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for

Metal-Free Triazole Formation as a Tool for Bioconjugation

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1. Experimental Section

General: Unless otherwise stated, all chemicals were obtained from commercial sources and used without further purification. Analytical thin layer chromatography (TLC) was performed on *Merck* precoated silica gel 60 F-254 plates (layer thickness 0.25 mm) with visualization by ultraviolet (UV) irradiation at l = 254 nm and/or l = 366 nm and/or staining with $KMnO_4$. Preparative thin layer chromatography (Prep-TLC) was performed on *Merck* precoated silica gel 60 F-254 plates (layer thickness 1.00 mm) with concentration zone and visualization by UV irradiation at l = 254 nm and/or l = 366 nm. Purifications by silica gel chromatography were performed using *Acros* (0.035 – 0.070 mm, pore diameter ca. 6 nm) silica gel. Unless otherwise stated, all experiments were performed under ambient atmosphere and temperature. The water used in the biological procedures was deionised using a Labconco Water Pro PS purification system. THF was distilled under nitrogen from sodium/benzophenone. CH_2Cl_2 was distilled under nitrogen from CaH_2. Hen egg white lysozyme (HEWL) was obtained from Sigma.

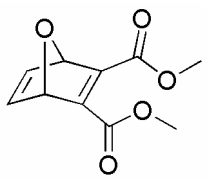
Infrared red spectroscopy (IR spectrometry): IR spectra were recorded on a ATI Matson Genesis Series FTIR spectrometer fitted with a ATR cell. The vibrations ($\tilde{n}$) are given in cm^{-1}.

Nuclear magnetic resonance (NMR): NMR spectra were recorded on Bruker DPX-200 (200 MHz and 50 MHz for ^{1}H and ^{13}C, respectively), Bruker DMX300 (300 MHz and 75 MHz for ^{1}H and ^{13}C, respectively) and Varian inova 400 spectrometers. ^{1}H NMR chemical shifts (d) are reported in parts per million (ppm) relative to a residual proton peak of the solvent, d = 3.31 for CD_3OD, d = 7.26 for $CDCl_3$, and d = 4.79 for D_2O. Broad peaks are indicated by the addition of br. Coupling constants are reported as a *J* value in Hertz (Hz). The number of protons (*n*) for a given resonance is indicated as nH, and is based on spectral integration values. ^{13}C NMR chemical shifts (d) are reported in ppm relative to CD_3OD (d = 49.0) or $CDCl_3$ (d = 77.0).

Size exclusion chromatography (SEC): Molecular weight distributions were measured with a Shimadzu SEC, equipped with a guard column and a PL gel 5 µm mixed D column (Polymer Laboratories) with differential refractive index and UV (l = 254 nm and l = 330 nm) detection using either THF or $CHCl_3$ as an eluent (1 mL/min at 35 °C). In both cases PS standards were used for calibration.

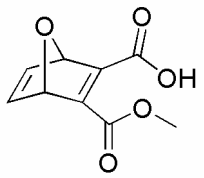
Mass spectrometry (MS): Electrospray LC/MS analysis was performed using a Shimadzu LC/MS 2010A system. MALDI-TOF spectra were measured on a Bruker Biflex III spectrometer and samples were prepared from MeOH solutions using indoleacrylic acid (IAA) (20 mg/mL) as a matrix. LCQ/MS analysis was performed using Thermo scientific Advantage LCQ Lineair-Iontrap Electrospray (ESI-MS). Electrospray ionisation time-of-flight (ESI-ToF) spectra were measured with a JEOL AccuToF.

2. Synthesis

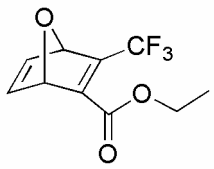


Dimethyl 7-oxa-bicyclo[2.2.1]hepta-2,5-diene-2,3-dicarboxylate (2a):[S1] Furan (0.64 mL, 10 mmol) and dimethyl acetylenedicarboxylate (DMAD, 1.22 mL, 10 mmol) were dissolved in 4 mL ether. The mixture was stirred for 7 days at RT. Water (10

mL) was added and the layers were separated. The water layer was extracted with ether (15 mL). The combined ether layers were washed with brine (20 mL). The organic layer was dried over Na_2SO_4 and concentrated in vacuo. The obtained liquid was purified by column chromatography (EtOAc/*n*-heptane, 1:1) resulting in the desired product (1.48 g (70%), light yellow liquid). R_f = 0.6 (EtOAc/*n*-heptane 1:1). FTIR ν_{max} film: (cm^{-1}) 2950, 1709, 1429, 1269, 1203, 1100, 875. ^{1}H NMR (300 MHz, $CDCl_3$) *d* (ppm): 7.22 (s, 2H), 5.68 (s, 2H), 3.83 (s, 6H). ^{13}C NMR (75 MHz, $CDCl_3$) *d* (ppm): 162.7, 152.5, 142.8, 84.6, 51.9. HRMS (ESI+) *m/z* calcd for $C_{10}H_{10}NaO_5$ $[M+Na]^+$ 233.0426, found 233.0426.

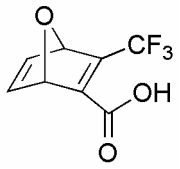


3-(methoxycarbonyl)-7-oxa-bicyclo[2.2.1]hepta-2,5-diene-2-carboxylic acid (3a):[S2] Oxanorbornadiene **2a** (0.13 g, 0.62 mmol) was dissolved in 4 mL THF. The mixture was cooled to 0 °C and NaOH (aq) (4 mL, 0.25 M) was added drop wise. The conversion of the reaction was monitored with TLC (100% EtOAc) and after full conversion the reaction was quenched with 1 mL HCl (aq) (1M) and subsequently extracted into EtOAc (10 mL). The combined organic layers were dried over Na_2SO_4 and concentrated in vacuo resulting in the desired product as a dark yellow oil (97 mg (80 %)). ^{1}H NMR (300 MHz, $CDCl_3$) *d* (ppm): 7.27 (dd, *J* = 1.8, 3.3 Hz, 1H), 7.19 (dd, *J* = 1.8, 3.3 Hz, 1H), 5.83 (t, *J* = 1.8 Hz, 1H), 5.78 (t, *J* = 1.8 Hz, 1H), 3.98 (s, 3H). ^{13}C NMR (75 MHz, $CDCl_3$) *d* (ppm): 166.3, 161.8, 161.3, 151.8, 143.1, 142.8, 85.2, 84.2, 54.1. LCQ MS(ESI-) *m/z* calcd for $C_9H_7NaO_5$ $[M-H]^-$ 195.03, found 194.93.

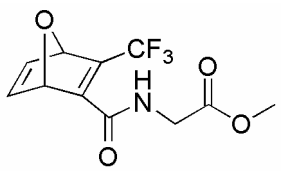


3-Trifluoromethyl-7-oxa-bicyclo[2.2.1]hepta-2,5-diene-2-carboxylic acid ethyl ester (2b):[S3] Ethyl 2-fluorobut-2-ynoate (1.00 g, 0.86 mL, 6.02 mmol) was placed in a Schlenk tube which was fitted with a stopper, evacuated and back-filled with argon. Furan (498 mg, 468 µL, 7.32 mmol) was added and the reaction mixture was heated to 40 °C. The reaction was stirred at 40 °C under an argon atmosphere for 4 days. The resulting mixture was washed out with ether and concentrated in vacuo. The

crude mixture was purified by column chromatography (EtOAc/*n*-heptane, 1:4) resulting in compound **2a** as a slightly yellow oil (1.00 g (71%)). R_f = 0.43 (EtOAc/*n*-heptane, 1:4). FTIR ν_{max} film: (cm^{-1}) 2975, 1733, 1288, 1147. ^{1}H NMR (400 MHz, $CDCl_3$) *d* (ppm): 7.30 (dd, *J* = 5.3, 1.9 Hz, 1H), 7.20 (dd, *J* = 5.3, 1.9 Hz, 1H), 5.70 (m, 1H), 5.66 (t, *J* = 1.7 Hz, 1H), 4.29 (m, 2H), 1.30 (t, *J* = 7.1 Hz, 3H). ^{13}C NMR (75 MHz, $CDCl_3$) *d* (ppm): 161.39, [151.14, 151.08, 150.99, 150.48] (q), 143.4, 142.2, 137.4, [126.5, 122.9, 119.4, 115.8] (q, CF_3), 84.7, 83.5, 61.4, 13.4. HRMS (EI+) *m/z* calcd for $C_{10}H_{10}F_3O_3$ $[M+H]^+$ 234.0506, found 234.0504.

