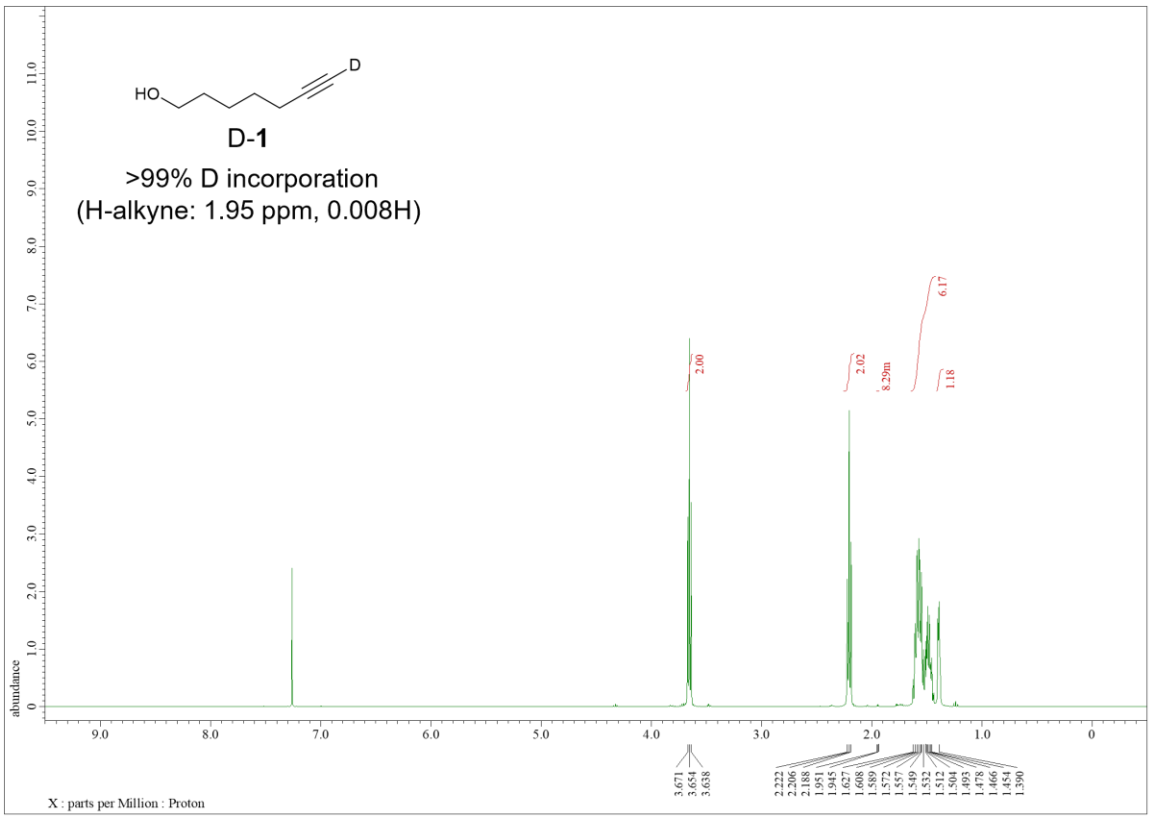
1 A. Mahadevan-Jansen and C. A. Lieber, *Appl. Spectrosc.*, 2003, **57**, 1363–1367.

2 Gaussian 16, Revision C.01, M. J. Frisch, G. W. Trucks, H. B. Schlegel, G. E. Scuseria, M. A. Robb, J. R. Cheeseman, G. Scalmani, V. Barone, G. A. Petersson, H. Nakatsuji, X. Li, M. Caricato, A. V. Marenich, J. Bloino, B. G. Janesko, R. Gomperts, B. Mennucci, H. P. Hratchian, J. V. Ortiz, A. F. Izmaylov, J. L. Sonnenberg, D. Williams-Young, F. Ding, F. Lipparini, F. Egidi, J. Goings, B. Peng, A. Petrone, T. Henderson, D. Ranasinghe, V. G. Zakrzewski, J. Gao, N. Rega, G. Zheng, W. Liang, M. Hada, M. Ehara, K. Toyota, R. Fukuda, J. Hasegawa, M. Ishida, T. Nakajima, Y. Honda, O. Kitao, H. Nakai, T. Vreven, K. Throssell, J. A. Montgomery, Jr., J. E. Peralta, F. Ogliaro, M. J. Bearpark, J. J. Heyd, E. N. Brothers, K. N. Kudin, V. N. Staroverov, T. A. Keith, R. Kobayashi, J. Normand, K. Raghavachari, A. P. Rendell, J. C. Burant, S. S. Iyengar, J. Tomasi, M. Cossi, J. M. Millam, M. Klene, C. Adamo, R. Cammi, J. W. Ochterski, R. L. Martin, K. Morokuma, O. Farkas, J. B. Foresman, and D. J. Fox, Gaussian, Inc., Wallingford CT, 2016.

