Electronic Supplementary Material (ESI) for RSC Advances.


**Supporting Information**

**for**

# Tetrafluoroaryl azide as an *N*-terminal capping group for click-to-dissolve diphenylalanine hydrogels

**Sumit Dadhwal, Jessica M. Fairhall, Sarah Hook, Allan B. Gamble***

*School of Pharmacy, University of Otago, Dunedin, 9054, New Zealand*

**allan.gamble@otago.ac.nz*

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## 1. Supporting Figures

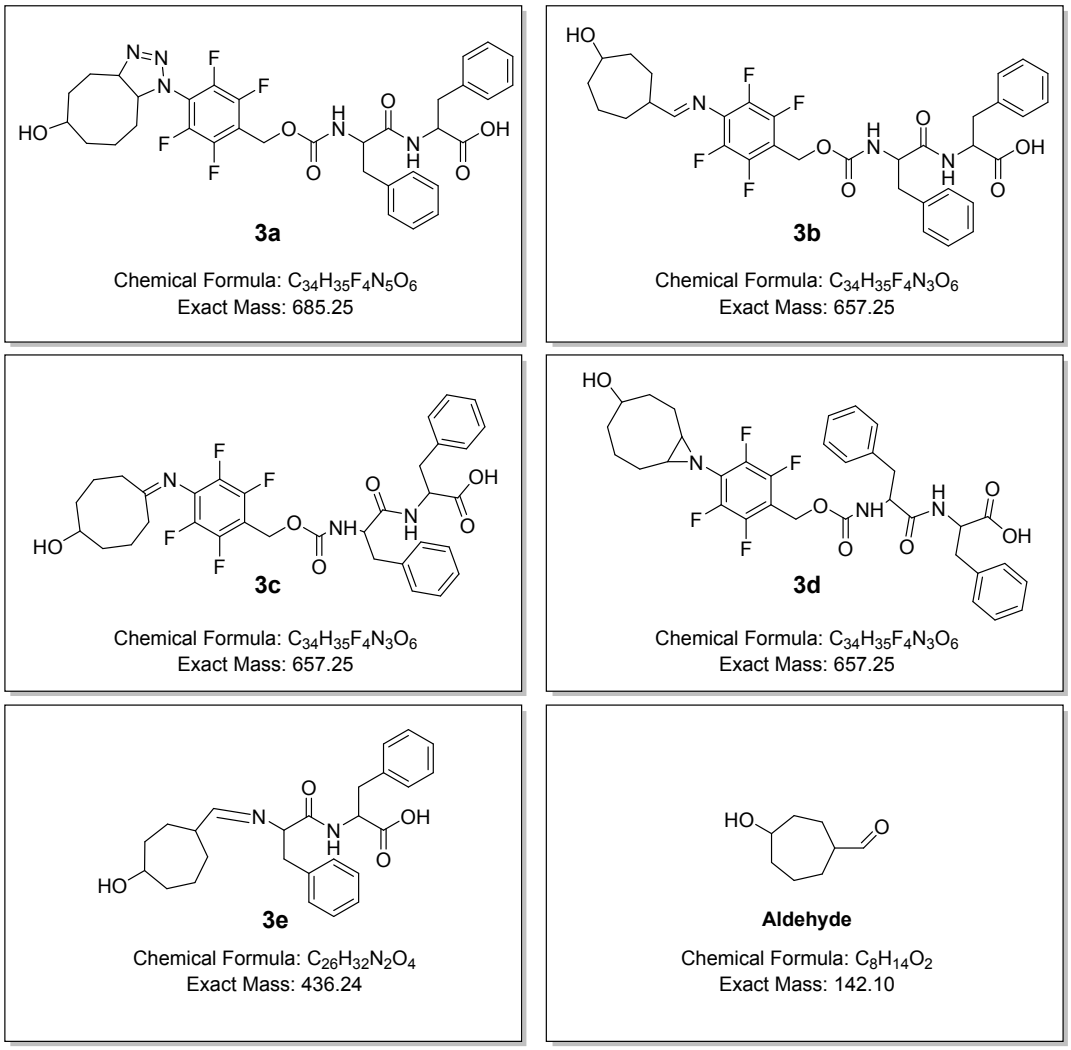



**Figure S1.** Structures of intermediates for the TCO-azide 1,3-dipolar cycloaddition. Molecular masses are shown for comparison in TCO induced gel-to-sol transition studies of $AzF_4$-PhePhe **3** hydrogels (HRMS spectra shown in Figures S13-S16 and S18).

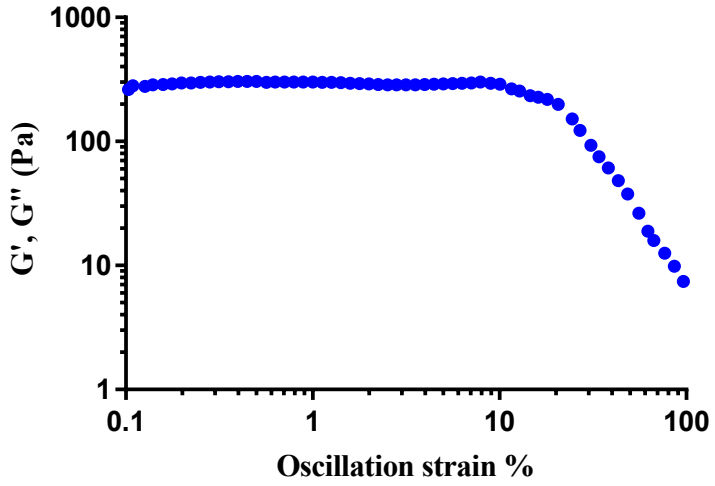



**Figure S2.** Linear viscoelastic region (LVER) of hydrogel (0.1 wt%) measured from 0.1 to 100% oscillation strain at 25 °C.