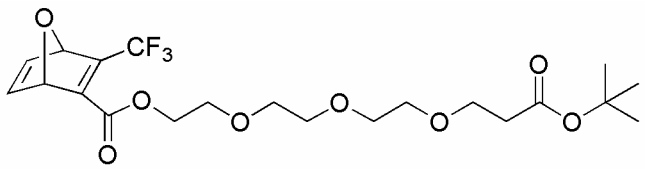


3-Trifluoromethyl-7-oxa-bicyclo[2.2.1]hepta-2,5-diene-2-carboxylic acid (3b): Oxanorbornadiene **2a** (500 mg, 2.13 mmol) was dissolved in THF (30 mL) and cooled to 0 °C. NaOH (aq) (4.85 mL, 1 M) was added drop wise. The mixture was stirred for 30 min. at 0 °C and 1-2 h at RT. After complete conver-sion the volume of the mixture was reduced to 50% of the original volume and H_2O (20 mL) and EtOAc (15 mL) were added. The layers were separated and the aque-ous layer was acidified to pH 4-5 with HCl (aq) (2 M). The water layer was extracted with EtOAc (2 × 20 mL). The combined organic layers were dried over $MgSO_4$ and evaporated to dryness. The solid was washed with CH_2Cl_2 (2 × 10 mL) to removed traces of EtOAc and THF. Compound **3b** was obtained as an off-white solid (363 mg (83%)). R_f = 0.1 (*n*-heptane/EtOAc, 2:1). FTIR ν_{max} film: (cm^{-1}) 3300, 2928, 1714, 1326, 1271, 1164, 1122, 880, 842, 709. ^{1}H NMR (400 MHz, $CDCl_3$) *d* (ppm): 8.80-7.57 (brs, 1H), 7.30 (dd, *J* = 5.3, 1.9 Hz, 1H), 7.22 (dd, *J* = 5.3, 1.9 Hz, 1H), 5.74 (s, 1H), 5.70 (d, *J* = 1.3 Hz, 1H). ^{13}C NMR (75 MHz, $CDCl_3$) *d* (ppm): 166.2, [155.1, 154.6, 154.1, 153.6] (q), [150.7, 150.6] (d), 143.9, 142.6, [126.7, 123.1, 119.5, 115.9] (q), 85.0, 84.2. HRMS (EI+) *m/z* calcd for $C_8H_6F_3O_3$ $[M+H]^+$ 206.0191, found 206.0199.



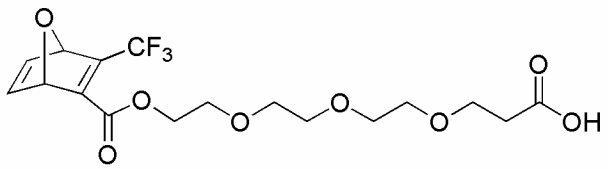
3-Trifluoromethyl-7-oxa-bicyclo[2.2.1]hepta-2,5-diene-2-carboxyl-Gly-OMe (7): Oxanorbornadiene carboxylic acid **3b** (20.6 mg, 0.1 mmol), H-Gly-OMe·HCl (13.8

mg, 0.11 mmol) and 4-(dimethylamino)-pyridine (DMAP, 24.2 mg, 0.2 mmol) were dissolved in 2 mL CH_2Cl_2 and cooled to 0 °C. 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC.HCl, 21 mg, 0.11 mmol) was added and the reaction mixture was stirred at 0 °C for 30 min. The mixture was allowed to warm to RT and was stirred for an additional 16 h. The reaction was quenched with HCl (aq) (2 mL, 2 M) and extracted with EtOAc (2 × 5 mL). The combined organic layers were washed with brine (5 mL), dried over Na_2SO_4 and concentrated in vacuo. The crude mixture was purified by preparative TLC (CH_2Cl_2/MeOH, 9:1) resulting in compound **7** as a slightly yellow solid (15.5 mg (56%)). R_f = 0.55 (CH_2Cl_2/MeOH, 9:1). FTIR ν_{max} film: (cm^{-1}) 2924, 1744, 1636, 1169, 1117, 886. ^{1}H NMR (400 MHz, $CDCl_3$) *d* (ppm): 7.33 (dd, *J* = 5.3, 2.0 Hz, 1H), 7.16 (dd, *J* = 5.3, 2.0 Hz, 1H), 6.41 (brs, 1H, NH), 5.68 (m, 2H), 4.15 (dq, *J* = 18.5, 18.5, 18.5, 5.2 Hz, 2H) 3.80 (s, 3H). ^{13}C NMR (50 MHz, $CDCl_3$) *d* (ppm): 166.6, 154.9, 154.2, 150.7, 144.0, 142.7, [124.8, 118.6] CF_3, 85.1, 84.3. HRMS (ESI+) *m/z* calcd for $C_{11}H_{10}F_3NaNO_4$ $[M+Na]^+$ 300.0460, found 300.0459.

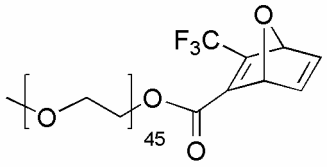


3,6,9-Trioxadodecan-12-oic acid, 1-[[3-trifluoromethyl-7-oxa-bicyclo[2.2.1]hepta-2,5-diene-2-carboxyl]oxy]-1,1-dimethylethyl ester (9): A mixture of **3b** (103 mg, 0.50 mmol), *tert*-butyl-12-hydroxy-4,7,10-trioxadodecanoate (139 mg, 0.50 mmol) and 4-(dimethylamino)-pyridine (DMAP, 121 mg, 1.00 mmol) in CH_2Cl_2 (6 mL) was cooled to 0 °C before adding 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC.HCl, 105 mg, 0.55 mmol). The mixture was stirred for 5 min at 0 °C and 18 h at RT. The reaction mixture was acidified with HCl (2 M) to a pH of 1-2 and extracted with CH_2Cl_2 (2 × 10 mL). The combined organic layers were dried over $MgSO_4$ and concentrated in vacuo. The crude mixture was purified by preparative TLC (CH_2Cl_2/MeOH, 9:1) resulting in compound **9** as a slightly yellow oil (65 mg (27%)). R_f = 0.81 (CH_2Cl_2/MeOH, 9:1). FTIR ν_{max} film: (cm^{-1}) 2971, 2868, 1731, 1666, 1359, 1338, 1273, 1117, 878. ^{1}H NMR (400 MHz, $CDCl_3$) *d* (ppm): 7.19 (dd, *J* = 5.3, 1.9 Hz, 1H), 7.29 (dd, *J* = 5.3, 1.9 Hz, 1H), 5.71 (dd, *J* = 2.9, 1.6 Hz, 1H), 5.66 (t, *J* = 1.7 Hz, 1H), 4.36 (ddt, *J* = 11.9, 11.9, 7.1, 4.9 Hz, 2H), 3.73 (t, *J* = 4.9, 2H), 3.70 (t, *J* = 6.6 Hz, 2H), 3.64 (s, 6H), 3.60 (m, 2H), 2.49 (t, *J* = 6.6 Hz, 2H), 1.44 (s, 9H). ^{13}C

NMR (75 MHz, $CDCl_3$) *d* (ppm): 170.8, 161.6 (q), 151.8, 151.3 (q), 143.9 (q), 142.6, [126.9, 123.3, 119.7, 116.2] (q of CF_3), 85.1, 83.9 (q) 80.4, 70.60, 70.55, 70.50, 70.3, 68.6, 66.8, 64.7, 36.2, 28.0. HRMS (ESI+) *m/z* calcd for $C_{21}H_{29}F_3NaO_8$ $[M+Na]^+$ 489.1712, found 489.1719.



