Electronic Supplementary Material (ESI) for ChemComm.

Easy access to triazolinedione-endcapped peptides for chemical ligation

Supporting Information

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Contents

1. Materials

1.1. Peptide Synthesis and ligation

N-α-Fmoc protected amino acids, 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium-hexafluorophosphate (HBTU) and *N*-methyl-2-pyrrolidone (NMP, 99.9 %, peptide synthesis grade) were used as received from IRIS Biotech GmbH (Marktredwitz, Germany). Wang resin (loading: 0.84 mmol/g) was obtained from novabiochem (Sandhausen, Germany). *N,N*-diisopropylethylamine (DIPEA, peptide grade, Acros), triethylsilane (TES, 98+ %, Alfa Aesar, Karlsruhe, Germany), α-cyano-4-hydroxy-cinnamic acid (α-CHCA, 99 %, Sigma Aldrich), *N*-bromosuccinimide (99%, ABCR, Karlsruhe, Germany) and pyridine (anhydrous, Fluka, Taufkirchen, Germany) have been applied as received. Trifluoroacetic acid (TFA, peptide grade, Acros Organics) was distilled prior to use. Dichloromethane (DCM, peptide grade, IRIS Biotech GmbH) was distilled from CaH_2 prior to use. *N,N*-Dimethylformamide (DMF, anhydrous) was purchased from VWR (Darmstadt, Germany) and used as received.

1.2. Polymer Synthesis

ε-Caprolactone (99%, Acros Organics), *trans,trans*-2,4-hexadien-ol (≥97%, Sigma-Aldrich), chloroform (99.8%, Sigma-Aldrich), methanol (99.9%, Sigma-Aldrich), toluene (>99.9%, Sigma-Aldrich) were used as received. A commercial lipase, Novozyme 435 (Lipase B from Candida antarctica immobilized on a macroporousacrylic resin, Sigma-Aldrich) was dried under reduced pressure at room temperature and stored under nitrogen before it was used as a catalyst for the polymerization reaction.

2. Instrumentation

2.1. Peptide synthesis

Synthesis of peptides was performed automatically on an Applied Biosystems ABI 433a peptide synthesizer with ABI-Fastmoc protocols.

2.2. Preparative HPLC

Peptides were purified via preparative HPLC at 22 mL/min on a Shimadzu (Germany) Prominence LC 20-AP system using a CBN-20A Communications Bus Module, a LC-20AP Preparative Liquid Chromatograph, a SIL-20A HT Auto Sampler and a FRC-10A Fraction

Collector. UV-Vis detection was conducted on a SPD-10A UV-Vis Detector. Separation of the products was achieved using a Synergy 4n Fusion-RP 80 A column (Phenomenx Synergi 4µ Fusion-RP 80A, Ax) and Solvent A/Solvent B-mixtures (Solvent A: 99.9 % MilliQ H_2O: 0.1 % formic acid; Solvent B: acetonitrile) as solvents.

2.3. MALDI-TOF-MS

MALDI-TOF-MS measurements were performed on a Confidence Spectrometer from Shimadzu (Kyoto, Japan).

2.4. UPLC-ESI-MS

UPLC measurements were performed on an Acquity-UPLC H-class CM Core system (Waters Corporation, Milford, USA) with an Acquity-UPLC PDA and QDa detector and a LCT Premier XE mass spectrometer for UPLC-HRMS. An Acquity-UPLC HSS T3 column (Waters) was used at 40 °C and Solvent A/Solvent B-mixtures (Solvent A: 99.9 % MilliQ H_2O: 0.1 % formic acid; Solvent B: acetonitrile) as solvents.

2.5. Size-exclusion chromatography (SEC)

Size-exclusion chromatography (SEC) with *N*,*N*-dimethylacetamide (DMA) as eluent was performed on a Agilent 1260-series HPLC system equipped with a 1260 online degasser, a 1260 ISO-pump, a 1260 automatic liquid sampler, a thermostated column compartment at 50°C equipped with a PSS Gram30 column in series with a PSS Gram1000 column, a 1260 diode array detector and a 1260 refractive index detector. The used solvent was DMA containing 50 mM of LiBr at a flow rate of 1 mL/min. Molar mass and dispersity values were calculated against Varian PMMA standards.

2.6. ^{1}H-NMR spectroscopy

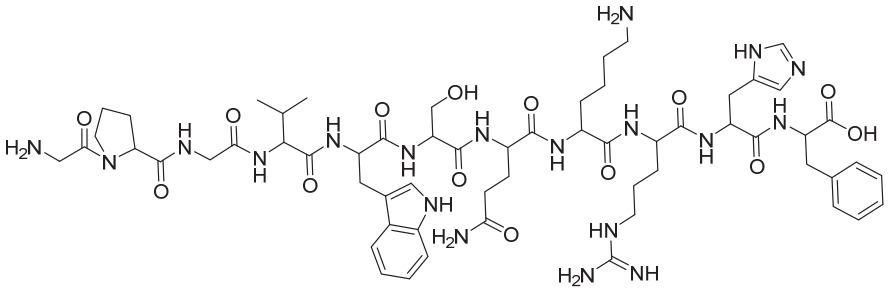
^{1}H nuclear magnetic resonance spectra (^{1}H-NMR) spectra for diene-functionalized poly(ε-caprolactone)s were recorded using a Bruker AM500 spectrometer at 500 MHz at room temperature. Deuterated chloroform ($CDCl_3$) was used as solvent. All other ^{1}H-NMR spectra were conducted on a Bruker AV 500 spectrometer at 500 MHz in TFA-d1 or $CDCl_3$ at room temperature

3. Synthesis of substances

3.1. Peptide synthesis

Peptide synthesis was performed automatically on an Applied Biosystems ABI 433a peptide synthesizer by standard Fmoc-strategy. The ABI-Fastmoc protocols (single coupling for amino acid 1 to 4, double coupling for amino acid 5 to end, capping) were applied and peptide synthesis was conducted in NMP using Wang resin (0.84 mmol/g, 0.1 mmol) as solid support. Fmoc-amino acid coupling was facilitated by HBTU/DIPEA. After final Fmoc removal the resin was transferred to a syringe reactor. Subsequently, the resin was washed five times with NMP followed by 10 times DCM and dried overnight in a vacuum oven at room temperature. Cleavage of the final urazole-functionalized peptide from the resin with 95:2.5:2.5 v/v/v TFA-H_2O-TES for 2 h resulted in the fully deprotected product. The product was isolated by precipitation in diethyl ether and centrifugation. Freeze-drying from water was optionally followed by preparative HPLC.