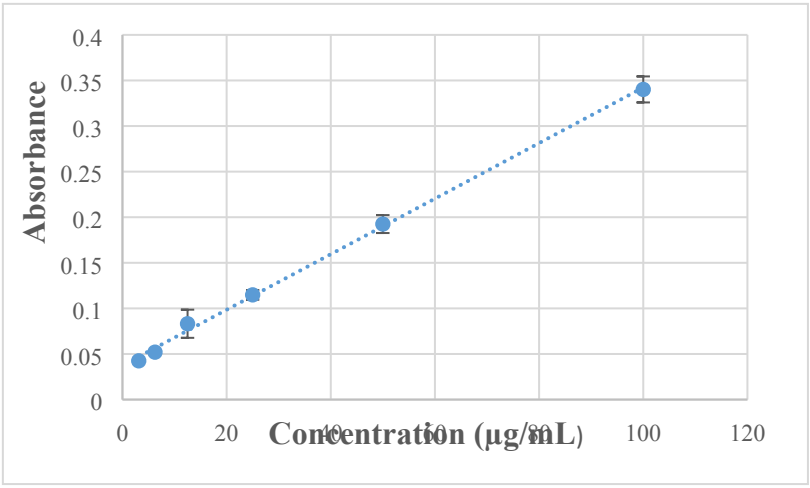



**Figure S3.** Doxorubicin standard curve measured at 485 nm. Error bars generated from triplicate data (n=3).

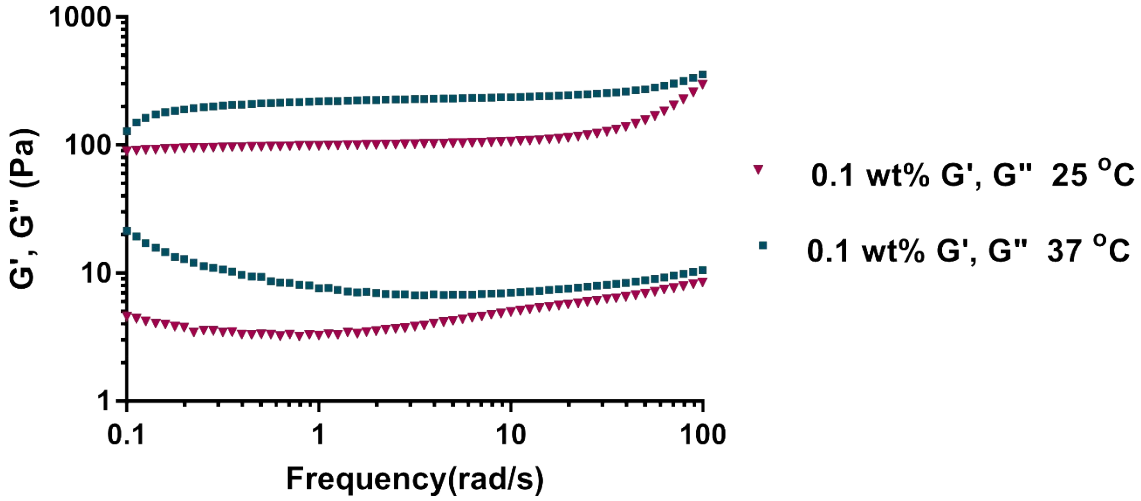



**Figure S4.** Frequency sweep measurements of hydrogel (0.1 wt%) with a fixed 1.0% strain at 25 °C and 37 °C.

**Figure S5**. Photograph of 0.1 wt% $AzF_4$-PhePhe **3** (with 5% DMSO) in buffer (PBS; final pH 3.9).

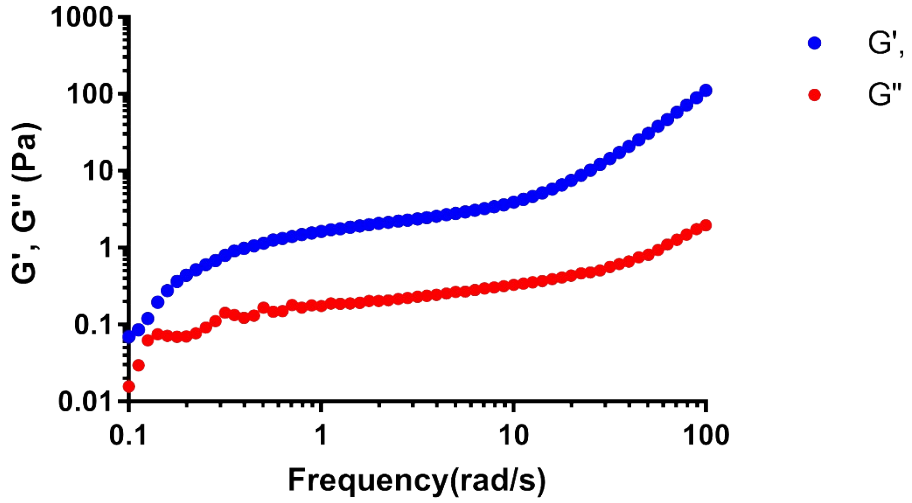



**Figure S6.** Frequency sweep measurements of $AzF_4$-PhePhe **3** (0.1 wt%) in PBS (final pH 3.9) with a fixed 1.0% strain at 25 °C.

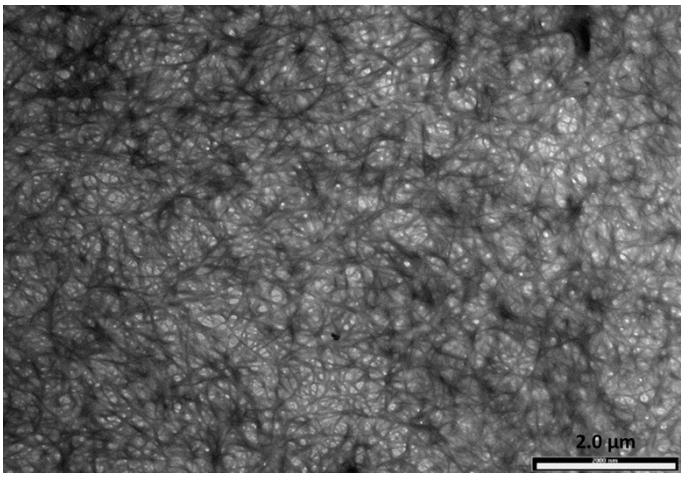



**Figure S7.** Transmission electron (TEM) microscope image of hydrogel formed with water showing fibrous network (Scale bar: 2.0 µm).

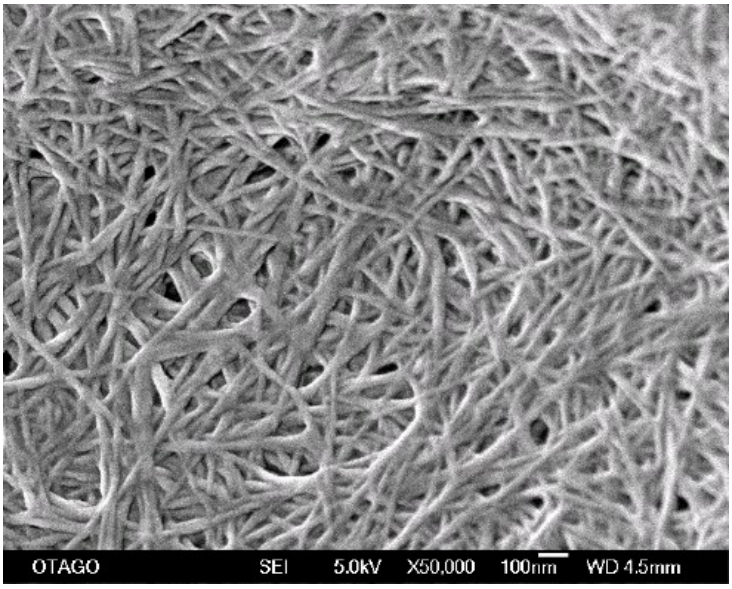



**Figure S8.** Scanning electron microscope (SEM) image of hydrogel formed with water showing fibrous network (White scale bar: 100 nm).

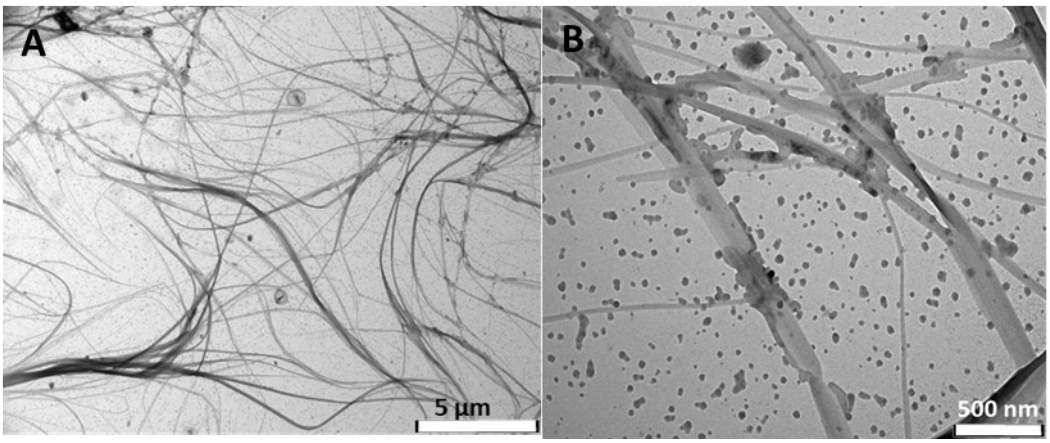



**Figure S9.** Transmission electron microscope (TEM) image of $AzF_4$-PhePhe **3** (0.1 wt% with 5% DMSO) in PBS (final pH 3.9); **A**) immediately after addition of PBS, and **B**) after 24 hours incubation of sample in PBS at 37 °C.

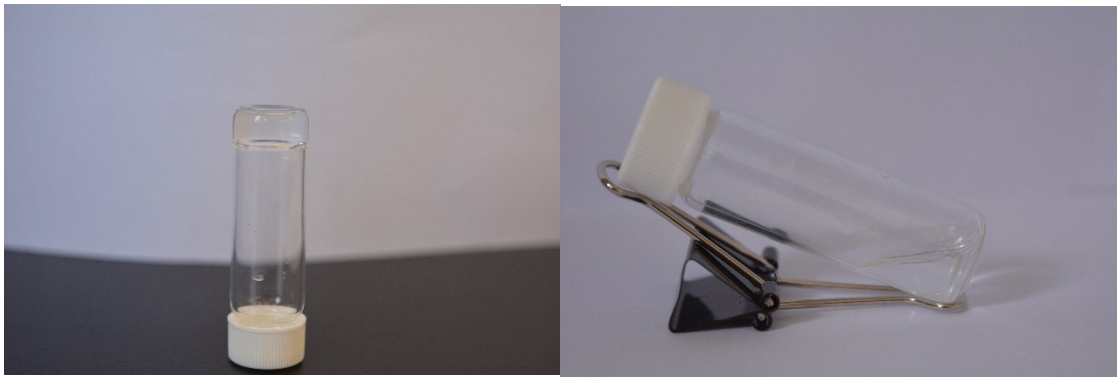


**Figure S10.** Hydrogel of $AzF_4$-PhePhe **3** (0.1 wt%), before (left) and after (right) addition of the trigger (TCO; 5 mM).