3,6,9-Trioxadodecan-12-oic acid, 1-[[3-trifluoromethyl-7-oxa-bicyclo[2.2.1]hepta-2,5-diene-2-carboxyl]oxy]-12-carboxylic acid (10): A solution of compound **9** (65 mg, 0.13 mmol) in CH_2Cl_2 (2 mL) was cooled to 0 °C before trifluoroacetic acid (TFA, 55 µL) was added drop wise. The mixture was stirred for 30 min. at 0 °C and 16 h at RT. The solvent was removed and the residue was dissolved in H_2O (5 mL) and dioxane (3 mL) and subsequently freeze-dried. The product was purified by preparative TLC (CH_2Cl_2/MeOH, 9:1), resulting in compound **10** as a colorless oil (44.6 mg (84%)). R_f = 0.25 (CH_2Cl_2/MeOH, 9:1). FTIR ν_{max} film: (cm^{-1}) 3434, 2928, 2846, 1731, 1455, 1260, 1104, 793. ^{1}H NMR (400 MHz, $CDCl_3$) *d* (ppm): 7.60 (brs, 1H), 7.30 (dd, *J* = 5.3, 1.9 Hz, 1H), 7.20 (dd, *J* = 5.3, 1.9 Hz, 1H), 5.72 (m, 1H), 5.66 (t, *J* = 1.72 Hz, 1H), 4.44-4.31 (m, 2H), 3.79-3.72 (m, 4H), 3.65 (s, 4H), 3.64 (s, 4H), 2.63 (t, *J* = 6.28 Hz, 2H). ^{13}C NMR (50 MHz, $CDCl_3$) *d* (ppm): 176.2, 161.7, 151.3, 144.0, 142.7, [124.3, 119.1] (CF_3), 109.7, 85.2, 84.1, 70.6, 70.4, 68.7, 66.4, 64.8, 34.9. HRMS (ESI-) *m/z* calcd for $C_{17}H_{20}F_3O_8$ $[M-H]^-$ 409.1110, found 409.1103.



α-methoxy ?-(trifluoromethyl-7-oxa-bicyclo[2.2.1.]hepta-2,5-diene-2-carbonyl) poly(ethylene glycol) (8): A mixture of oxanorbornadiene carboxylic acid **3b** (80 mg, 0.39 mmol), α-methoxy poly(ethylene glycol) (mPEG, 152 mg, 0.076 mmol) and 4-(dimethylamino)-pyridine (DMAP, 22 mg, 0.18 mmol) in anhydrous CH_2Cl_2 (4 mL) was cooled to 0 °C. 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC.HCl, 94.5 mg, 0.49 mmol) was added and the mixture was stirred for 1.5 h at 0 °C. The mixture was allowed to warm to RT and stirred for another 36 h. After dilution with CH_2Cl_2 (50 mL) the reaction mixture was washed with a saturated aqueous

$NaHCO_3$ solution (2 × 50 mL) and a saturated aqueous NH_4Cl solution (2 × 50 mL). Subsequently, the organic phase was dried over Na_2SO_4 and concentrated in vacuo, affording a yellowish solid (142 mg (86%)). ^{1}H NMR (400 MHz, $CDCl_3$) *d* (ppm): 7.27 (dd, *J* = 1.9, 5.3 Hz, 1H), 7.17 (dd, *J* = 1.9, 5.3 Hz, 1H, oxanorbornadiene), 5.72 (m, 1H, oxanorbornadiene), 5.63 (t, *J* = 1.7, 1H, oxanorbornadiene), 4.34 (m, 2H, O-CH_2-CH_2-CO_2), 3.62 (br s, 180H, O-(CH_2CH_2) -O), 3.35 (s, 3H, CH_3-O).

ESI-ToF (ESI+) analysis (Figure S1) shows a clear shift of the molecular weight distribution towards higher molecular weight. The [*M*+Na] peaks for n = 38 were assigned for both compounds; ToF (ESI+) [*M*+Na] hydroxyl functionalized mPEG *m/z* = 1727.94 (calc *m/z* = 1728.02), [*M*+Na] oxanorbornadiene functionalized mPEG (**8**) *m/z* = 1915.86 (calc *m/z* = 1916.03). By subtracting the two peaks, the expected difference of *m/z* = 187.92 (calc *m/z* = 188.01) is found. Note: No fresh calibration sample was acquired while measuring the samples.

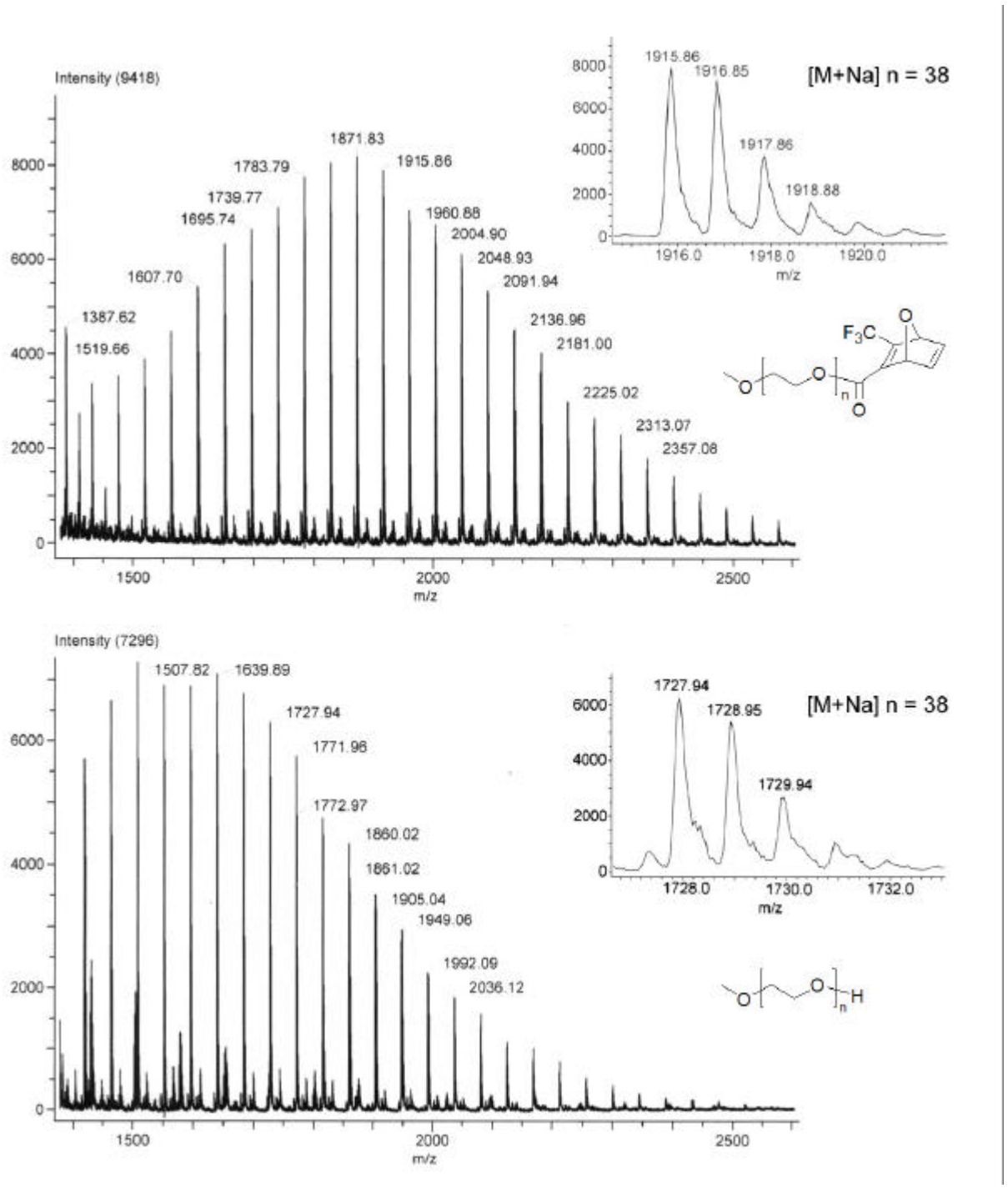
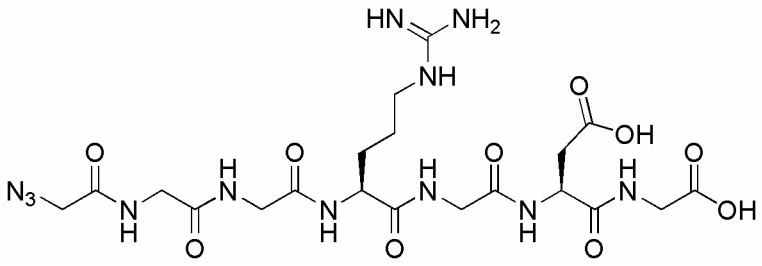
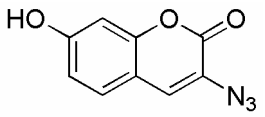


Figure S1. ESI-ToF spectra of hydroxyl functionalized mPEG and oxanorbornadiene functionalized mPEG. Note: The extra distributions on the lower molecular weight side (only slightly visible) belong to the double charged species.