Analysis of GPGVWSQKRHF (1)



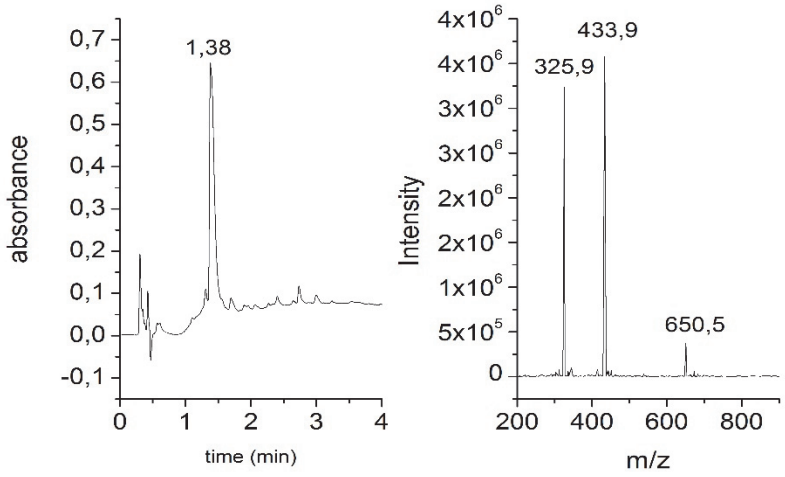


Figure S1. UPLC of GPGVWSQKRHF (**1**) using a gradient of 5 % - 40 % acetonitrile in ultrapure water within 4 minutes (t_R = 1.78 min) (left) and ESI-MS of the product peak (right).

UPLC/ESI-MS m/z[calc.] = 650.24 $(M+2H)^{2+}$, 433.82 $(M+3H)^{3+}$, 325.62 $(M+4H)^{4+}$

m/z[found] = 650.5 $(M+2H)^{2+}$, 433.9 $(M+3H)^{3+}$, 325.9 $(M+4H)^{4+}$

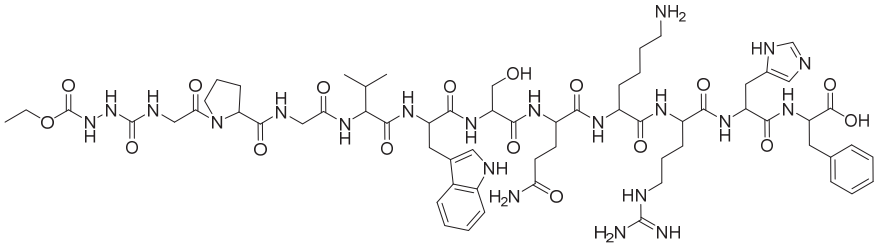
MALDI-TOF-MS m/z[calc.] = 1299.47 $(M+H)^+$

m/z[found] = 1299.8 $(M+H)^+$

The signals can be assigned within ± 1.0 g/mol accuracy.

Purity The peptide was obtained with a purity of 85% calculated from UPLC measurements.

3.2. Preparation of ethylcarbazate 2



The resin-bound peptide was transferred into a syringe reactor and rinsed with DMF twice. A solution of bis(4-nitrophenyl)carbonate (5 eq.) in 4 mL of DMF was added to the syringe reactor and the resulting mixture was shaken at room temperature for 2 h. The solution was discarded and the resin washed with DMF twice. Subsequently, a mixture of triethylamine (7.5 eq.) and ethyl carbazate (7.5 eq.) in 3 mL of DMF was added. The resulting reaction mixture was shaken at room temperature overnight. Afterwards, the resin was washed five times with DMF and DCM each and dried under vacuum. For quantification, a small part of the peptide was cleaved from the resin by treatment with 95:2.5:2.5 v/v/v (TFA/H_2O/TES) for 2 h. The product was obtained with a purity of 81% calculated from UPLC measurements resulting in a yield of 95%.

Analysis of EC+GPGVWSQKRHF (2)

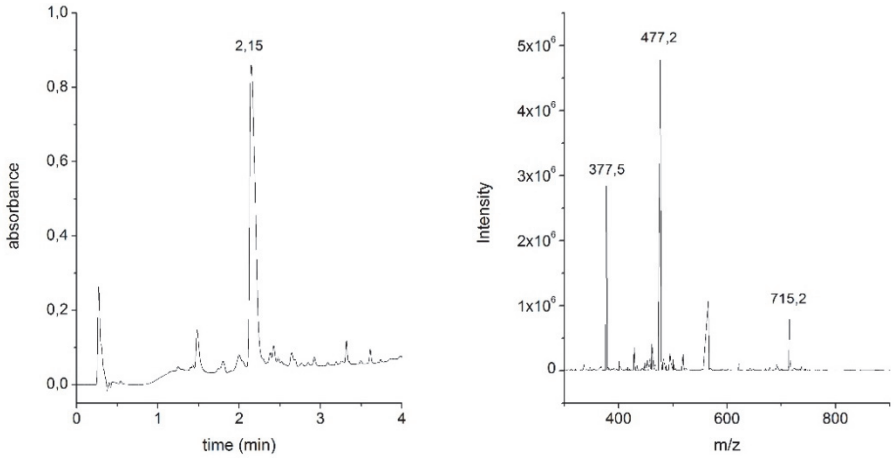


Figure S2. UPLC of EC+GPGVWSQKRHF (**2**) using a gradient of 5 % - 40 % acetonitrile in ultrapure water within 4 minutes (t_R = 2.15 min) (left) and ESI-MS of the product peak (right).

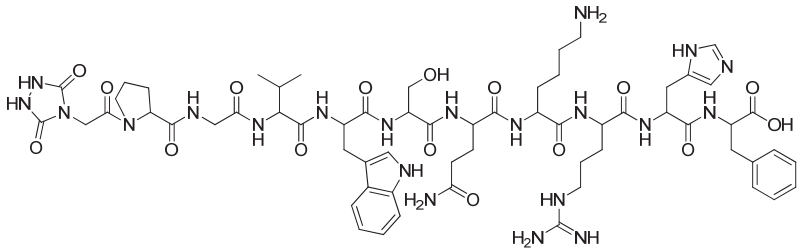
UPLC/ESI-MS m/z [calc.] = 714.79 $(M+2H)^{2+}$, 476.86 $(M+3H)^{3+}$, 357.90 $(M+4H)^{4+}$
m/z [found] = 715.2 $(M+2H)^{2+}$, 477.2 $(M+3H)^{3+}$, 377.5 $(M+4H)^{4+}$

MALDI-TOF-MS m/z [calc.] = 1428.58 $(M+H)^{+}$
m/z [found] = 1428.8 $(M+H)^{+}$

The signals can be assigned within ± 1.0 Da accuracy.