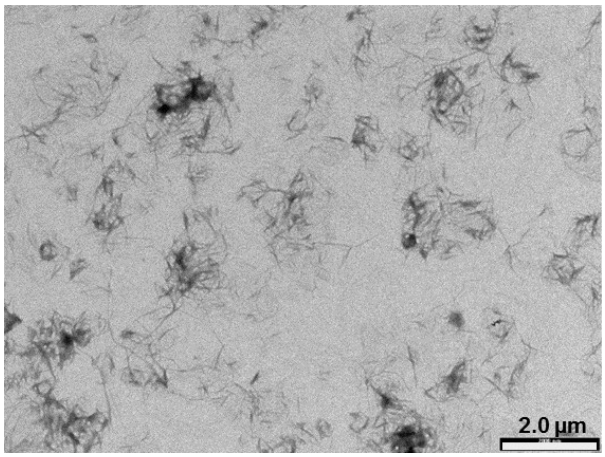



**Figure S11.** Transmission electron microscope (TEM) image of hydrogel (0.1 wt%) with TCO (5 mM) showing broken/disrupted fibrous network (Scale bar: 2.0 µm).

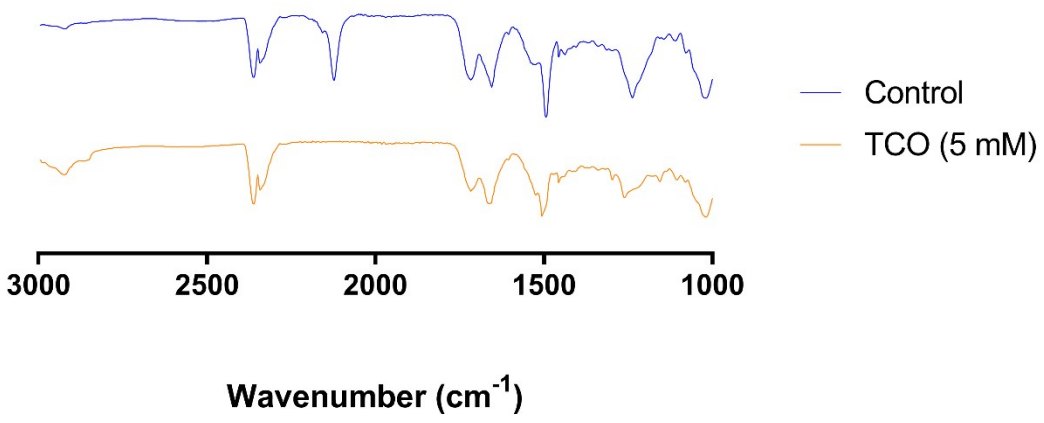
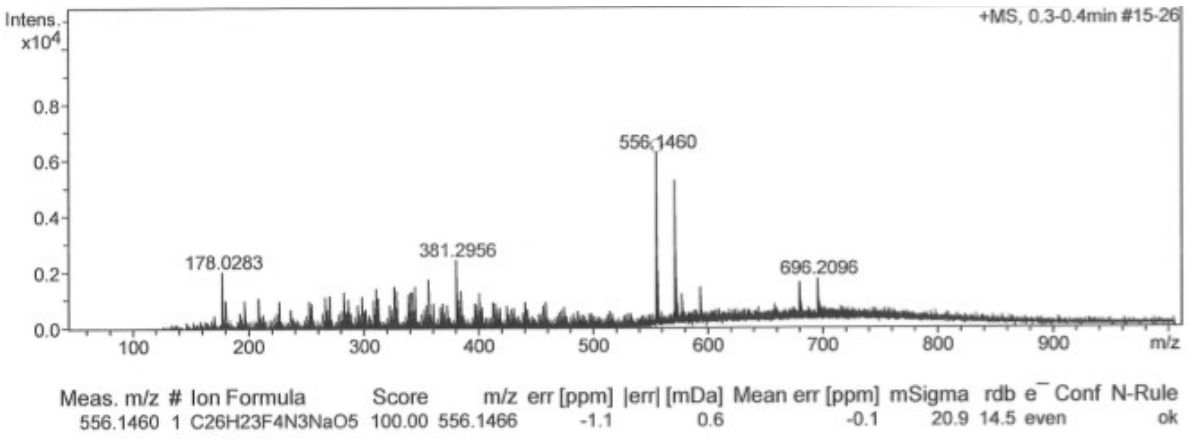
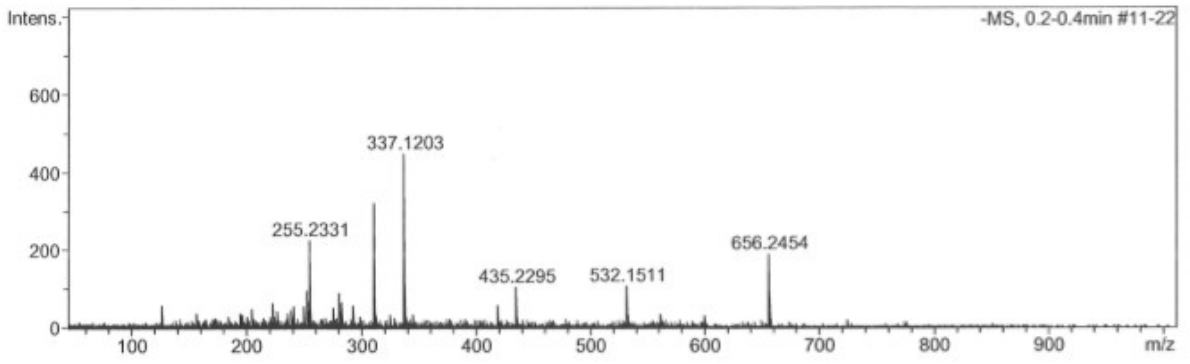



**Figure S12.** ATR-FTIR spectra of hydrogel (0.1 wt%) incubated with the trigger (TCO; 5 mM). After addition of TCO, a rapid loss of the azide peak at 2100 $cm^{-1}$ is evident, indicating that the 1,3-dipolar cycloaddition has transpired.



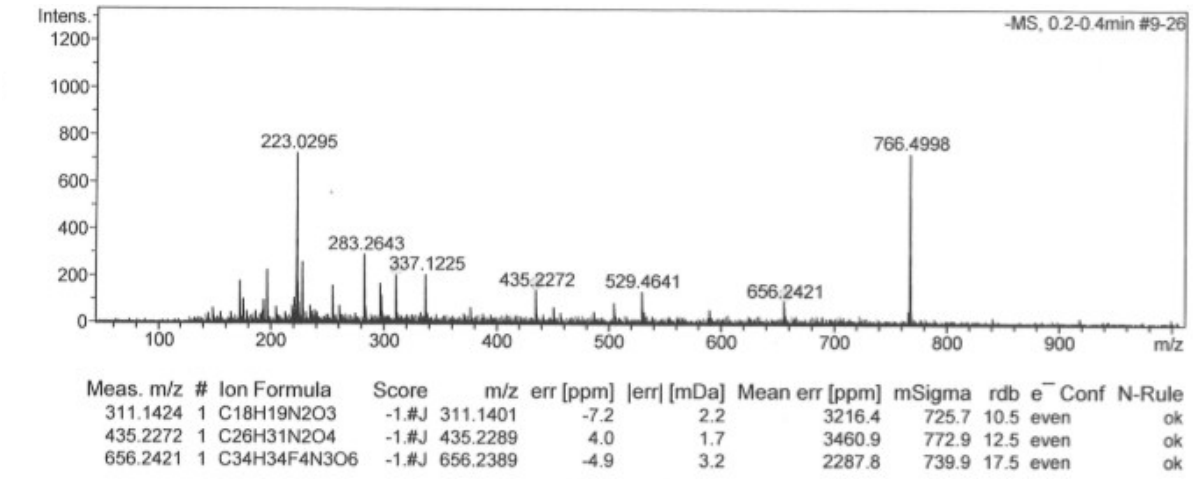

| Meas. m/z | # | Ion Formula | Score | m/z | err [ppm] | \|err\| [mDa] | Mean err [ppm] | mSigma | rdb | e⁻ Conf | N-Rule |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 556.1460 | 1 | C26H23F4N3NaO5 | 100.00 | 556.1466 | -1.1 | 0.6 | -0.1 | 20.9 | 14.5 | even | ok |