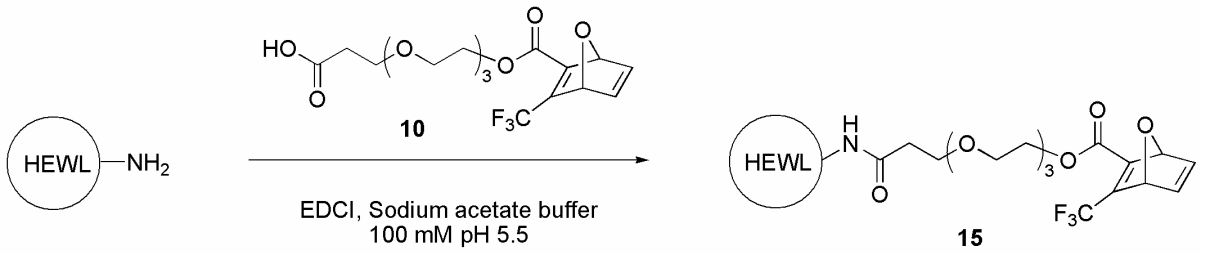
Azidoacetyl-Gly-Gly-Arg-Gly-Asp-Gly-OH (12): Azide functionalized GGRGDG (**12**) was synthesized by standard solid-phase methods using a 'Wang' resin.[S4,S5] A suspension of Wang resin (30 g) in DMF (300 mL) was cooled in an ice bath, after which Fmoc-Gly-OH (13.5 g, 45 mmol), 1-hydroxybenzotriazole hydrate (HOBt, 9.2 g, 60 mmol) and *N,N*'-diisopropylcarbodiimide (DIPCDI, 4.3 g, 34 mmol) were added. This mixture was shaken for 6 h. The functionalized resin was filtered and washed repeatedly with CH_2Cl_2, DMF, and isopropyl alcohol. Unfunctionalized groups on the resin were capped by adding benzoyl chloride (10.2 mL) and pyridine (8.4 mL) to a suspension of the resin in CH_2CL_2 (300 mL) at 0°C. The mixture was shaken for 30 min, filtered and washed repeatedly with CH_2Cl_2, DMF, and isopropyl alcohol. Then the Fmoc-Gly funtionalized Wang resin (1 g, loading; 0.67 mmol/g) was swollen in DMF (20 mL) and filtered three times. Subsequently, the mixture was shaken in a 20% (v/v) solution of piperidine in DMF (20 mL) for 30 min to remove the Fmoc protecting group. A positive Kaiser test [S6,S7] indicated the completeness of this reaction. After filtering and washing with DMF (3 × 20 mL), the next amino acid was coupled by adding a mixture of Fmoc-Asp(O*t*Bu)-OH (500 mg, 1.22 mmol), HOBt (405 mg, 3.00 mmol) and DIPCDI (340 mg, 2.70 mmol) in DMF (20 mL). The mixture was shaken for 45 min., after which it was filtered and washed with DMF (3 × 20 mL). A negative Kaiser test indicated the completeness of the reaction. The deprotection-coupling sequence was repeated with the following amino acids: Fmoc-Gly-OH (210 mg, 0.706 mmol), Fmoc-Arg(PMC)-OH (700 mg, 1.77 mmol), and Fmoc-Gly-OH (210 mg, 0.706 mmol) twice. After deprotection of the terminal Fmoc group, 2-azidoacetic acid[S8] (158 mg, 1.56 mmol) was coupled to the peptide by shaking the mixture with HOBt (405 mg, 3.00 mmol) and DIPCDI (340 mg, 2.70 mmol) in DMF (20 mL) for 45 min. The mixture was washed repeatedly with DMF and MeOH. Subsequently, the resin was stirred in a mixture of TFA/triisopropyl silane/water (95/2.5/2.5, 3.5 mL) to cleave the peptide from the resin. The peptide was precipitated in Et_2O and stirred in TFA/water (95/5, 3 mL) for 4 h to achieve a complete deprotection of the amino acid residues. Upon precipitation in Et_2O and drying in vacuo the peptide was obtained as

an off-white solid (350 mg (87%)) ^{1}H NMR (400 MHz, D_2O) *d* (ppm): 4.82 (m, 1H), 4.35 (dd, *J* = 5.7, 8.7 Hz, 1H), 4.11 (s, 2H), 4.07 – 3.89 (m, 8H), 3.21 (t, *J* = 6.7 Hz, 2H), 2.92 (ddd, *J* = 6.6, 17.2, 25.0 Hz, 2H), 1.97 – 1.85 (m, 1H), 1.85 – 1.73 (m, 1H), 1.73 – 1.56 (m, 2H). FTIR ν_{max} film: (cm^{-1}) 3285, 3092, 2928, 2114, 1651, 1540, 1186 cm^{-1}. ESI-ToF (ESI-): calc. *m/z* = 599.229 [*M*-H]$^-$, found *m/z* = 599.233 [*M*-H]$^-$.



Azido-7-hydroxycoumarin (11): This compound was prepared according to a literature procedure.[S9] ^{1}H NMR (DMSO, 400 MHz): *d* ≥ 10.53 (s, 1H), 7.60 (s, 1H), 7.48 (d, *J* = 8.5 Hz, 1H), 6.81 (dd, *J* = 8.5, 2.3 Hz, 1 H), 6.76 (d, *J* = 2.3 Hz, 1H). FTIR ν_{max} film: (cm^{-1}) 3291, 2115, 1679, 1616, 1303.

Hen Egg White Lysozyme (HEWL) was functionalized with an oxanorbornadiene moiety by employing an EDC peptide coupling between oxanorbornadiene **10** and one of the primary amines on the surface of HEWL (Scheme S1).



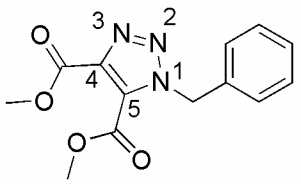
Scheme S1. Functionalization of HEWL with an oxanorbornadiene moiety.

Typical procedure for the functionalization of Hen Egg White Lysozyme (HEWL) via an EDCI coupling at pH 5.5: Hen Egg White Lysozyme (5.7 mg, 4.0×10^{-4} mmol) was dissolved in sodium acetate buffer (1 mL, 100 mM, pH 5.5). After the addition of an azide or oxanorbornadiene functionalized carboxylic acid (14×10^{-3} mmol, as a solution in 100 µL THF), EDCI (3.8 mg, 19×10^{-3} mmol) was added as a solution in sodium acetate buffer (100 mM, pH 5.5, 200 µL). The reaction mixture was shaken at RT for 14 h. Subsequently, the protein was separated from low molecular weight compounds by a sephadex G50 column using a sodium acetate solution (20 mM, pH 5.5) as the eluent.

3. Cycloaddition reactions with oxanorbornadiene derivatives.

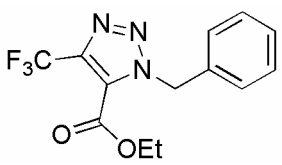
General procedure for reactions between oxanorbornadiene derivatives and azido compounds monitored by ^{1}H NMR spectroscopy: A solution of an oxanorbornadiene derivative (0.05 mmol) in a deuterated solvent (0.5 mL) was added to a test tube containing an azido compound (various equivalents). The mixture was briefly stirred using a vortex and then added to an NMR tube. Directly after the addition, the tube was placed in a Varion inova 400 NMR apparatus at 25 or 37 °C, and the reaction was monitored following a preset measurement schedule.

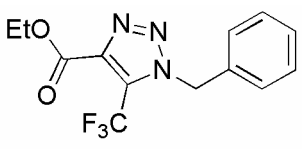
Cycloaddition of oxanorbornadiene **2a** with benzyl azide.