Optical rotation $[\alpha, H_2O]_{D,\ 25\ °C}$ = -69,3°

3.3. Preparation of Urazole 3



The resin-bound semicarbazate-containing peptide (**2**) was swollen in DMF for 30 min. prior to reaction. Triazabicyclodecene (TBD, 5 eq.) was dissolved in 2 mL of DMF and added to the resin. The mixture was shaken at room temperature for 3 h. Afterwards, the resin was washed five times with DMF and DCM each and dried under vacuum. Subsequent cleavage from resin and deprotection was realized via treatment with 95:2.5:2.5 v/v/v (TFA/H_2O/TES) for 2 h. The crude product was precipitated by addition of cleavage solution to ice cold ether. The peptide could be used without further purification (yield 78%). The product was obtained with a purity of 68% calculated from UPLC measurements resulting in a yield of 84%.

Analysis of Urazol + GPGVWSQKRHF

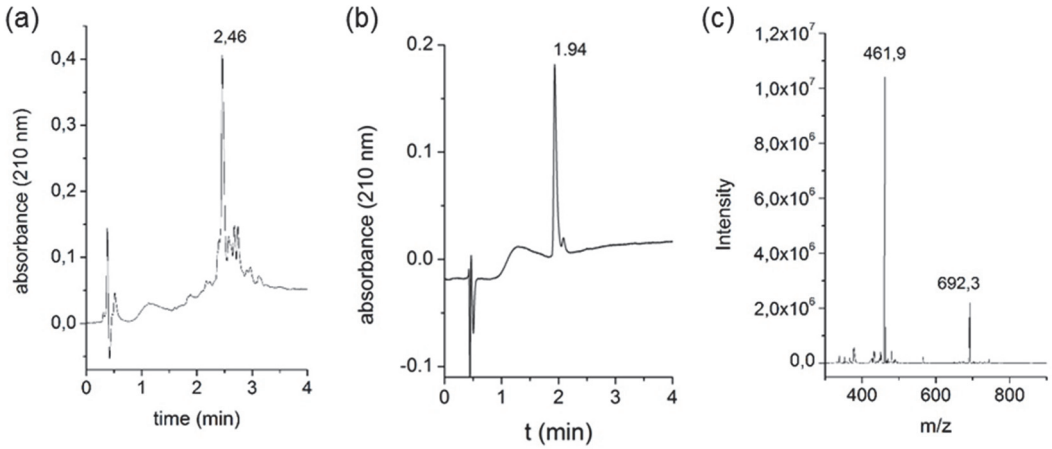


Figure S3. (a) UPLC of crude Urazole-GPGVWSQKRHF (**3**) using a gradient of 5 % - 40 % acetonitrile in ultrapure water within 4 minutes (t_R = 2.46 min); (b) UPLC of purified Urazole-GPGVWSQKRHF (**3**, 96%) using a gradient of 5 % - 50 % acetonitrile in ultrapure water within 4 minutes (t_R = 1.94 min); (c) ESI-MS of the product peak.

UPLC/ESI-MS m/z [calc.] = 692.24 $(M+2H)^{2+}$, 461.82 $(M+3H)^{3+}$

m/z [found] = 692.3 $(M+2H)^{2+}$, 461.9 $(M+3H)^{3+}$

MALDI-TOF-MS m/z [calc.] = 1383.52 $(M+H)^{+}$

m/z [found] = 1383.0 $(M+H)^{+}$

The signals can be assigned within ± 1.0 Da accuracy.

Optical rotation $[\alpha, H_2O]_{D,\ 25\ °C}$ = -71,4°

FT-IR [υ in cm^{-1}]: 3291 (broad, medium), 2934 (weak), 2875 (weak), 1654 (strong), 1533 (medium), 1450 (medium), 1199 (medium), 1131 (medium), 1049 (weak), 835 (weak), 800 (weak), 748 (weak), 720 (weak), 622 (weak).

^{1}H-NMR

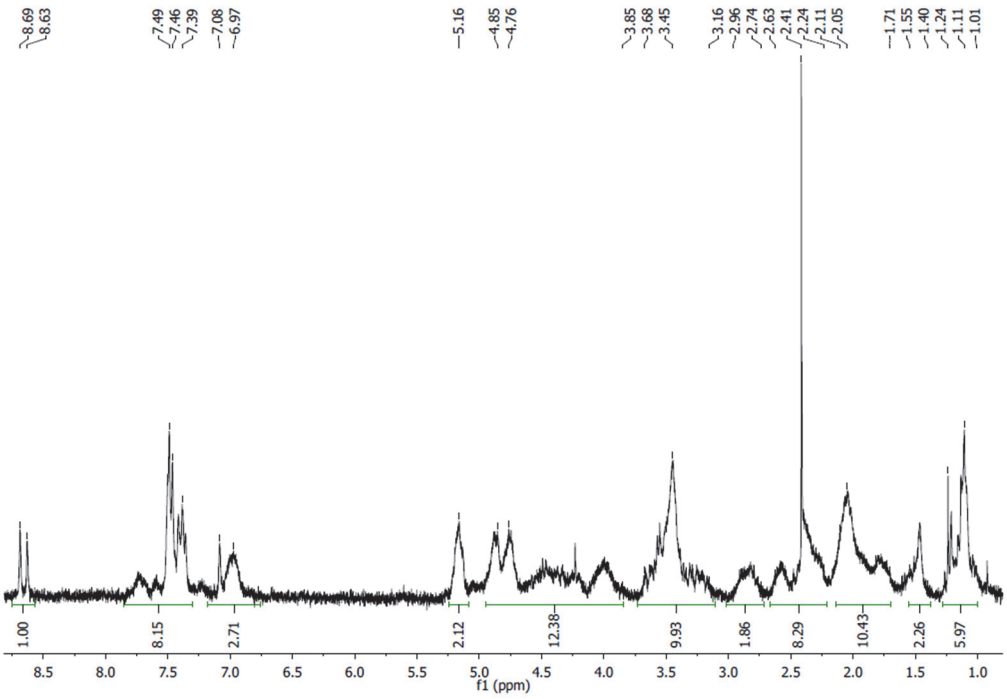
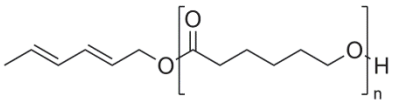


Figure S4. NMR of crude Urazole-GPGVWSQKRHF (**3**) in TFA-d_1.