**Figure S13.** HRMS (ESI+) of TCO triggered hydrogel (pH 3.7) incubated for 4 hours.



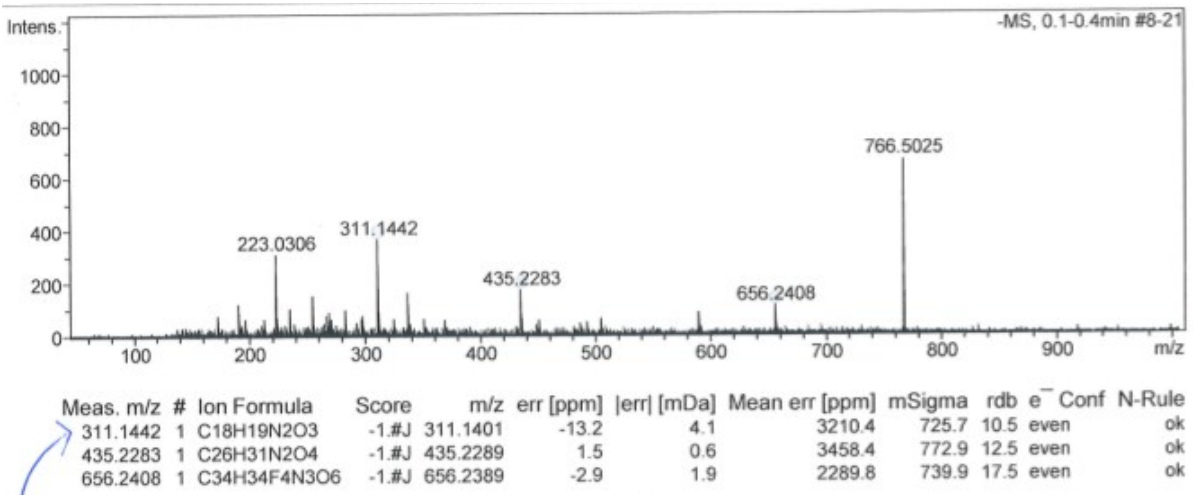

| Meas. m/z | # | Ion Formula | Score | m/z | err [ppm] | \|err\| [mDa] | Mean err [ppm] | mSigma | rdb | e⁻ Conf | N-Rule |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 311.1403 | 1 | C18H19N2O3 | -1.#J | 311.1401 | -0.7 | 0.2 | 3222.9 | 725.7 | 10.5 | even | ok |
| 435.2295 | 1 | C26H31N2O4 | -1.#J | 435.2289 | -1.3 | 0.6 | 3455.6 | 772.9 | 12.5 | even | ok |
| 532.1511 | 1 | C26H22F4N3O5 | -1.#J | 532.1501 | -1.8 | 1.0 | 2825.2 | 771.1 | 15.5 | even | ok |
| 656.2454 | 1 | C34H34F4N3O6 | -1.#J | 656.2389 | -9.9 | 6.5 | 2282.7 | 739.9 | 17.5 | even | ok |

**Figure S14.** HRMS (ESI-) of TCO triggered hydrogel (pH 3.7) incubated for 12 hours.




| Meas. m/z | # | Ion Formula | Score | m/z | err [ppm] | \|err\| [mDa] | Mean err [ppm] | mSigma | rdb | e⁻ Conf | N-Rule |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 311.1424 | 1 | C18H19N2O3 | -1.#J | 311.1401 | -7.2 | 2.2 | 3216.4 | 725.7 | 10.5 | even | ok |
| 435.2272 | 1 | C26H31N2O4 | -1.#J | 435.2289 | 4.0 | 1.7 | 3460.9 | 772.9 | 12.5 | even | ok |
| 656.2421 | 1 | C34H34F4N3O6 | -1.#J | 656.2389 | -4.9 | 3.2 | 2287.8 | 739.9 | 17.5 | even | ok |

**Figure S15.** HRMS (ESI-) of TCO triggered hydrogel (pH 7.4) incubated for 12 hours.




| Meas. m/z | # | Ion Formula | Score | m/z | err [ppm] | \|err\| [mDa] | Mean err [ppm] | mSigma | rdb | e⁻ Conf | N-Rule |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 311.1442 | 1 | C18H19N2O3 | -1.#J | 311.1401 | -13.2 | 4.1 | 3210.4 | 725.7 | 10.5 | even | ok |
| 435.2283 | 1 | C26H31N2O4 | -1.#J | 435.2289 | 1.5 | 0.6 | 3458.4 | 772.9 | 12.5 | even | ok |
| 656.2408 | 1 | C34H34F4N3O6 | -1.#J | 656.2389 | -2.9 | 1.9 | 2289.8 | 739.9 | 17.5 | even | ok |

**Figure S16.** HRMS (ESI-) of TCO triggered hydrogel (pH 6.5) incubated for 12 hours.

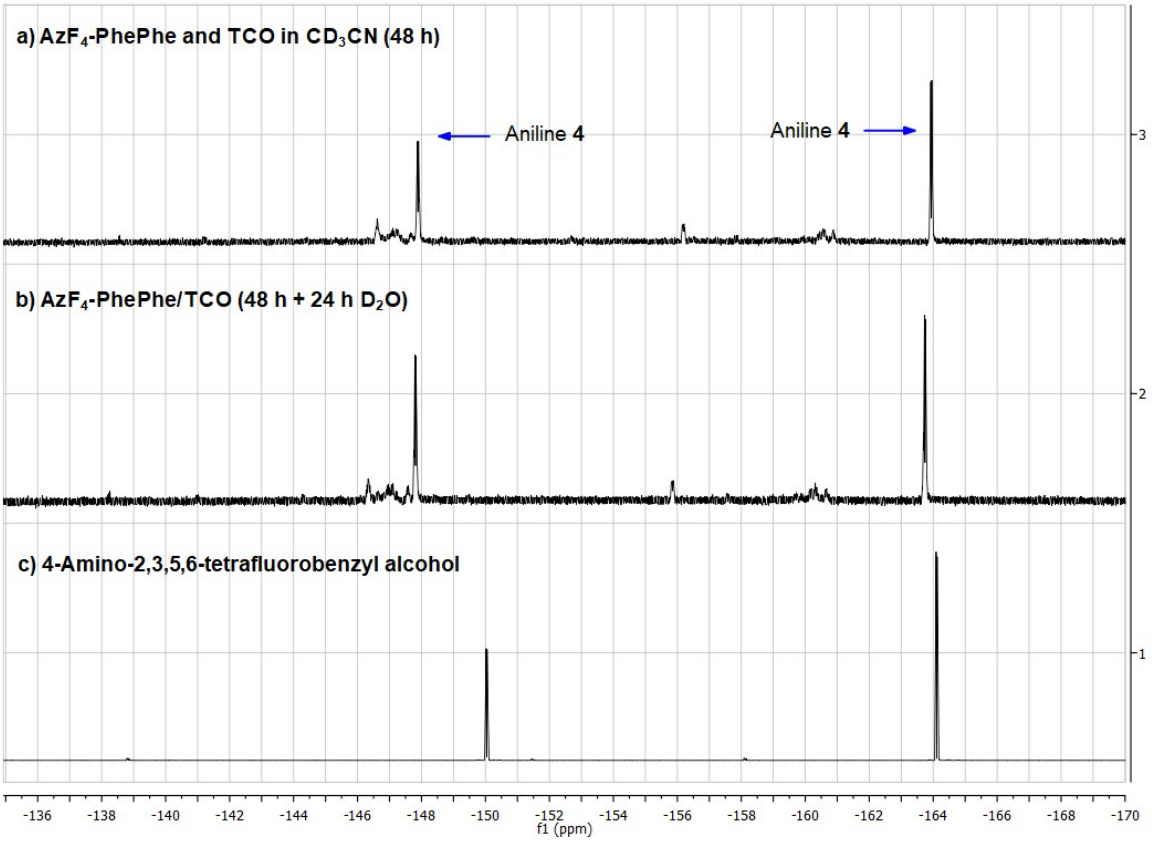
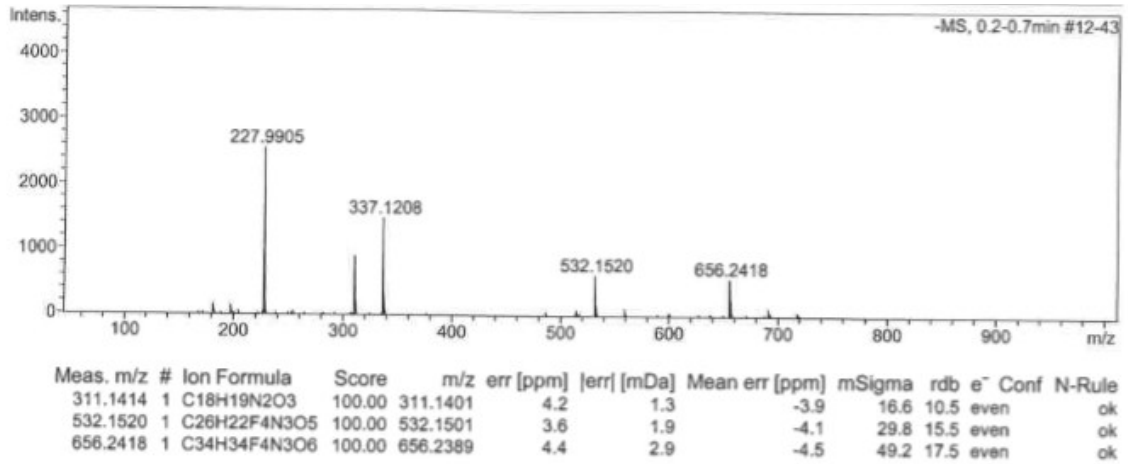