Dimethyl 1-benzyl-1*H*-1,2,3-triazole-4,5-dicarboxylate (5a): A solution of azanorbornadiene **2a** (15.4 mg, 0.05 mmol) in CD_3OD (0.5 mL) was added to a test tube containing benzyl azide (31.3 μL, 0.25 mmol). The mixture was briefly stirred using a vortex and then added to an NMR tube. Directly after the addition, the tube was placed in a *Varion inova 400* NMR apparatus at 25 °C, and reaction was monitored following a preset measurement schedule. The conversion to triazole **5a,** determined by ^{1}H NMR, was found to be 90% after 14 h. R_f = 0.3 (EtOAc/*n*-heptane, 3:1). FTIR n_{max} film: (cm^{-1}) 2946, 1731, 1558, 1457, 1219, 1057. ^{1}H NMR (CD_3OD, 400 MHz) *d* (ppm): 7.34 (m, 3H), 7.27 (d, *J* = 7.5, 2.0 Hz, 2H), 5.81 (s, 2H), 3.91 (s, 3H), 3.87 (s, 3H). ^{13}C NMR (75 MHz, $CDCl_3$) *d* (ppm): 159.9, 158.3, 139.7, 133.4, 129.3, 128.5, 128.4, 127.5, 53.5, 52.8, 52.2. HRMS (ESI+) *m/z* calcd for $C_{13}H_{13}N_3NaO_4$ 298.0804 [*M*+H]$^+$, found 298.0780.

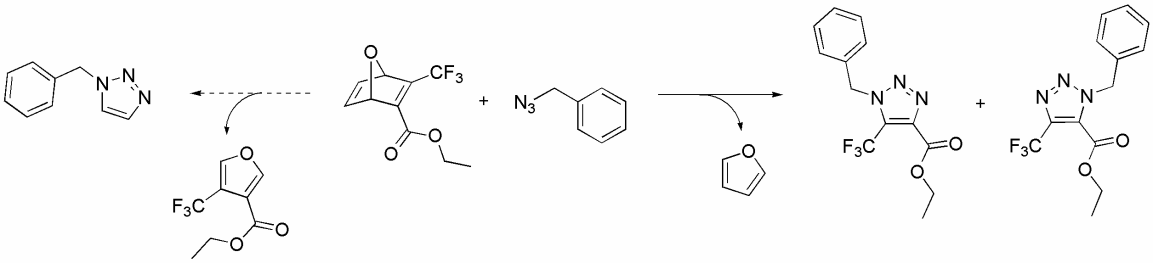
Cycloaddition of oxanorbornadiene **2b** with benzyl azide.





Ethyl 1-benzyl-4-(trifluoromethyl)-1*H*-1,2,3-triazole-5-carboxylate (5b) and ethyl 1-benzyl-5-(trifluoromethyl)-1*H*-1,2,3-triazole-4-carboxylate (6b): Benzyl azide (39.9 mg, 0.3 mmol) was added to a solution of oxanorbornadiene **2b** (70.2 mg, 0.3 mmol) in CD_3OD (3 mL) and the reaction mixture was stirred at RT for 16 h. The solvent was removed under reduced pressure and the crude mixture was purified by preparative TLC (EtOAc/*n*-heptane, 3:1) resulting in compounds **5b** (24 mg (27%)), R_f = 0.75 (EtOAc/*n*-heptane, 3:1), ^{1}H NMR (400 MHz, $CDCl_3$) *d* (ppm): 7.34 (s, 5H), 5.94 (s, 2H), 4.39 (q, *J* = 7.2, 7.2 Hz, 2H), 1.35 (t, *J* = 7.2 Hz, 3H) **6b** (53 mg (60%)), R_f = 0.70 (EtOAc/*n*-heptane, 3:1), ^{1}H NMR (400 MHz, $CDCl_3$) *d* (ppm): 7.35 (m, 3H), 7.22 (dd, *J* = 6.6, 2.8 Hz, 2H), 5.76 (s, 1H), 4.45 (q, *J* = 7.1, 7.1 Hz, 2H), 1.41 (t, *J* = 7.1 Hz, 3H) as slightly yellow oils.

An overview of the reactions that were carried out is presented in Tables S1 and S2. Below, an example of the calculation of the kinetics for entry 3 (Table S1) is presented.



Scheme S2. Reaction of oxanorbornadiene **2b** with benzyl azide (1:0.99) in CD_3OD at 25 °C (entry 3 of Table S1).

The ^{1}H NMR spectra of the reaction between oxanorbornadiene **2b** and benzyl azide at *t* = 1 and 850 min are partially depicted in Figure S2. The spectra clearly indicate a decrease of starting material corresponding to the consumption of oxanorbornadiene shown by the bridgehead signals (*d* = 5.72, 5.69 ppm) decreasing in time. At the same moment, new distinct signals rise, belonging to the C*H*$_2$-triazole (*d* = 5.96, 5.87, 5.62 ppm) of the products. By comparing the integrals of the C*H*$_2$-triazole signals with the integrals of the bridgehead signals, the total molar fraction of the products can be determined. Subsequently, the molar fraction of the products can be plotted as a function of the reaction time resulting in the conversion plot of the reaction (Figure S3).

The amount of side product (1-benzyl-1,2,3-triazole) that was formed, could easily be determined by comparing the integral value of the C*H*$_2$-triazole (*d* = 5.62 ppm) with the sum of all C*H*$_2$-triazole products. In this example, only 3% of the undesired product following cycloaddition-retro Diels-Alder pathway B was formed.

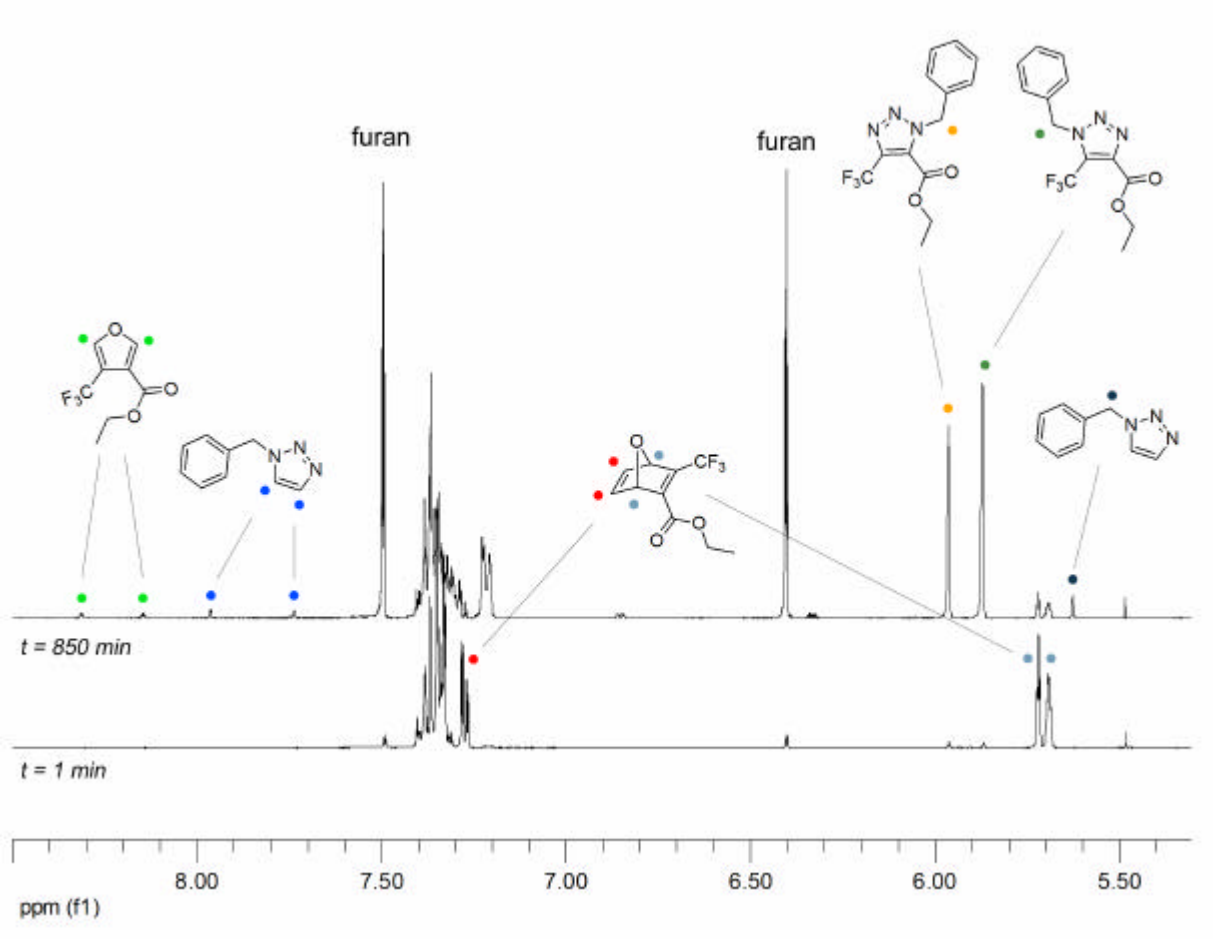


Figure S2. ^{1}H NMR spectra of the reaction between **2b** and benzyl azide at *t* = 1 and 850 min.