^{1}H-NMR (500 MHz, TFA-d_1) [δ in ppm]: 8.66 (d, *J = 17.7 Hz*, 1H, $CH_{arom.,H}$), 7.84 – 7.29 (m, 8H, 5x$CH_{arom.,F}$, 2x$CH_{arom.,W}$, $CH_{arom.,H}$), 7.04 – 6.83 (m, 3H, 3×$CH_{arom.,W}$), 5.26 – 5.08 (m, 2H, $CH_{\alpha(H,W)}$), 4.94 – 3.85 (m, 12H, $CH_{\alpha(F,S,P,Q,R,K,V)}$, 2x$CH_{2(G)}$, $CH_{(V)}$), 3.68 – 3.16 (m, 10H, $CH_{2(P,H,S,W,F)}$), 2.96 – 2.74 (m, 2H, $CH_{2(R)}$), 2.63 – 2.24 (m, 8H, $CH_{2(K,2×Q,R)}$), 2.11 – 1.71 (m, 10H, $CH_{2(P)}$, $CH_{2(R,2×K,2×P)}$), 1.55 – 1.40 (m, 2H, $CH_{2(K)}$), 1.24 – 1.01 (m, 6H, 2×$CH_{3(V)}$).

3.4. Synthesis of diene end-functionalized poly(ε-caprolactone)s (5b,c)



A solution of *trans,trans*-2,4-hexadien-ol (HDEO) (see Table S1) in dry toluene was added to a solution of ε-caprolactone (1 g, 8.76 mmol) in dry toluene (5 ml) in a dry 50 ml two-neck round-bottom flask. The polymerization was initiated by the addition of Novozyme 435 (100 mg, 10 wt%) and the mixture was stirred for several hours (see Table S1) at 50°C in the dark under a nitrogen atmosphere. After reaction, the mixture was cooled and chloroform (~ 10 ml) was added to quench the reaction. Novozyme 435 was removed by filtration. Afterwards, the solvents (toluene and chloroform) were removed *in vacuo* and the PCL was purified by precipitation in cold methanol. The synthetic procedure was applied to synthesize PCL with two different molecular weights.

Table S1. Overview of amounts of HDEO used for each PCL synthesis. The amounts of toluene used to dissolve HDEO and reaction times are included.

Product	Amount of initiator HDEO (mg)	Amount of initiator HDEO (mmol)	Reaction time (h)
PCL[$M_n = 3{\cdot}10^3$]	32.4	0.33	5
PCL[$M_n = 8{\cdot}10^3$]	9.80	0.10	6

The polymers were characterized by ^{1}H NMR, FT-IR and SEC.

Table S2. Molecular weight analysis by NMR and SEC.

Polymer PCL-expected molecular weight	Molecular weight (SEC)	Molecular weight (analyzed by ^{1}H-NMR)
$M_n = 3.0*10^3$ g/mol	$M_n = 4.5*10^3$ g/mol, Đ = 1.5	$M_n = 3.5*10^3$ g/mol
$M_n = 8.0*10^3$ g/mol	$M_n = 8.6*10^3$ g/mol, Đ = 1.6	$M_n = 8.9*10^3$ g/mol

Characterization of diene end-functionalized PCL_{30} (5b)

^{1}H-NMR (500 MHz, $CDCl_3$) [δ in ppm]: 6.18 (dd, 1H, *J* = 15.2, 10.6 Hz, CH_{HDEO}), 6.05 - 5.92 (m, 1H, CH_{HDEO}), 5.73 - 5.63 (m, 1H, CH_{HDEO}), 5.61 - 5.50 (m, CH_{HDEO}), 4.50 (d, 2H, *J* = 6.64 Hz, $CH_{2,HDEO}$), 3.99 (t, 60H, *J* = 6.63 Hz, $CH_{2,PCL}$), 2.28 - 2.20 (m, 60H, $CH_{2,PCL}$), 1.70 (d, 3H, *J* = 6.66 Hz, $CH_{3,HDEO}$) 1.64 - 1.52 (m, 120H, $CH_{2,PCL}$), 1.36 - 1.27 (m, 60H, $CH_{2,PCL}$).

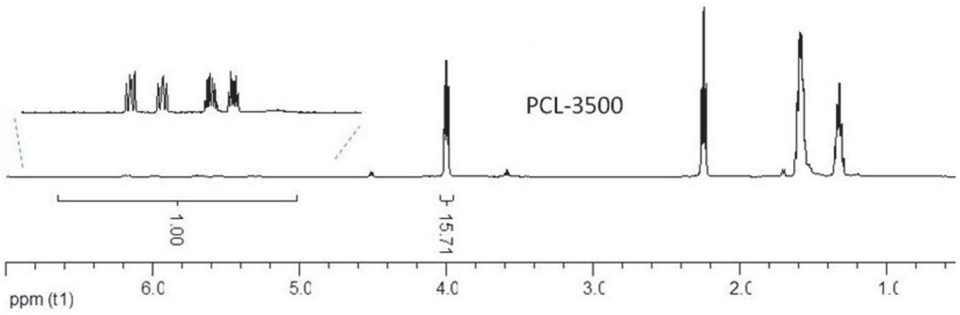


Figure S5. ^{1}H NMR spectrum of diene-functionalized PCL_{30}.

FT-IR [υ in cm^{-1}]: 2946 (strong), 2867 (medium), 1722 (strong), 1471 (medium), 1417 (weak), 1398 (weak), 1366 (medium), 1294 (medium), 1239 (medium), 1168 (strong), 1107 (weak), 1045 (medium), 960 (medium), 935 (weak), 732 (medium).