**Figure S17.** $^{19}$F NMR spectra of the 1,3-dipolar cycloaddition reaction (a) after 48 hours in acetonitrile-$d_6$, (b) after addition of 16% $D_2O$ and a further 24 hours of incubation. (c) The $^{19}$F NMR spectrum of 4-amino-2,3,5,6-tetrafluorobenzyl alcohol **6** (synthesis in Section S2). This is the product of the linker that would be expected if the 1,6-self-immolation had occurred under the NMR experimental conditions (generated by reaction of the azaquinone methide with advantageous water).




| Meas. m/z | # | Ion Formula | Score | m/z | err [ppm] | \|err\| [mDa] | Mean err [ppm] | mSigma | rdb | e⁻ Conf | N-Rule |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 311.1414 | 1 | C18H19N2O3 | 100.00 | 311.1401 | 4.2 | 1.3 | -3.9 | 16.6 | 10.5 | even | ok |
| 532.1520 | 1 | C26H22F4N3O5 | 100.00 | 532.1501 | 3.6 | 1.9 | -4.1 | 29.8 | 15.5 | even | ok |
| 656.2418 | 1 | C34H34F4N3O6 | 100.00 | 656.2389 | 4.4 | 2.9 | -4.5 | 49.2 | 17.5 | even | ok |

**Figure S18.** HRMS (ESI-) of NMR sample in $CD_3CN$ after 48 hours of 1,3-dipolar cycloaddition.

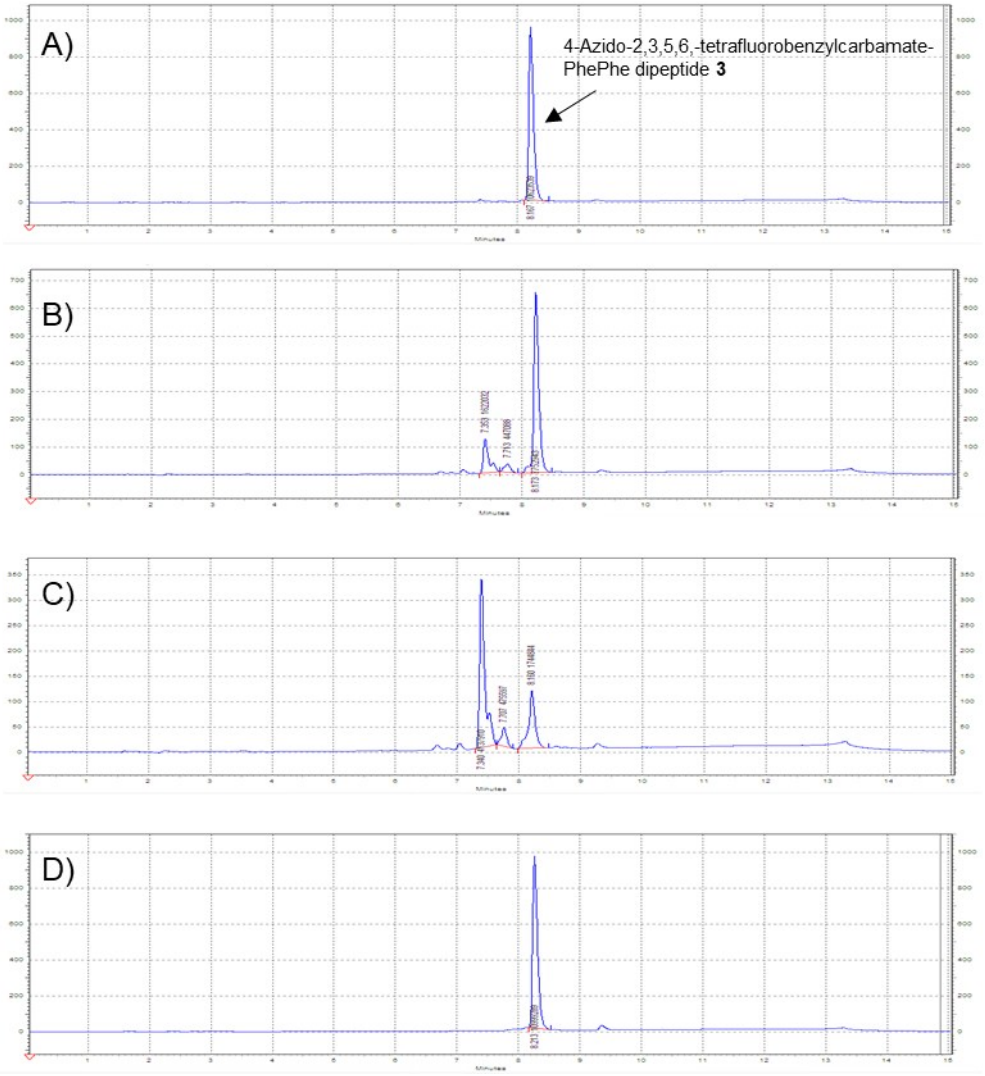



**Figure S19.** Example HPLC trace of the 1,3-dipolar cycloaddition kinetic experiment between compound **3** ($R_T$ = 8.1 min) and *trans*-cyclooctenol (6 mM) at **(A)** 0 min, **(B)** 20 min, **(C)** 100 min, and **(D)** Control 24 hours (no *trans*-cyclooctene). Absorbance is measured at 254 nm, and the area under curve was used for *pseudo* first-order calculations

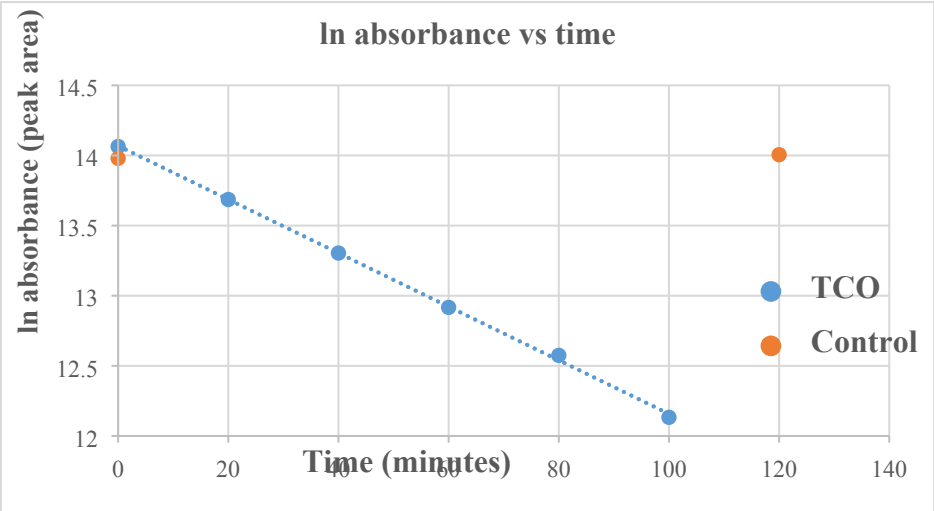
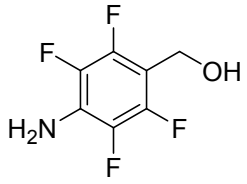



**Figure S20.** *Pseudo* first-order kinetic data obtained for the reaction of compound **3** (0.5 mM) with TCO-major (3 mM). Example of data from one experiment. Second-order rate constant, from triplicate runs, was calculated as 0.0947 ± 0.0098 $M^{-1}s^{-1}$ (n=3).