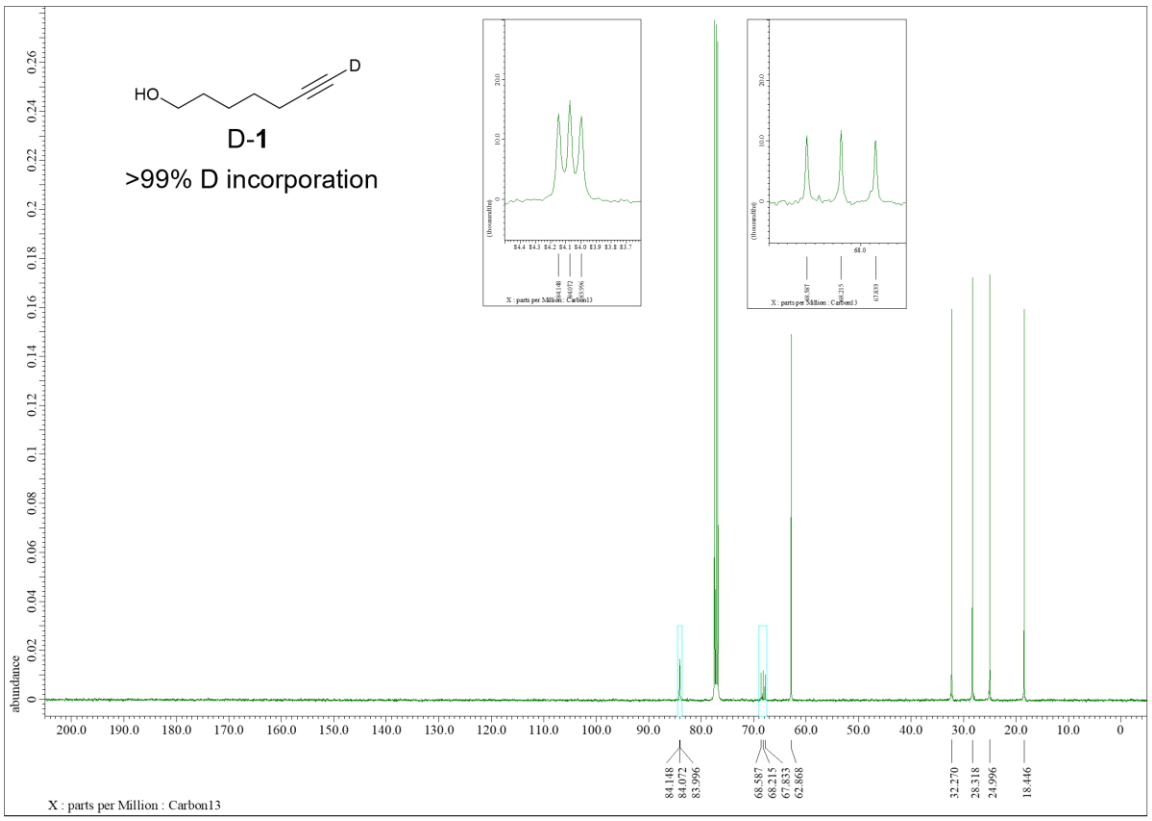
3 H. J. Van Manen, Y. M. Kraan, D. Roos and C. Otto, *J. Phys. Chem. B*, 2004, **108**, 