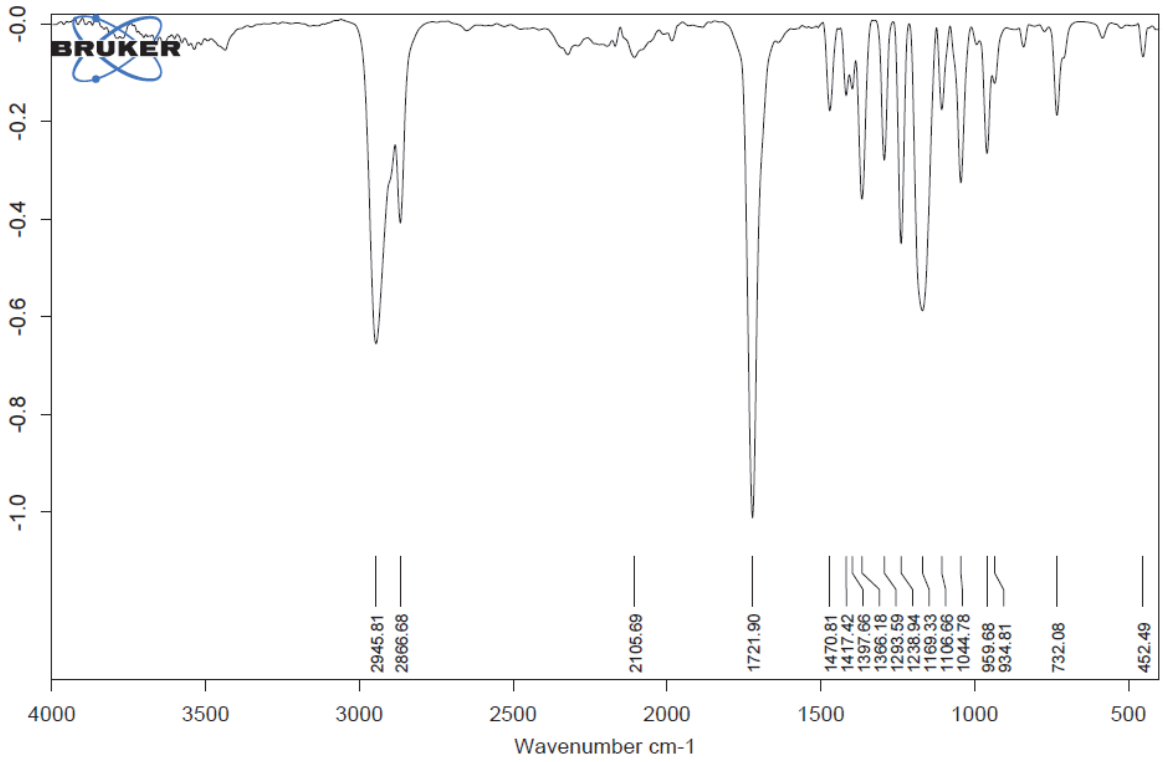


Figure S6. FT-IR-spectrum of diene-functionalized PCL_{30}.

Characterization of diene end-functionalized PCL_{77} (5c)

^{1}H-NMR (500 MHz, $CDCl_3$) [δ in ppm]: 6.18 (dd, 1H, *J* = 15.1, 10.6 Hz, CH_{HDEO}), 6.03 - 5.91 (m, 1H, CH_{HDEO}), 5.73 - 5.64 (m, 1H, CH_{HDEO}), 5.59 - 5.51 (m, CH_{HDEO}), 4.50 (d, 2H, *J* = 6.60 Hz, $CH_{2,HDEO}$), 3.99 (t, 156H, *J* = 6.7 Hz, $CH_{2,PCL}$), 2.24 (t, 156H, *J* = 7.5 Hz, $CH_{2,PCL}$), 1.74 - 1.67 (m, 3H, $CH_{3,HDEO}$) 1.66 - 1.40 (m, 312H, $CH_{2,PCL}$), 1.40 - 1.22 (m, 156H, $CH_{2,PCL}$).

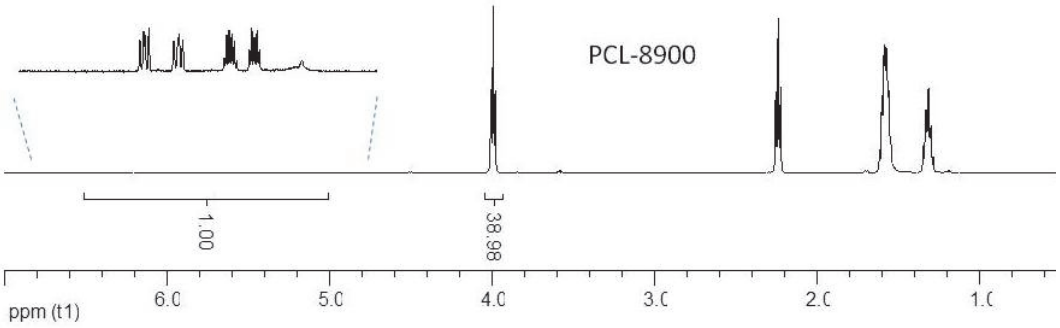


Figure S7. ^{1}H NMR spectrum of diene-functionalized PCL_{77}.

FT-IR [υ in cm^{-1}]: 2946 (strong), 2867 (medium), 1722 (strong), 1471 (medium), 1417 (weak), 1398 (weak), 1366 (medium), 1294 (medium), 1239 (medium), 1168 (strong), 1107 (weak), 1045 (medium), 960 (medium), 935 (weak), 732 (medium).

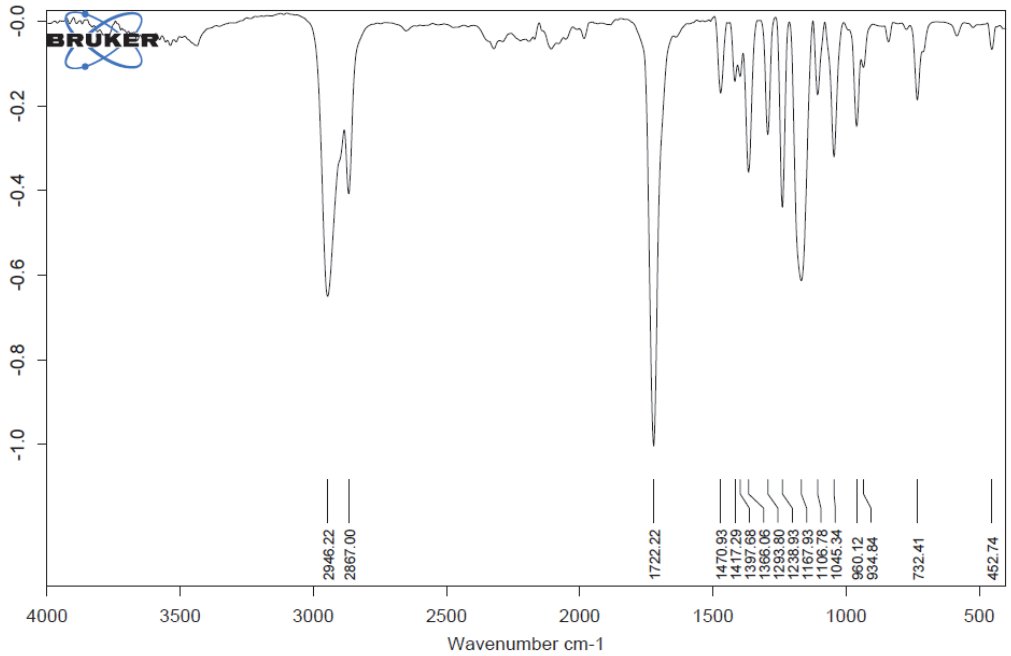
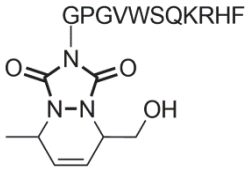


Figure S8. FT-IR-spectrum of diene-functionalized PCL_{77}.