## 2. Synthesis

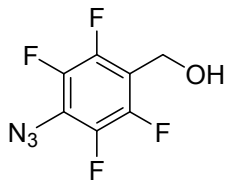
The synthesis of 4-azido-2,3,5,6-tetrafluorobenzyl alcohol **7**[1] *trans*-cyclooct-4-enol (TCO)[2,3] were conducted according to literature procedures.

### Synthesis of 4-amino-2,3,5,6-tetrafluorobenzyl alcohol 6

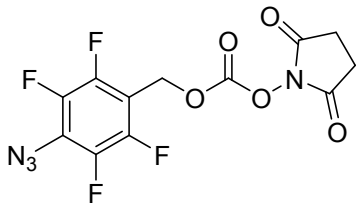



Using our previously reported procedure,[1] a dispersion of $LiAlH_4$ (0.181 g, 4.76 mmol) in dry THF (40 mL) under nitrogen was stirred at 0 °C for 20 min. The benzoic acid **5** (0.5 g, 2.39 mmol) was dissolved in dry THF (10 mL) and added to the $LiAlH_4$ dispersion dropwise over 20 min. After addition of the benzoic acid **5**, the reaction was brought to room temperature and stirred overnight. The progress of the reaction was monitored by TLC, and after completion of the reaction, the mixture was quenched by slow addition of saturated $Na_2SO_4$ at 0 °C on ice. The reaction mixture was passed through Celite and extracted with ethyl acetate. The combined organic layer was washed with water (2 × 100 mL), brine (2 × 100 mL) and dried over $MgSO_4$ and evaporated. The crude reaction mixture was passed through a small plug of silica gel (60% ethyl acetate:hexanes) which provided the title compound as brown amorphous solid (0.255 g, 54%) which was spectroscopically similar to that reported in the literature.[1] $^1$H NMR ($CDCl_3$, 400 MHz) δ 4.73 (t, $J$ = 1.6 Hz, 2H).

### Synthesis of 4-azido-2,3,5,6-tetrafluorobenzyl alcohol 7



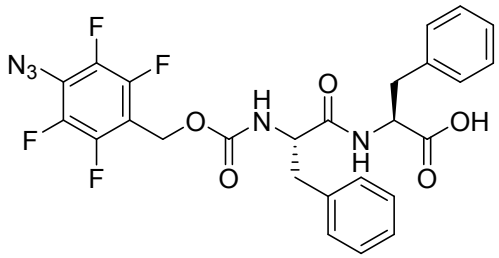

Using our previously reported procedure,[1] benzyl alcohol **6** (0.6 g, 3.07 mmol) and *p*-toluenesulfonic acid (5.2 g, 27.33 mmol) were dissolved in water (15 mL) at 0 °C, followed by slow addition of $NaN_3$ (0.397 g, 6.01 mmol). The reaction mixture was stirred for 10 min followed by addition of $NaNO_2$ (0.844 g, 12.23 mmol) in portions over 15 min. The reaction mixture was stirred for 30 min and the progress of the reaction was monitored by TLC. After completion of the reaction, the pH of the reaction mixture was adjusted to ~8 using saturated $NaHCO_3$, and extracted with ethyl acetate (3 × 50 mL). The combined organic layers were washed with water (2 × 100 mL), brine (2 × 100 mL) and dried over $MgSO_4$ and concentrated under vacuum. The reaction mixture was triturated with hexanes which resulted in a brown solid (0.640 g, 94%), which was spectroscopically similar to that reported in the literature.[1,4] $^1$H NMR ($CDCl_3$, 400 MHz) δ 4.85-4.70 (m, 2H).



**Synthesis of 4-azido-2,3,5,6-tetrafluorobenzylsuccinic carbonate 8**

Synthesised using a previously reported literature procedure.[5,6] To a solution of *N,N'*-disuccinimidyl carbonate (DSC) (0.230 g, 0.898 mmol) in dry acetonitrile (3 mL), was added 4-azido-2,3,5,6-tetrafluorobenzyl alcohol **7** (0.1 g, 0.452 mmol) and triethylamine (TEA) (0.068 g 0.679 mmol). The mixture was stirred under nitrogen at 0 °C for 30 min and then room temperature for an additional 4 h. The reaction mixture was monitored by TLC and after complete consumption of starting material, the solvent was evaporated under vacuum. The crude reaction mixture was dissolved in DCM (50 mL) and washed with water (2 × 100 mL). The organic layer was collected and dried over $MgSO_4$. The crude reaction mixture was loaded on silica gel and subjected to flash silica gel column chromatography (20% ethyl acetate:hexanes) to afford the title compound as a white solid (0.072 g, 44%). $^1$H NMR ($CDCl_3$, 400 MHz): δ 5.42 (s, 2H); 2.85 (s, 4H). $^{13}$C NMR ($CDCl_3$, 100 MHz): 168.7, 151.7, 146.1 (dm, $^1J_{C\text{-}F}$ = 247 Hz), 140.9 ($^1J_{C\text{-}F}$ = 249 Hz), 122.8 (m), 107.7 (t, $J$ = 17.0 Hz), 59.7 (m), 25.9.

**Synthesis of (4-azido-2,3,5,6-tetrafluorobenzyloxycarbonyl)-L-phenylalanyl-L-phenylalanine 3**



Synthesis of **3** was carried out using a modified literature procedure.[5,6] To a solution of 4-azido-2,3,5,6-tetrafluorobenzylsuccinic carbonate **8** (0.150 g, 0.414 mmol) in THF (8 mL) was added L-Phe-L-Phe (0.194 g, 0.621 mmol) and DIPEA (0.133 g, 0.618 mmol) followed by addition of deionised water (2 mL). The reaction was stirred for 2 h at room temperature. After TLC analysis revealed completion of the reaction, the solvent was removed and the aqueous mixture was acidified (pH 2-3) with 5% citric acid solution. The acidified reaction mixture was extracted with ethyl acetate (3 × 25 mL) and the combined organic layer was washed with water (2 × 50 mL). The organic layer was dried ($MgSO_4$) and filtered. The filtrate was concentrated *in vacuo* and precipitated in hexanes twice to provide **3** as a pure white solid (0.189 g, 82%). $^1$H NMR (DMSO–$d_6$, 400 MHz): δ 8.27 (d, $J$ =7.6 Hz, 1H), 7.52 (d, $J$ = 8.8 Hz, 1H), 7.29-7.13 (m, 10H), 4.99 (s, 2H), 4.45-4.40 (m, 1H), 4.25-4.19 (m, 1H), 3.07 (dd, $J$ = 14.0, 4.8 Hz, 1H), 2.96-2.90 (m, 2H), 2.64 (dd, $J$ = 13.6, 11.2 Hz, 1H). $^{13}$C NMR (DMSO–$d_6$, 100 MHz): 172.7, 171.4, 155.0, 144.8 (dm, $^1J_{C\text{-}F}$ = 246.0 Hz), 139.9

(dm, $^{1}J_{C\text{-}F}$ = 262.0 Hz), 137.9, 137.3, 129.18, 129.15, 128.2, 127.9, 126.4, 126.2, 120.3 (m), 110.3 (t, $J$ = 18.0 Hz), 56.1, 53.5, 53.1, 37.3, 36.7. $^{19}$F NMR ($CD_3CN$, 376 MHz) δ -144.6 (m), -154.1 (m). HRMS (ESI+) calculated for: $C_{26}H_{21}F_4N_5O_5Na$: 582.1371, found: 582.1380. IR: $\upsilon_{max}/cm^{-1}$ 2121, 1697, 1659, 1533, 1490, 1234, 1029, 697.