18762–18771.

NMR spectra of deuterated terminal alkynes:

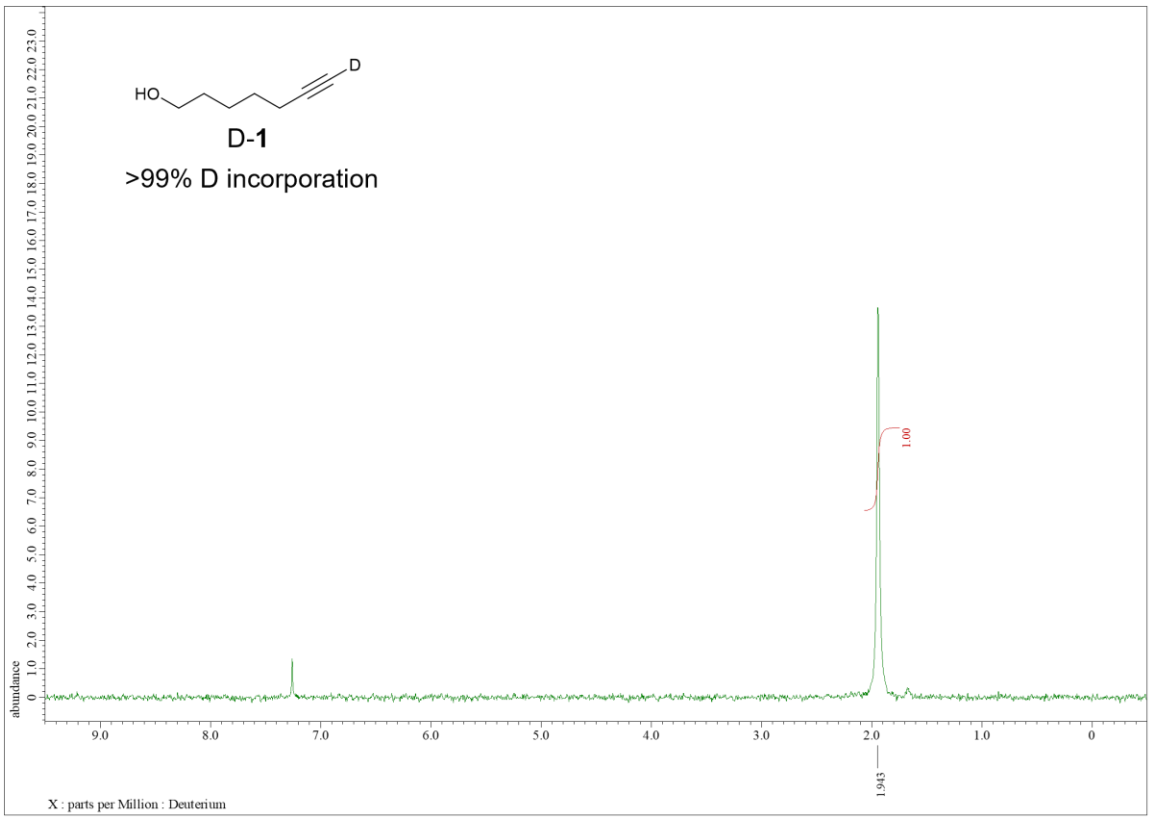
^{1}H NMR of D-**1**