3.5. Activation of Urazole and ligation to *trans,trans*-2,4-hexadien-ol (HDEO)



To show the efficiency of the TAD-diene ligation, purified urazole-containing peptide **3** was used. Activation to triazolinedione was achieved by addition of the peptide (5 mg, 3.6 µmol, 10 eq.) to a solution of 1.1 eq. (0.7 mg, 4.0 µmol) *N*-Bromosuccinimide and 1.1 eq. (0.32 µL, 4.0 µmol) of pyridine in 20 µL of dry DMF. The solution was then shaken at 4°C for 15 minutes under inert atmosphere. The solution was then added to 1 eq. (0.04 µL, 36 nmol) of HDEO in 10 µL DMF/DCM (9:1) and incubated at room temperature for 30 min. UPLC-ESI-MS experiments showed a quantitative reaction to the Diels-Alder adduct **6a** (cf. main text Fig. 3a).

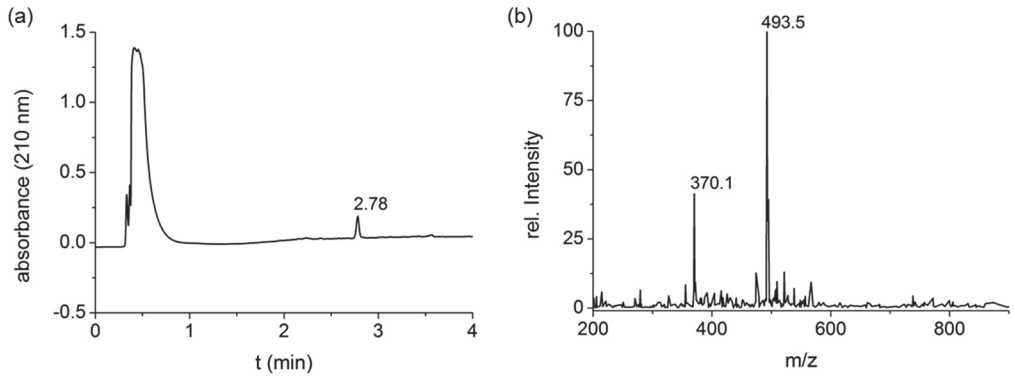


Figure S9. (a) UPLC chromatogram of the reaction mixture after 30 min. showing a complete converstion to the Diels-Alder adduct. Measurements were performed using a gradient of 5 % - 40 % acetonitrile in ultrapure water within 4 minutes. The large initiation peak results from DMF/DCM in the reaction mixture. (b) ESI-MS spectra of the product peak.

UPLC/ESI-MS m/z[calc.] = 493.87 $(M+3H)^{3+}$, 370.65 $(M+4H)^{4+}$

m/z[found] = 493.5 $(M+3H)^{3+}$, 370.1 $(M+4H)^{4+}$

The signals can be assigned within ± 1.0 Da accuracy.

3.6. Tyrosine reference experiment

The urazole-containing peptide **3** (5 mg, 3.6 µmol, 5 eq.) was activated as previously described (cf. chapter 3.5.) and was then added to tyrosine (0.13 mg, 0.72 µmol, 1 eq.) in 100 µl of 20% acetonitrile (%v/v, in ultrapure water). The resulting solution was then shaken for 30 min. at room temperature. Subsequent evaluation was performed via UPLC-ESI-MS experiments from the reaction mixture. No formation of a tyrosine-peptide adduct was apparent.

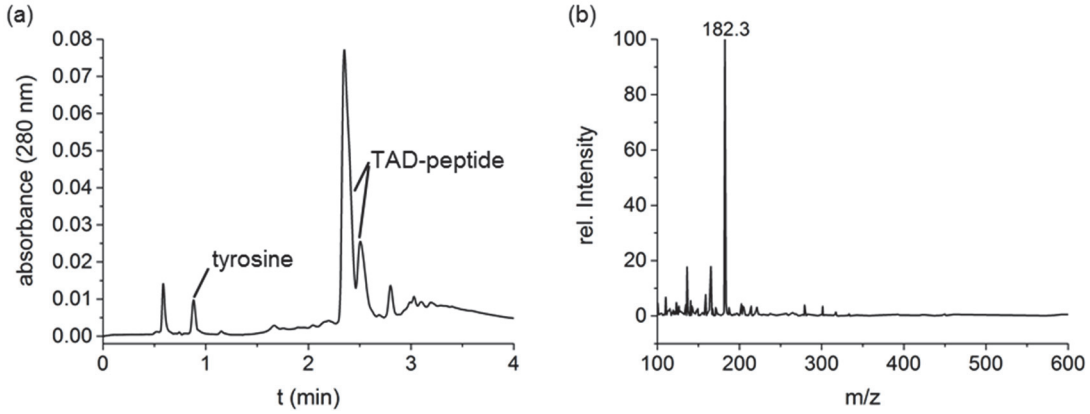
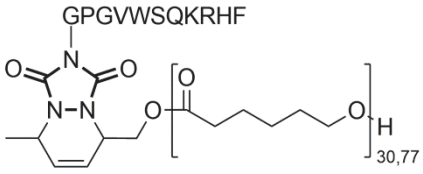


Figure S10. (a) UPLC chromatogram of the reaction mixture of **4** with tyrosine after 30 min. showing no formation of the corresponding Diels-Alder adduct. Measurements were performed using a gradient of 5 % - 50 % acetonitrile in ultrapure water within 4 minutes. Additional peaks result from the reaction mixture and cannot be assigned to a tyrosine-peptide adduct. (b) ESI-MS spectra of the tyrosine peak.

3.7. Activation of urazole and ligation to PCL_{30} and PCL_{77}



The urazole-containing peptide **3** (5 mg, 3.6 µmol, 5 eq.) was activated as previously described (cf. chapter 3.5.) and was then added to poly(ε-caprolactone) (PCL_{30}: 2.5 mg, 0.7 µmol, 1 eq.; PCL_{77}: 6.4 mg, 0.7 µmol, 1 eq.) in 10-20 µl of anhydrous DMF/DCM (9:1). The resulting solution was then shaken for 30 min. at room temperature accompanied by a reduced intensity of the red color of TAD. The product was precipitated by addition of 1:1 v/v (MeOH/H_2O) and discarding of the solvent. The crude peptide-polymer conjugate was then redissolved in a minimal amount of DCM and precipitated by addition of 1:1 v/v (MeOH/H_2O). After removal of the solvent the purification step was repeated once more and the final product dried under vacuum. The conjugates were characterized by SEC, NMR and FT-IR.

Analysis of Peptide-PCL_{30} (6b)

For SEC analysis cf. main text Fig. 4c.

Table S3. Analysis of the bioconjugates by SEC.