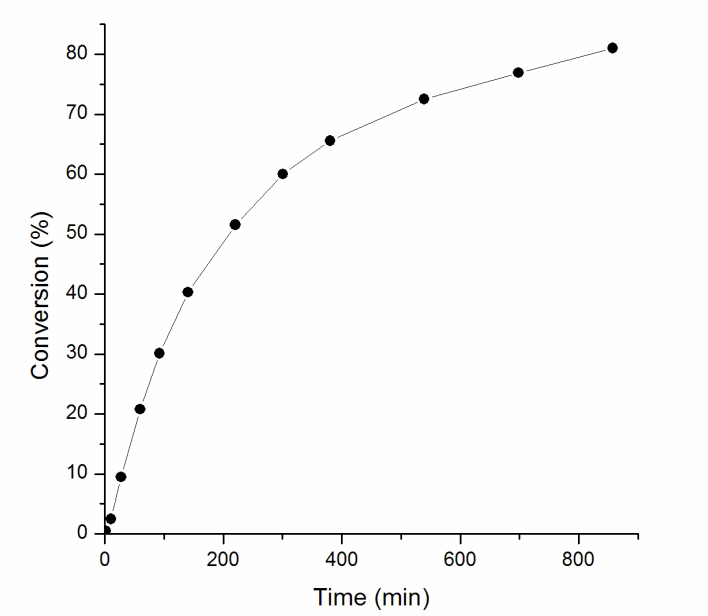


Figure S3. Conversion plot for the reaction between oxanorbornadiene **2b** with benzyl azide (1:0.99) in CD_3OD at 25 °C (entry 3 of Table S1).

From the conversion plot (Figure S3) the second order rate plot can be deduced, by fitting the data to Equation (S1).

$$kt = \frac{1}{[B]_0 - [A]_0} \times \ln \frac{[A]_0([B]_0 - [P])}{([A]_0 - [P])[B]_0} \qquad \text{(S1)}$$

Herein, k = 2nd order rate constant ($M^{-1} \cdot s^{-1}$), t = reaction time (s), $[A]_0$ = the initial concentration of substrate A (M), $[B]_0$ = the initial concentration of substrate B (M), and $[P]$ = the concentration of the products (M).

The initial concentration of the oxanorbornadiene compound was weighted to be 0.10 M. By using the integral values from the ^1H NMR spectrum at t = 0, the initial concentration of the azido compound was adapted to this 0.10 M. In this particular example the initial concentration of benzyl azide was calculated to be 0.099 M. Using these initial concentrations and the data obtained from Figure S3 (typically up to 60% conversion), the second order rate plot was constructed as depicted in Figure S4. Linear regression (using Origin 6.1 software) of this dataset resulted in the following equation:

$$\frac{1}{[B]_0 - [A]_0} \times \ln \frac{[A]_0([B]_0 - [P])}{([A]_0 - [P])[B]_0} = (8.77 \pm 0.09) \times 10^{-4}\, t - 0.31 \pm 0.1$$

Affording,

$k = (8.77 \pm 0.09) \times 10^{-4}$ м$^{-1}$·s^{-1}

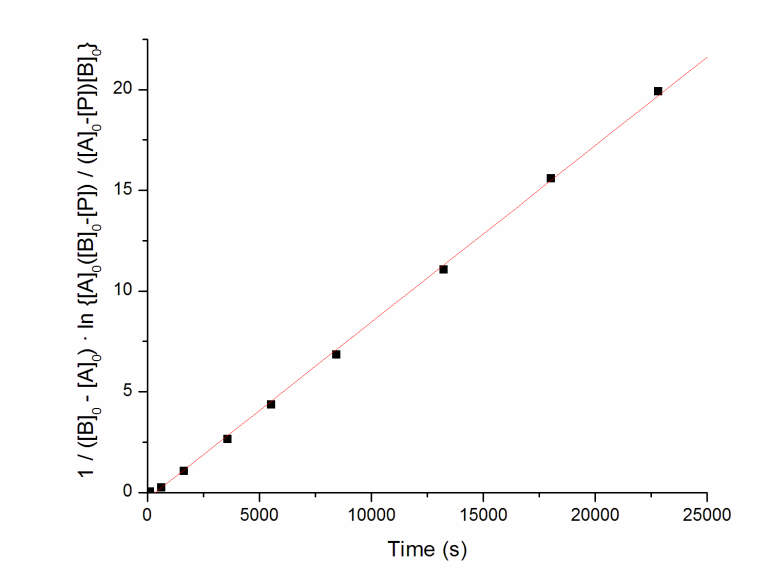
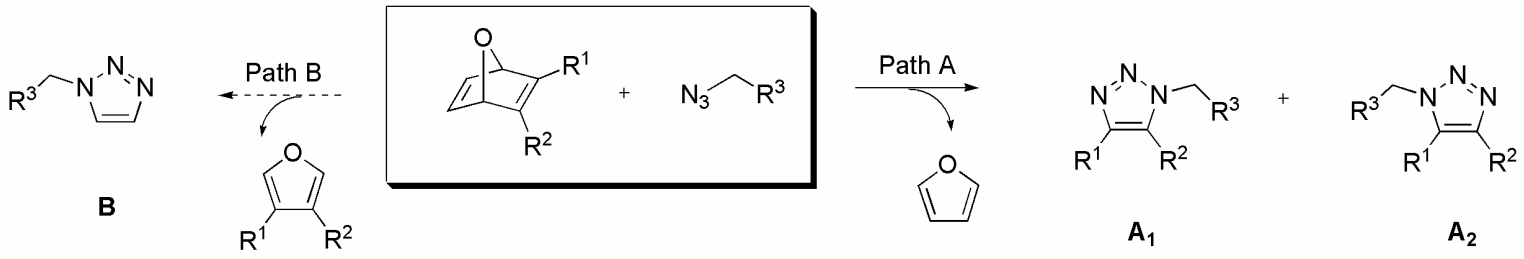
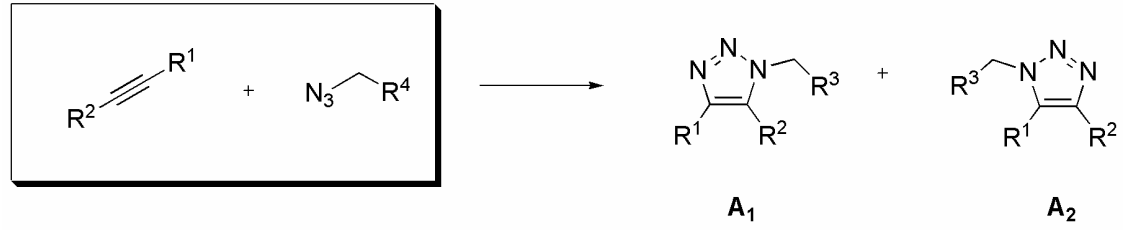


Figure S4. Second order rate plot for the reaction between oxanorbornadiene **2b** with benzyl azide (1:0.99) in CD_3OD at 25 °C (entry 3 of Table S1).



Scheme S3. Reaction pathway of oxanorbornadiene derivatives and azido compounds.



Scheme S4. Reaction pathway of activated alkynes and azido compounds.

Table S1. Products and kinetic data of reactions between oxanorbornadiene derivatives and azido compounds (at 25 ºC and 100 mM) obtained by monitoring the reactions with ^{1}H NMR spectroscopy (400 MHz).