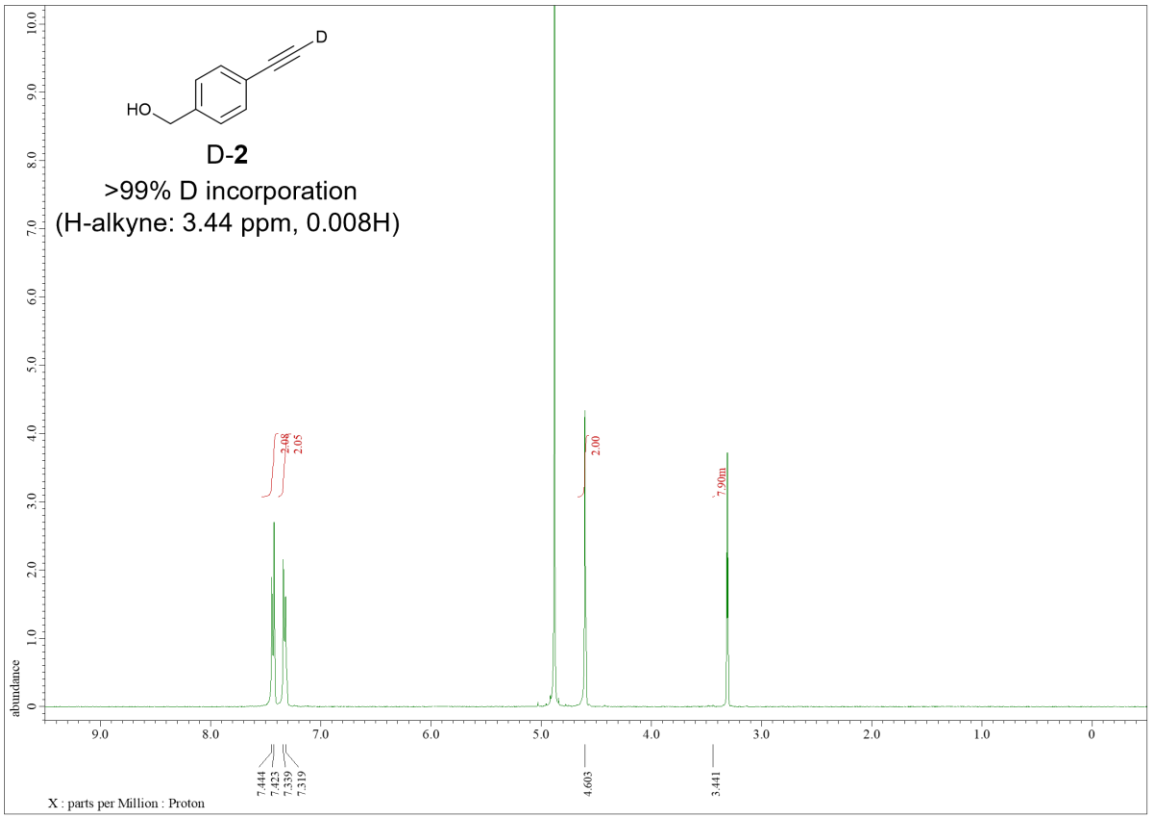
^{13}C NMR of D-**1**



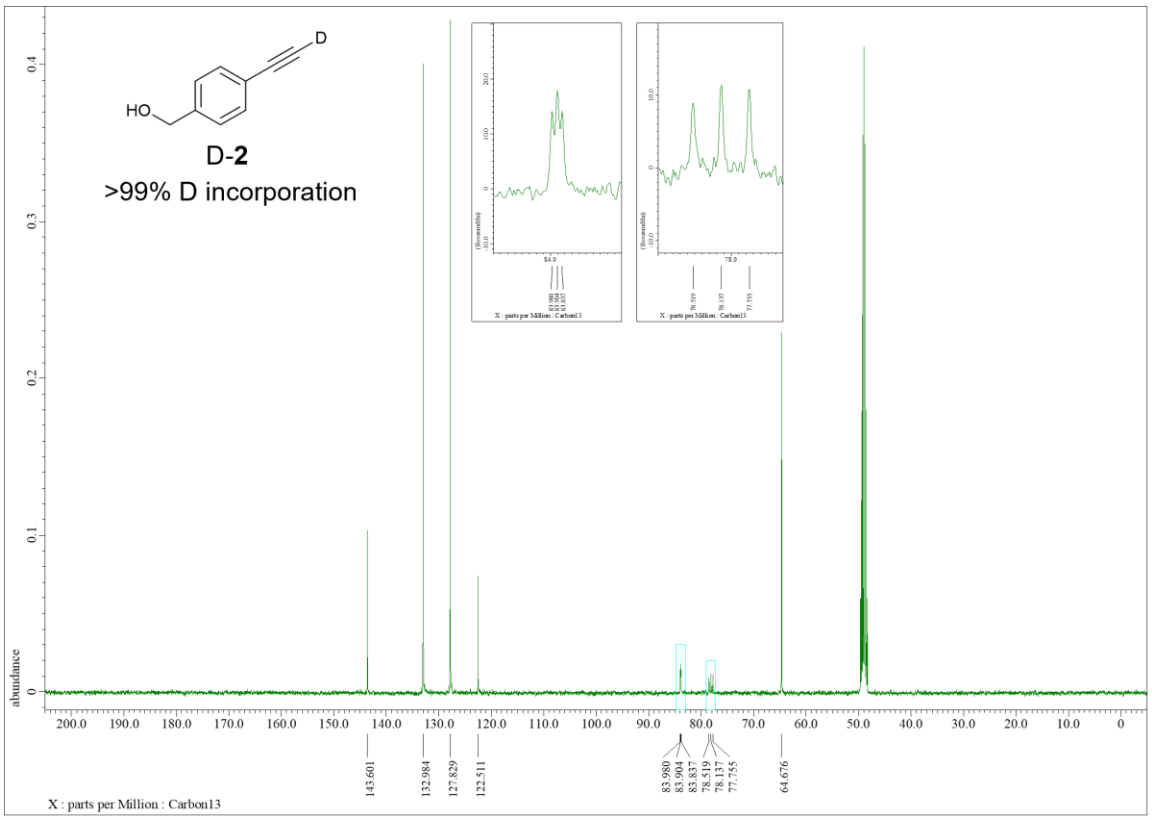