## 3. NMR spectra of 8 and 3

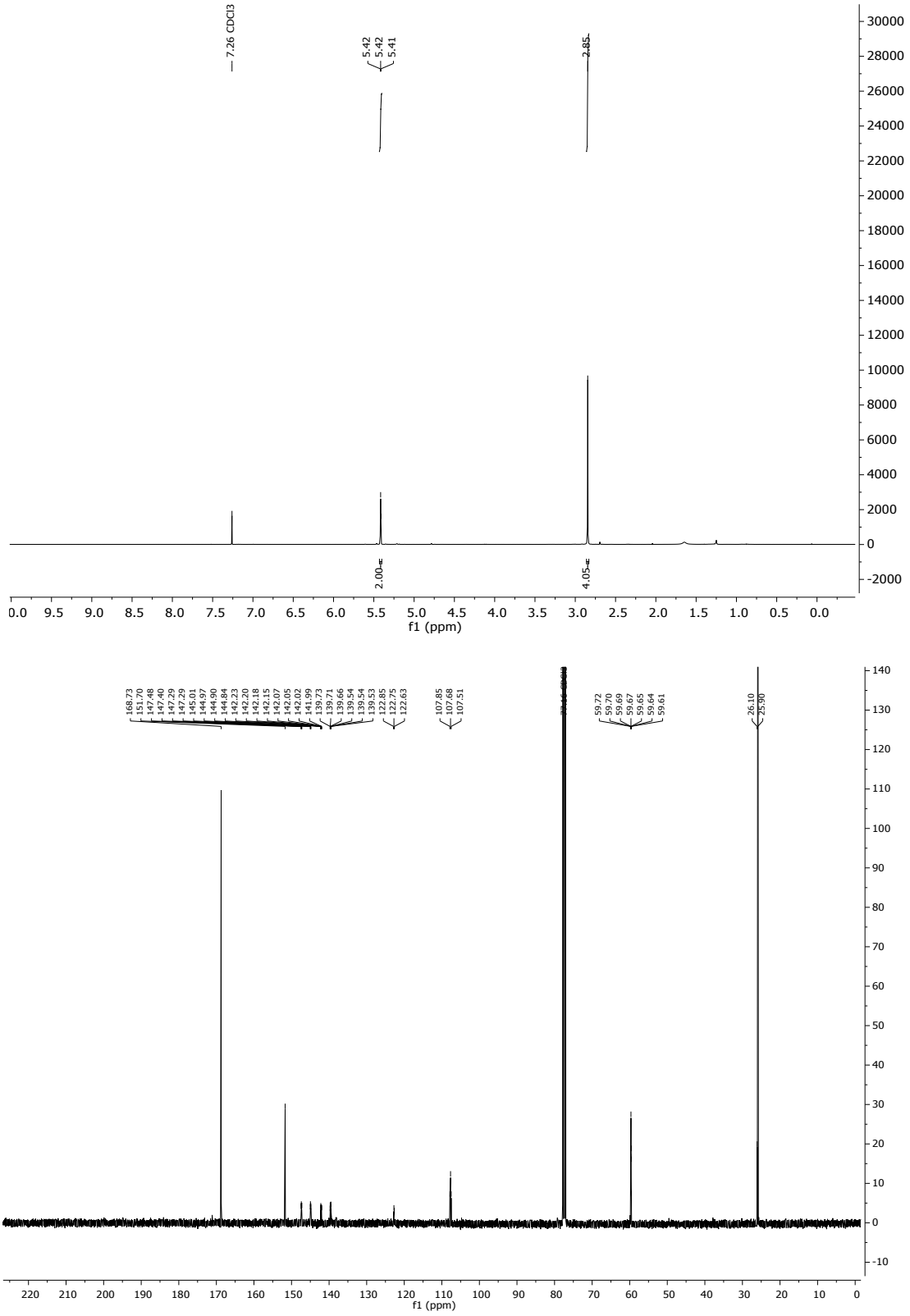
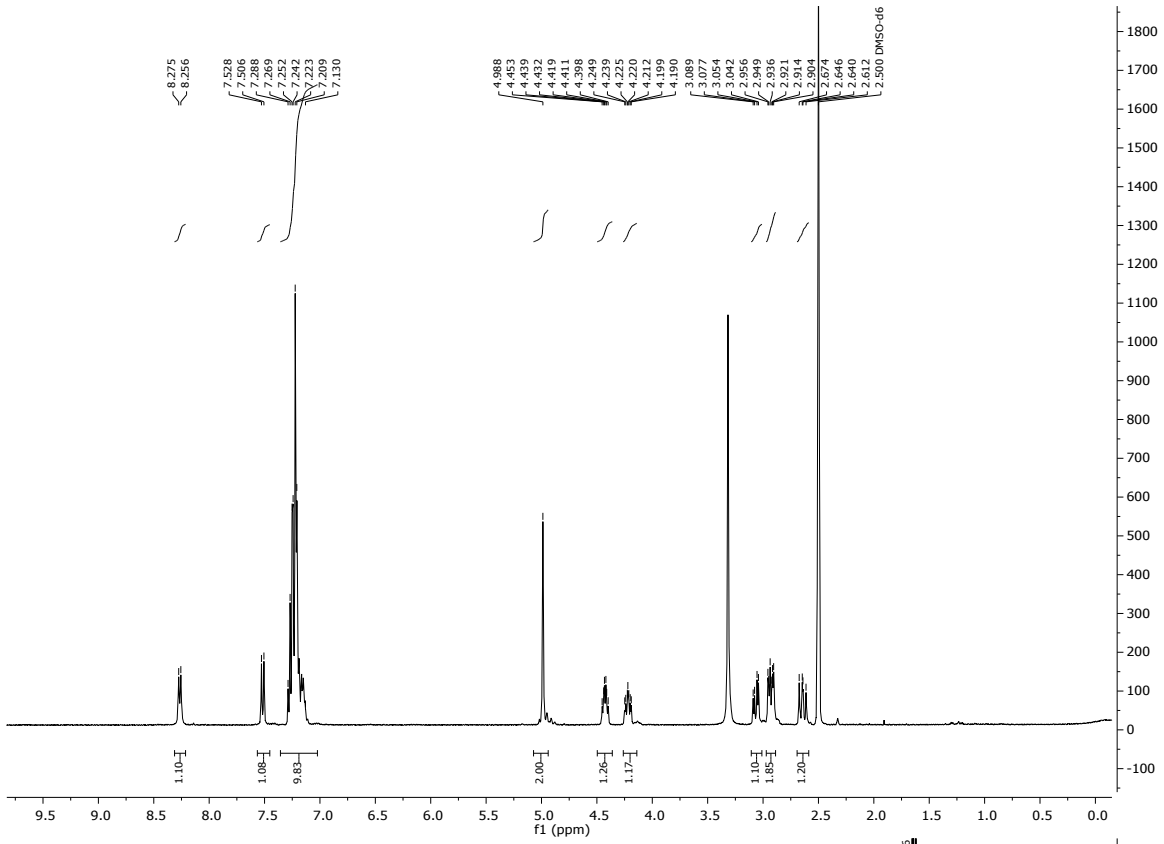