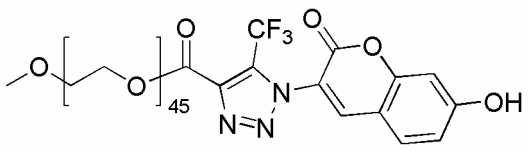
	R^1	R^2	R^3	Eq. N_3	Solvent	**A** (%)	$\mathbf{A}_1$: $\mathbf{A}_2$	$t_{1/2}$ (min)	**B** (%)	Rate $\times 10^4$ ($M^{-1} \cdot s^{-1}$)	Conv. at 14 h (**A**) (%)
1	CO_2Me	CO_2Me	Ph	0.93	CD_3OD	95	-	284	5	6.9 ± 0.05	71
2	CF_3	CO_2Et	Ph	0.98	CD_3OD	98	1 : 1.5	210	2	8.8 ± 0.14	83
3	CF_3	CO_2Et	Ph	0.99	CD_3OD	97	1 : 1.4	205	3	8.7 ± 0.14	82
4[a]	CF_3	CO_2Et	Ph	0.85	CD_3OD	97	1 : 1.4	90	3	23.8 ± 0.43	84
5	CF_3	CO_2Et	Ph	10.0	CD_3OD	98	1 : 1.4	19	2	6.0 ± 0.10	98
6	CF_3	CO_2H	Ph	0.93	CD_3OD	96	1 : 1.4	230	4	8.5 ± 0.15	78
7	CF_3	CO_2H	$EtNH_2$	1.39	D_2O	84	nd	180	16	7.0 ± 0.10	75
8	CF_3	CO_2H	CO_2H	1.09	D_2O	> 98	-[b]	140	trace	10.6 ± 0.05	86
9	CF_3	CO-Gly-OMe	Ph	1.32	CD_3OD	84	1 : 2.4	590	16	1.9 ± 0.03	50

[a] Reaction performed at 37 °C, [b] Exclusively one regio-isomer was observed. nd = not determined.

Table S2. Products and kinetic data of reactions activated alkynes and azido compounds (100 mM) obtained by monitoring the reactions with ^{1}H NMR spectroscopy (400 MHz).

	R^1	R^2	R^3	Eq. N_3	Solvent	*T* (°C)	**A** (%)	$\mathbf{A}_1$: $\mathbf{A}_2$	$t_{1/2}$ (min)	**B** (%)	Rate $\times 10^4$ ($M^{-1} \cdot s^{-1}$)	Conv. at 14 h (**A**) (%)
1	CO_2Me	CO_2Me	Ph	0.93	CD_3OD	25	100	-	> 2000	-	0.6 ± 0.005	21
2	CF_3	CO_2Et	Ph	0.99	CD_3OD	25	100	1 : 1.2	> 1000	-	1.4 ± 0.06	37

Cycloaddition of oxanorbornadiene functionalized PEG (**8**) with 3-azido-7-hydroxy-coumarin (**11**).



a-**Methoxy-ω-(3-(5-(trifluoromethyl)-1*H*-1,2,3-triazol-4-carbonyl)-7-hydroxy-coumarin) poly(ethylene glycol) (13)**: A mixture of oxanorbornadiene functionalized PEG (**8**) (7.7 mg, 3.5 $\times 10^{-3}$ mmol) and 3-azido-7-hydroxycoumarin (**11**) (2.6 mg, 0.013 mmol) in CH_2Cl_2 (3 mL) was stirred for 15 h at RT. The presence of fluorescence (when irradiated by UV light of λ = 366 nm) indicated that the reaction took place. The mixture was concentrated in vacuo and characterized without further purification. From ^{1}H NMR analysis the level of functionalization was determined to be 88%. ^{1}H NMR (400 MHz, $CDCl_3$,) δ (ppm): 8.12 (s, 1H), 7.52 (d, J = 8.5 Hz, 1H), 7.00 (d, J = 1.9 Hz, 1H), 6.96 (dd, J = 2.2, 8.4 Hz, 1H), 4.46 – 4.40 (m, 2H, O-CH_2-CH_2-CO_2), 3.63 (br s, 180H, O-(CH_2CH_2) -O), 3.37 (s, 3H, CH_3-O).

After the cycloaddition reaction, SEC analysis using UV detection at λ = 340 nm (Figure S5 right) showed a clear signal at the elution time of PEG. As the oxanorbornadiene functionalized PEG shows almost no UV response, this confirms that the coumarin is covalently attached to the PEG. As expected, in the RI traces no significant differences could be observed before and after the cycloaddition reaction (Figure S5 left).

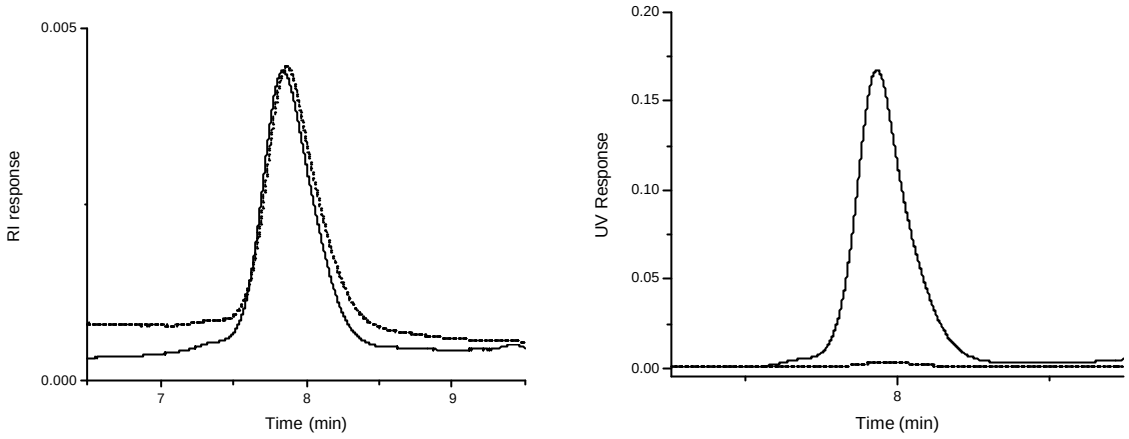
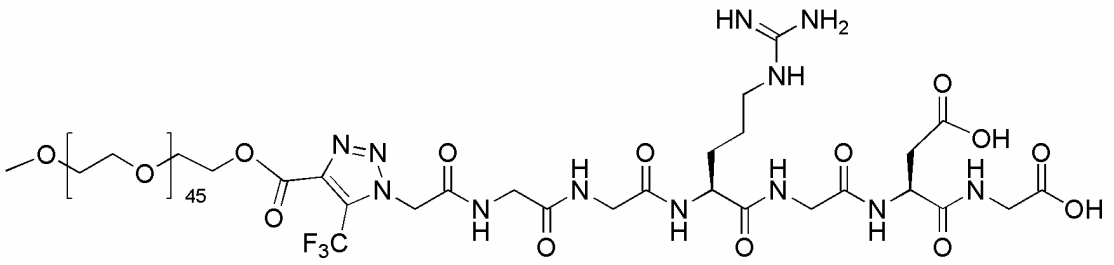


Figure S5. SEC ($CHCl_3$) traces of PEG before (dashed) and after (solid) cycloaddition with 3-azido-7-hydroxycoumarin, RI (left) and UV (340 nm, right).

Cycloaddition of oxanorbornadiene functionalized PEG (**8**) with 2-azidoactyl-Gly-Gly-Arg-Gly-Asp-Gly-OH (**12**).



a **-Methoxy-? -(3-(5-(trifluoromethyl)-1*H*-1,2,3-triazol-4-carbonyl)-acetyl-Gly-Gly-Arg-Gly-Asp-OH) poly(ethylene glycol) (14)**: A mixture of oxanorbornadiene functionalized PEG (**8**) (14.7 mg, 6.7 $\times 10^{-3}$ mmol) and 2-azidoactyl-Gly-Gly-Arg-Gly-Asp-Gly-OH (**12**) (10.7 mg, 0.018 mmol) in H_2O (2 mL) was stirred for 36 h at 37 °C. The mixture was concentrated in vacuo and cha-racterized without further purification. By comparing the ^{1}H NMR integral of an oxanorbornadiene bridgehead signal (d = 5.83) with the -CH_2-triazole signals (d = 5.68 – 5.62) of the product, the conversion was determined to be 80%.

MALDI-ToF analysis (using indoleacrylic acid (IAA) as a matrix) of the mixture clearly showed a shift of the molecular weight distribution towards higher mass (Figure S6).