Peptide-polycaprolactone bioconjugations	Molecular weight of the diene functionalized polycarolactone	Molecular weight of the conjugate
Peptide-TAD + PCL_{30}	M_n= 4.5*10^3 g/mol, Đ = 1.5	M_n= 7.0*10^3 g/mol, Đ = 1.4

FT-IR [υ in cm^{-1}]: 3306 (weak), 2946 (strong), 2867 (medium), 1722 (strong), 1661 (medium), 1536 (weak), 1469 (medium), 1417 (weak), 1398 (weak), 1366 (medium), 1294 (medium), 1239 (medium), 1180 (strong), 1101 (weak), 1045 (medium), 960 (medium), 935 (weak), 732 (medium), 709 (weak).

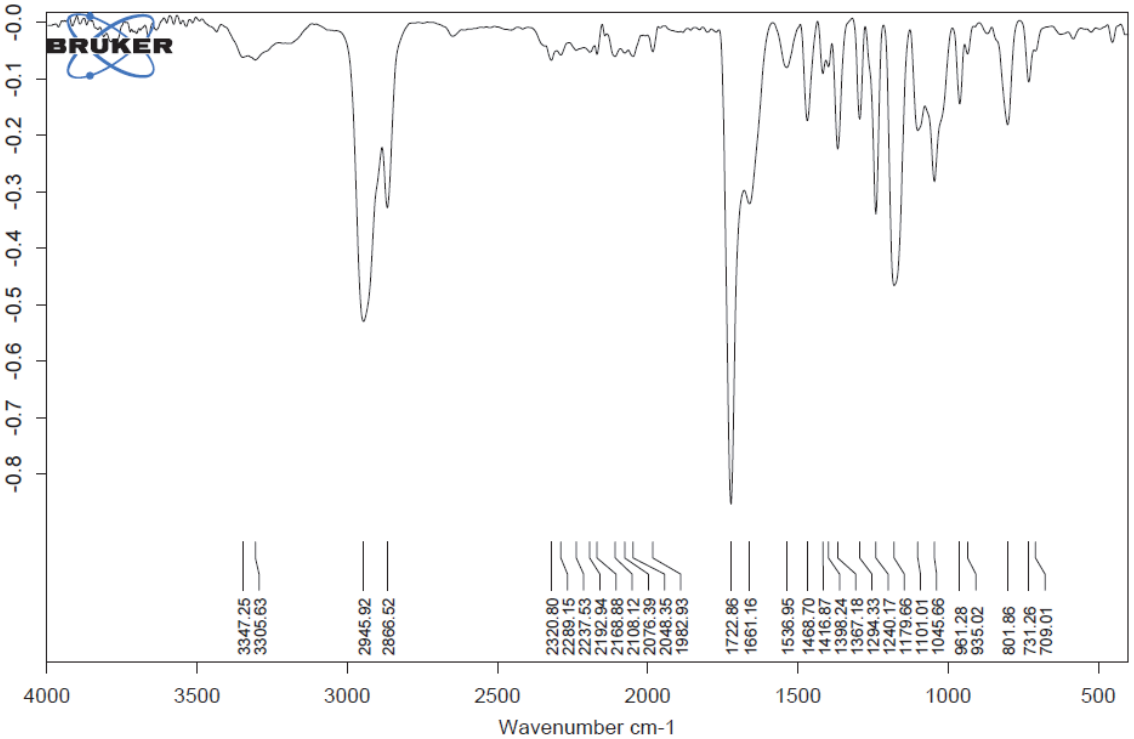


Figure S9. FT-IR-spectrum of the peptide-PCL_{30} conjugate (**6b**).

^{1}H-NMR experiments revealed the typical peaks for PCL and peptide. A quantitative shift of the PCL_{30} diene signals between 6.3 - 5.4 ppm to the newly formed double bond at 5.85 - 5.65 ppm indicates successful conversion to the peptide-PCL_{30} conjugate. Further comparison of the integrals for the newly formed double bond and the characteristic histidine C-2 proton at ~8 ppm reveals a 1:1 composition of the conjugate.

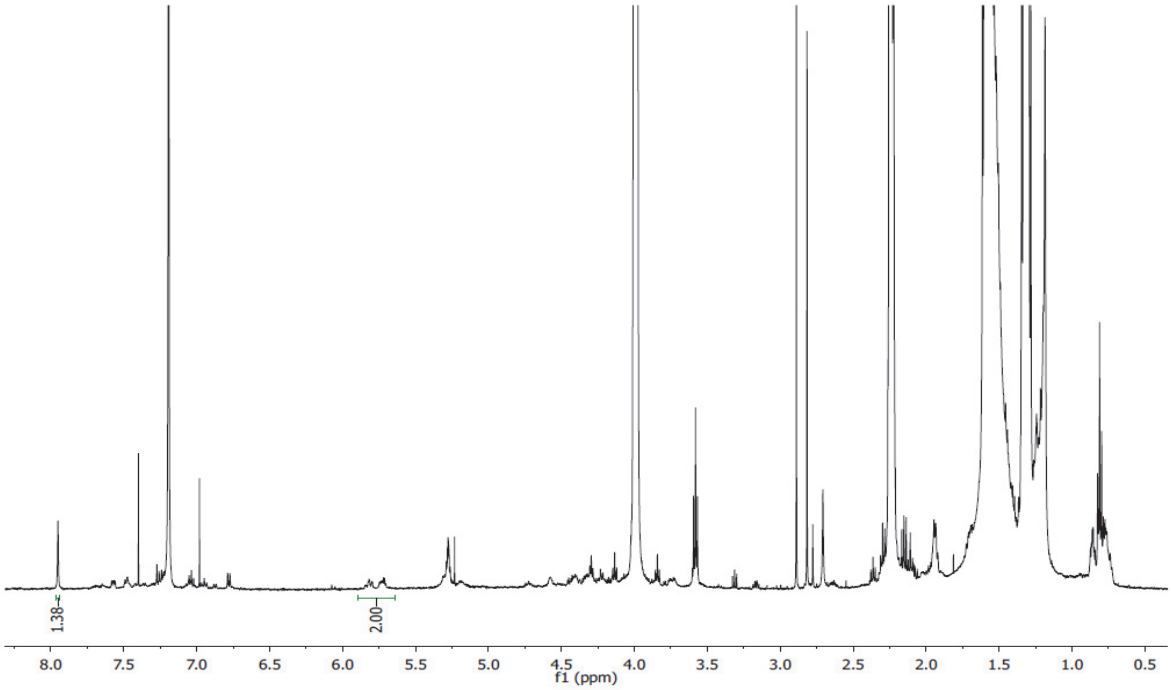


Figure S10. ^{1}H-NMR spectrum of peptide-PCL_{30} conjugate (**6b**).

Analysis of Peptide-PCL_{77} (6c)

For SEC analysis cf. main text Fig. 4c.