**Figure S21.** $^{1}H$ NMR and $^{13}C$ NMR for 4-azido-2,3,5,6-tetrafluorobenzylsuccinic carbonate **8**.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7.506
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7.269
7.252
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7.223
7.209
7.130
4.988
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4.439
4.432
4.419
4.411
4.398
4.249
4.239
4.225
4.220
4.212
4.199
4.190
3.089
3.077
3.054
3.042
2.956
2.949
2.936
2.921
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2.904
2.674
2.646
2.640
2.612
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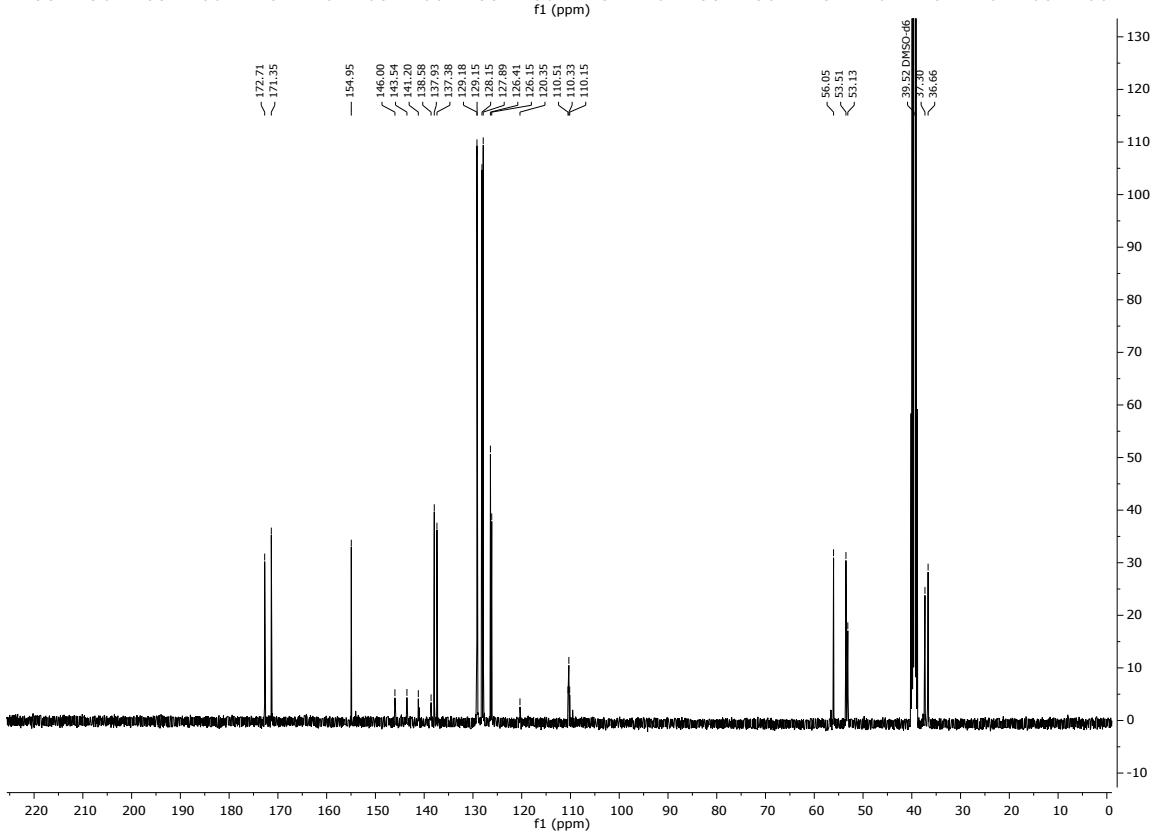
1.26
1.17
1.10
1.85
1.20
f1 (ppm)


172.71
171.35
154.95
146.00
143.54
141.20
138.58
137.93
137.38
129.18
129.15
128.15
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126.41
126.15
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53.13
39.52 DMSO-d6
37.30
36.66
f1 (ppm)

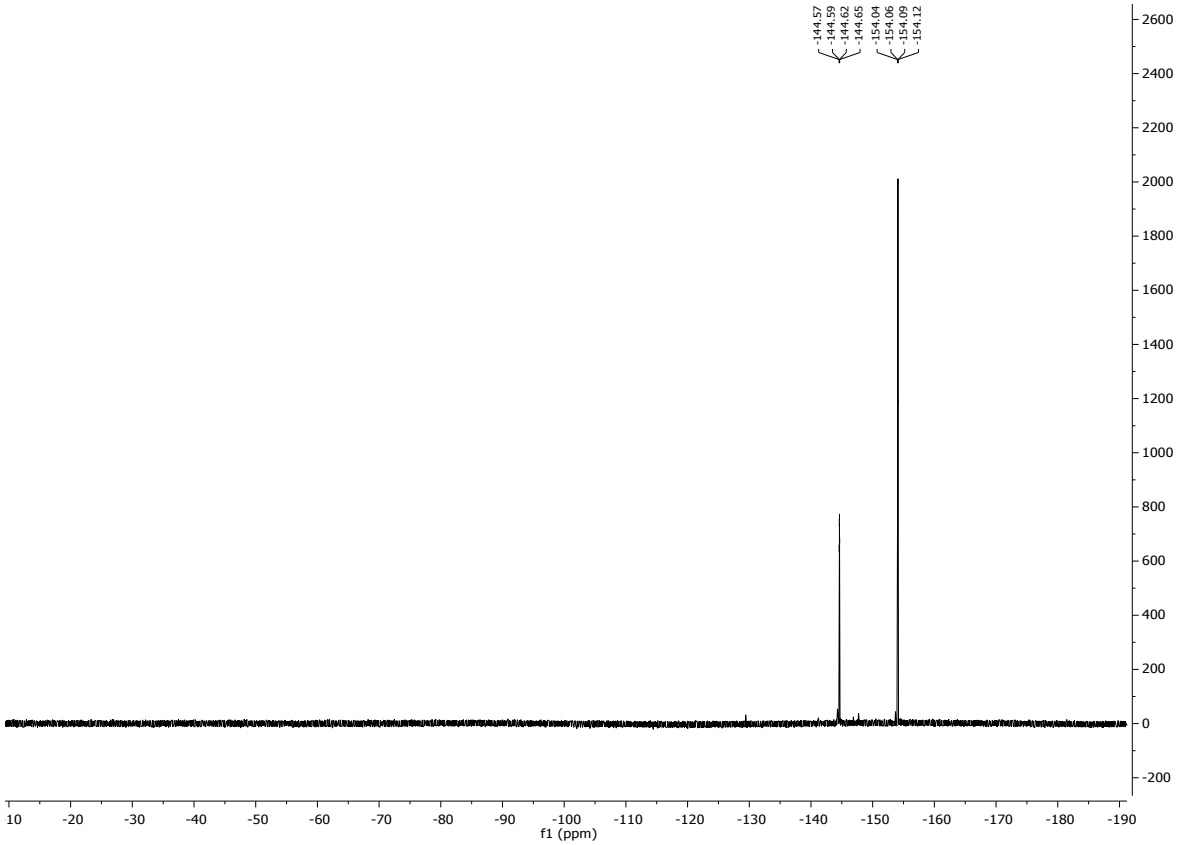


**Figure S22.** $^{1}H$, $^{13}C$ and $^{19}F$ NMR for (4-azido-2,3,5,6-tetrafluorobenzyloxycarbonyl)-L-phenylalanyl-L-phenylalanine **3.**

## References


1. S. S. Matikonda, J. M. Fairhall, F. Fiedler, S. Sanhajariya, R. A. J. Tucker, S. Hook, A. L. Garden, A. B. Gamble, Mechanistic Evaluation of Bioorthogonal Decaging with trans-Cyclooctene: The Effect of Fluorine Substituents on Aryl Azide Reactivity and Decaging from the 1,2,3-Triazoline. *Bioconjugate Chem.*, 2018, **29**, 324-334.
2. S. S. Matikonda, D. L. Orsi, V. Staudacher, I. A. Jenkins, F. Fiedler, J. Y. Chen, A. B. Gamble, Bioorthogonal prodrug activation driven by a strain-promoted 1,3-dipolar cycloaddition. *Chem. Sci.*, 2015, **6**, 1212-1218.
3. M. Royzen, G. P. A. Yap, J. M. Fox, A Photochemical Synthesis of Functionalizedtrans-Cyclooctenes Driven by Metal Complexation. *J.Am. Chem. Soc.*, 2008, **130**, 3760-3761.
4. X. Wu, L. Hu, Design and synthesis of peptide conjugates of phosphoramide mustard as prodrugs activated by prostate-specific antigen. *Bioorg. Med. Chem.*, 2016, **24**, 2697-2706.
5. M. Ikeda, T. Tanida, T. Yoshii, I. Hamachi, Rational Molecular Design of Stimulus-Responsive Supramolecular Hydrogels Based on Dipeptides. *Adv. Mater.*, 2011, **23**, 2819-2822.
6. S. Dadhwal, J. M. Fairhall, S. K. Goswami, S. Hook, A. B. Gamble, Alkene-azide 1,3-dipolar cycloaddition as a trigger for ultrashort peptide hydrogel dissolution. *Chem. Asian J.*, 2019, **14**, 1143-1150.