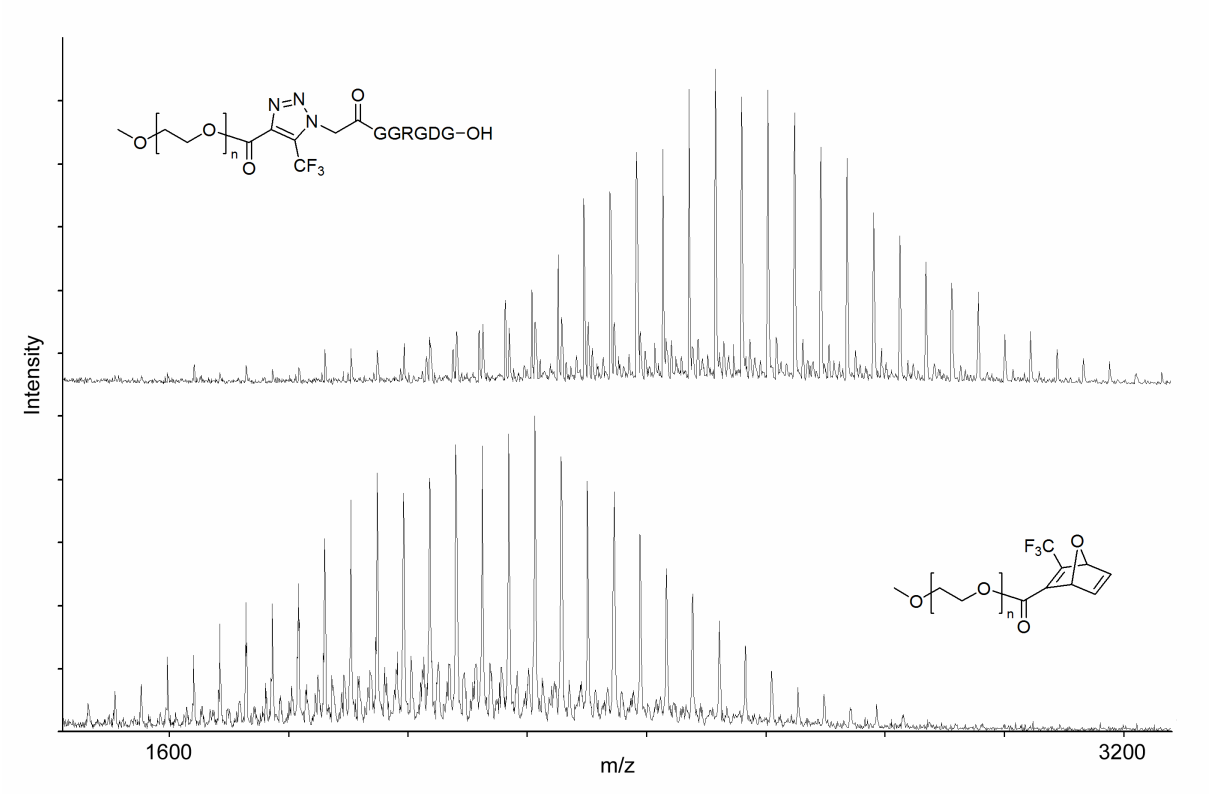


Figure S6. MALDI-ToF spectra of oxanorbornadiene functionalized PEG (**8**) (bottom) and reaction mixture of the cycloaddition between oxanorbornadiene functionalized PEG (**8**) and 2-azidoactyl-Gly-Gly-Arg-Gly-Asp-Gly-OH (**14**) (top)

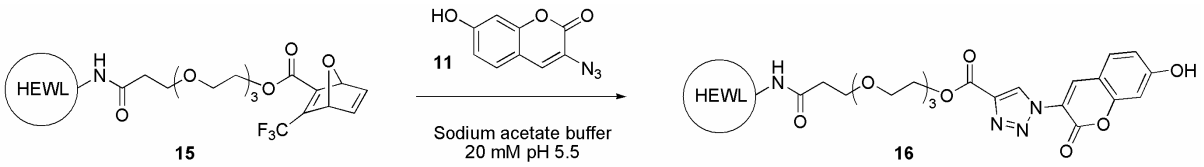
In principle the mass of a single polymer chain can be defined as the sum of masses of the end groups and the number (N) of repeating units. Therefore, the mass of the end groups can be derived from a molecular weight distribution by plotting the molecular mass against N. Consequently, the intercept (N = 0) affords the mass of the end groups. Prior knowledge to the likely nature of these end groups gives an appropriate choice of N, which in case of the obtained PEG-GlyGlyArgGlyAspGly-OH was defined as 34 for the peak assigned with *m/z* = 2293.696. Using this peak as a reference also N for the other peaks was determined and a plot of mass versus N was constructed. Linear regression (using *Origin 6.1* software) of this dataset resulted in the following equation:

$$\text{Mass} = 44.08 \times N + 794.67$$

Herein, 44.08 is in line with the mass of one repeating unit of PEG (calc 44.03) and the intercept of 794.67 corresponds to the α-methoxy (calc 31.02) and ?-Gly-Gly-Arg-Gly-Asp-Gly-OH (calc 721.23) end groups, plus an additional proton, sodium and water.

Cycloaddition reaction of functionalized Hen Egg white Lysozyme with 3-azido-7-hydroxycoumarin (11): Functionalized HEWL (typically, 300 µL of a 1.5 mg/mL solution, 3.3 $\times 10^{-5}$ mmol) and an azido compound or oxanorbornadiene derivative (depending on the functionality on HEWL) (1.6 $\times 10^{-3}$ mmol) were shaken at RT for 36 h. The mixtures were analyzed without further purification.

Employing the oxanorbornadiene-azide ligation in bioconjugation to proteins: The oxanorbornadiene functionalized HEWL (**15**) was mixed with 3-azido-7-hydroxy-coumarin (**11**) and shaken for 36 h (Scheme S5). As a control experiment, unfunctionalized HEWL was also incubated with 3-azido-7-hydroxycoumarin (**11**) under the same conditions. After 36 h the crude mixtures were analyzed by SDS-PAGE (15%), and for the reaction a clear fluorescent band was observed at the position of HEWL. As expected the control experiment showed no fluorescent band (Figure S7A). Since 3-azidocoumarin derivatives are known to become strongly fluorescent upon undergoing a cycloaddition[S9] the observed fluorescent band furthermore proved that the coumarin is covalently attached to the HEWL. Upon staining with coomassie blue, as expected, no mass differences were observed between the reaction mixture and the control experiment (Figure S7B).



Scheme S5. Cycloaddition reaction between oxanorbornadiene functionalized HEWL (**15**) and 3-azido-7-hydroxycoumarin (**11**).

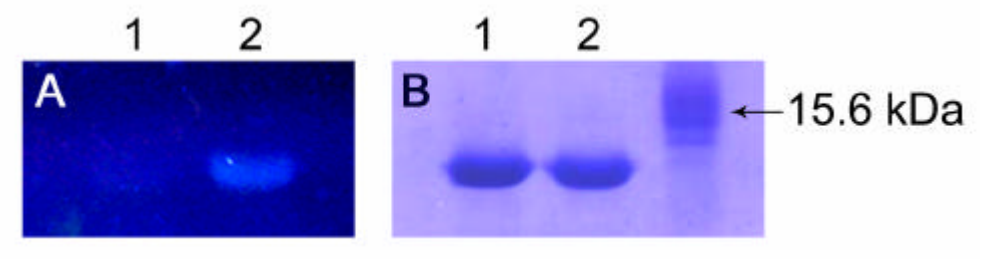


Figure S7. SDS-PAGE (15%) analysis of Cycloaddition reaction between oxanorbornadiene functionalized HEWL and 3-azido-7-hydroxycoumarin. Lane 1: Control experiment, Lane 2: Reaction mixture. Right lane: molecular weight marker. **A**: Fluorescence image by UV irradiation at λ = 366 nm. **B**: Image after staining with Coomassie blue.

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S8 Synthesis of azidoacetic acid: 2-bromoacetic acid (2.00 g, 14.4 mmol) was dissolved in water (15 mL) and cooled using an ice-bath. After the addition of NaN_3 (4.00 g, 61.5 mmol) the mixture was allowed to warm to RT during a period of 16 h. The reaction mixture was acidified to pH 1 by the addition of concentrated HCl, and subsequently extracted with EtOAc (3 × 75 mL). The combined organic phases were dried over Na_2SO_4 and concentrated in vacuo. After co-evaporation with CH_2Cl_2 (4 × 50 mL) the product was obtained as a slightly yellow liquid (1.36 g (93%)). ^{1}H NMR (400 MHz, $CDCl_3$) *d* (ppm): 8.25 (s, 1H), 3.98 (s, 2H). ^{13}C NMR ($CDCl_3$, 75 MHz) *d* (ppm): 174.4, 50.1. FTIR ν_{max} film: (cm^{-1}) 3451, 2110, 1724, 1215.

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