Table S4. Analysis of the bioconjugates by SEC.

Peptide-polycaprolactone bioconjugations	Molecular weight of the diene functionalized polycarolactone	Molecular weight of the conjugate
Peptide-TAD + PCL_{77}	M_n= 8.6*10^3 g/mol, Đ = 1.6	M_n= 11.5*10^3 g/mol, Đ = 1.6

FT-IR [υ in cm^{-1}]: 2946 (strong), 2867 (medium), 1722 (strong), 1661 (weak), 1471 (medium), 1417 (weak), 1398 (weak), 1366 (medium), 1294 (medium), 1239 (medium), 1168 (strong), 1107 (weak), 1045 (medium), 960 (medium), 935 (weak), 732 (medium).

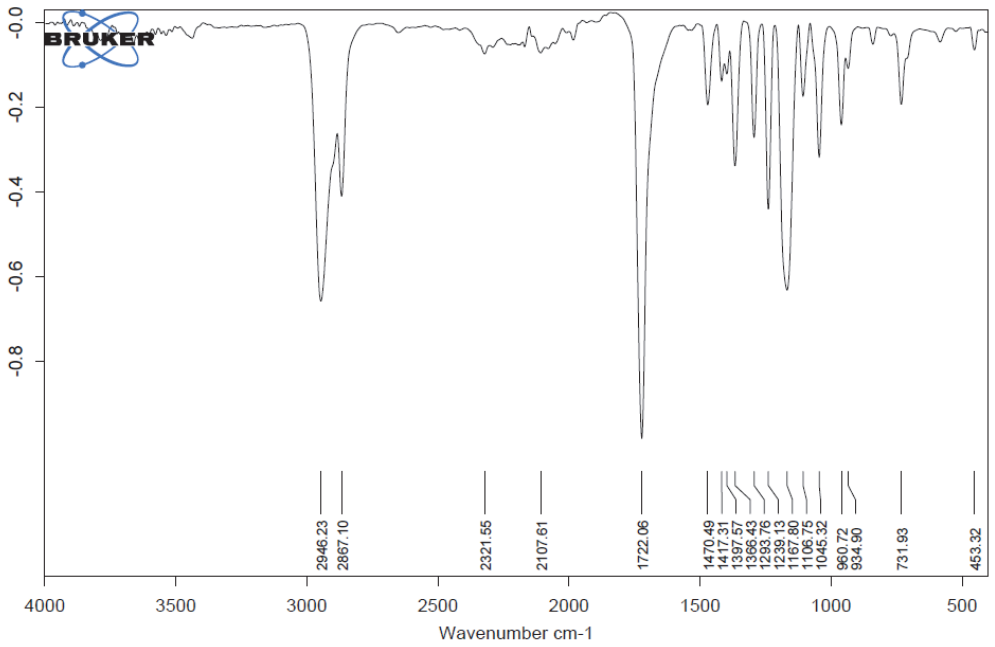


Figure S11. FT-IR-spectrum of the peptide-PCL_{77} conjugate (**6c**).

^{1}H-NMR experiments revealed the typical peaks for PCL. Peptide peaks as well as peaks for the newly formed double bond are broadened, which can be expected from higher molecular weight conjugates. Characteristic peaks corresponding to the diene-carrying educt were not observed, indicating an effective conjugation to the peptide-PCL_{77} conjugate **6c**.

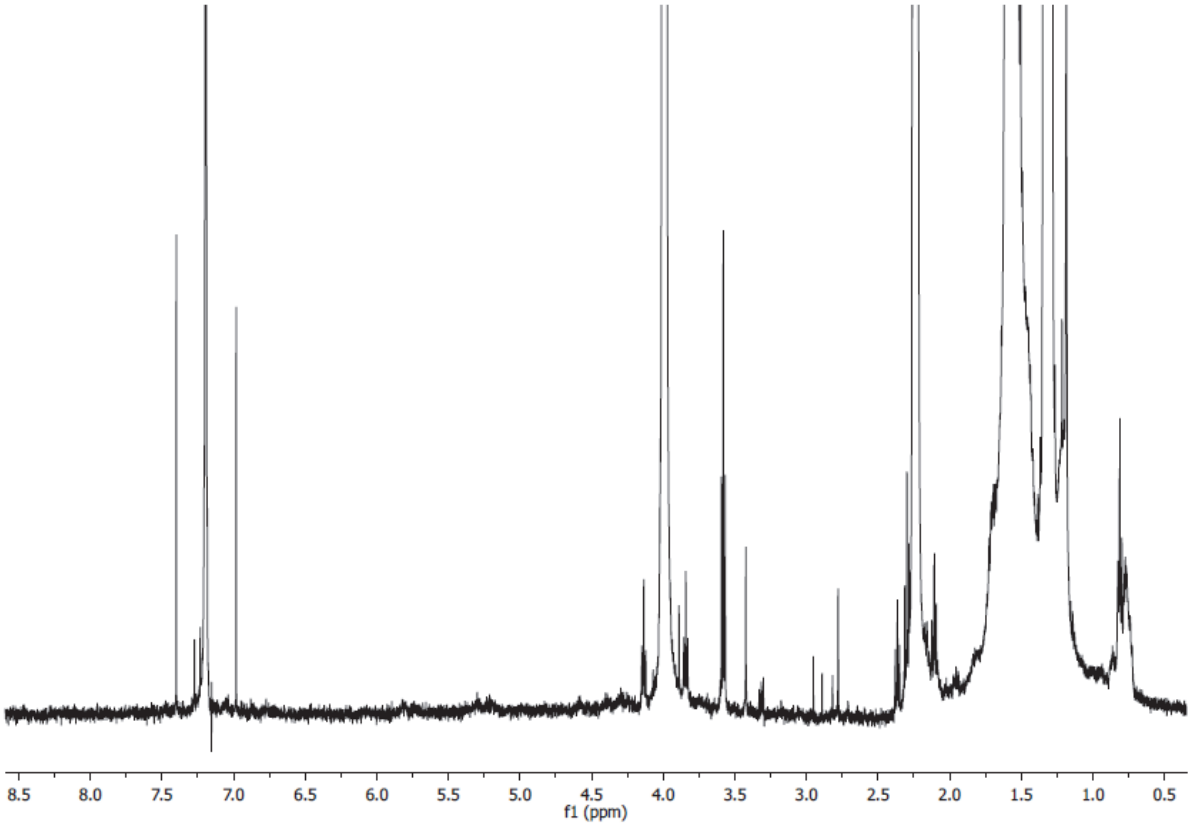


Figure S12. ^{1}H-NMR spectrum of peptide-PCL_{77} conjugate (**6c**).