^{2}H NMR of D-**1**



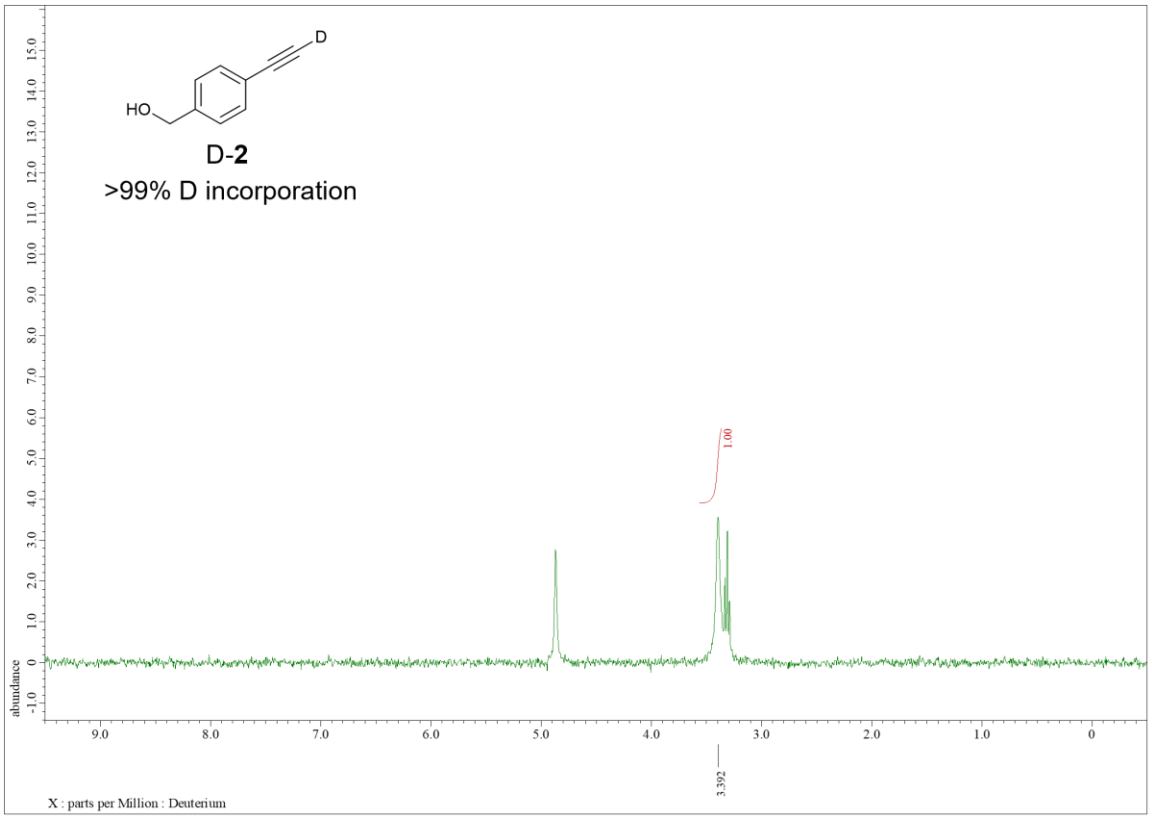
^{1}H NMR of D-**2**



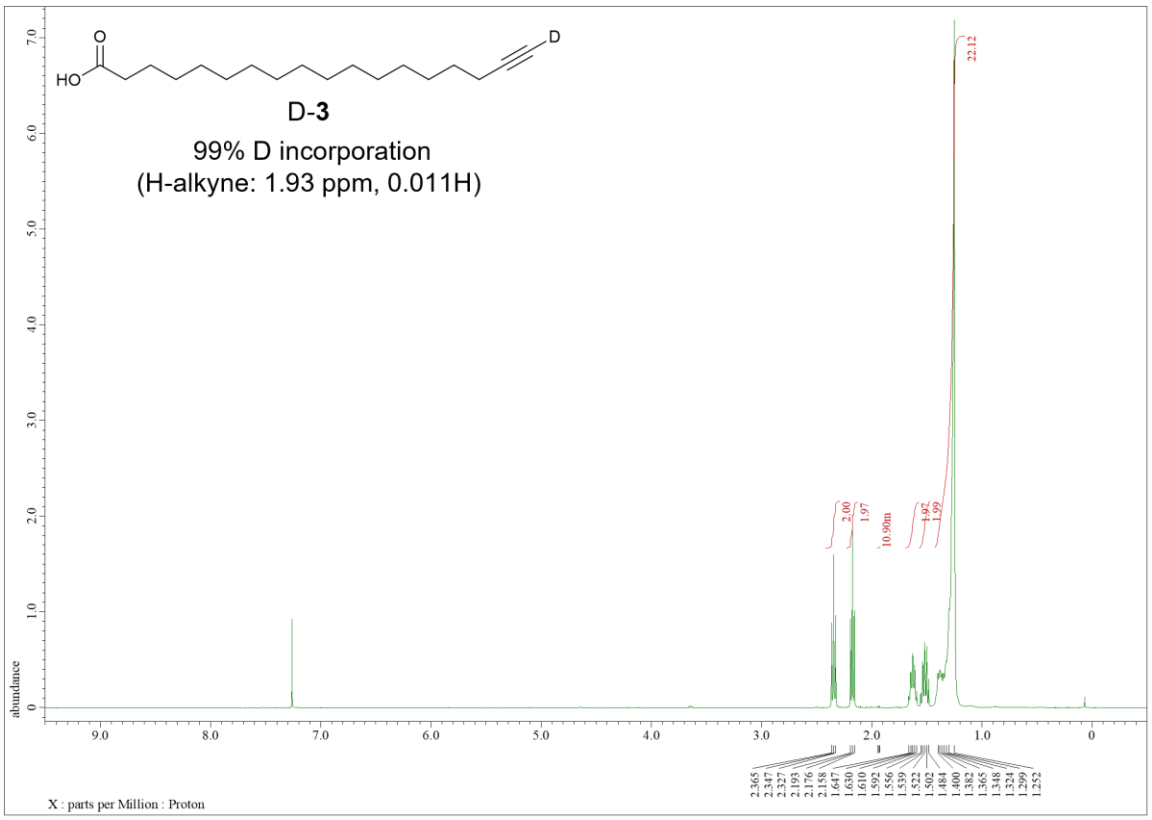
^{13}C NMR of D-**2**



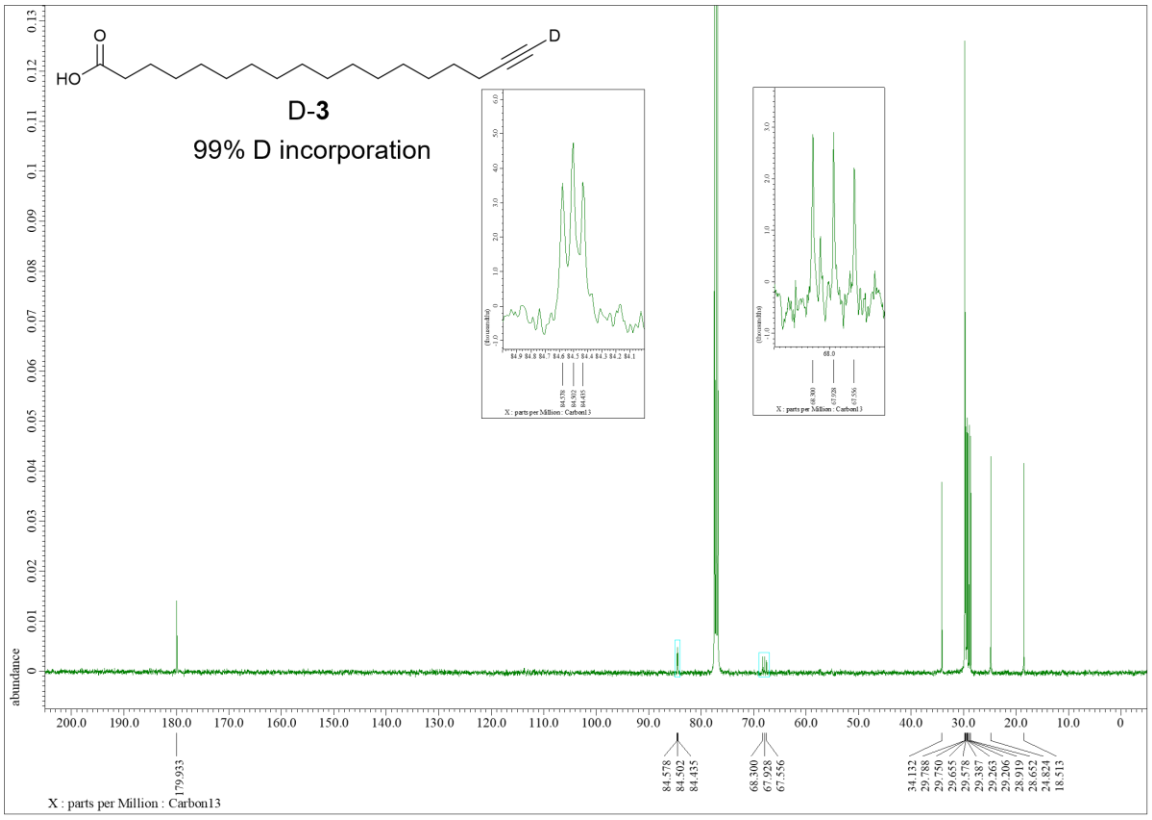
^{2}H NMR of D-**2**



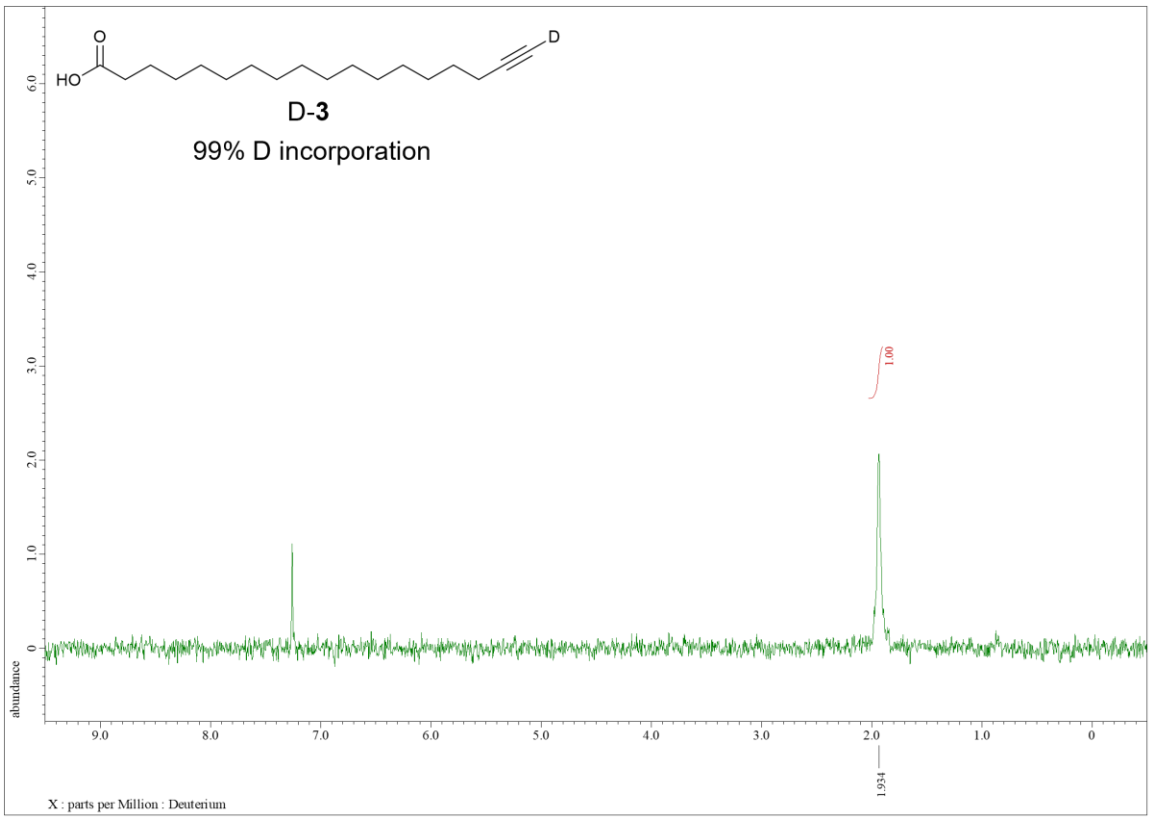
^{1}H NMR of D-**3**



^{13}C NMR of D-**3**



^{2}H NMR of D-**3**


O
HO
D
D-3
99% D incorporation
abundance
6.0
5.0
4.0
3.0
2.0
1.0
0
1.00
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0
1.934
X : parts per Million : Deuterium