# Supporting Information

## Construction of homologous branched oligomer megamolecules based on linker-directed protein assembly

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## Table of Contents

## Experimental section

### 1 Materials

The chemical and biological reagents used in linker syntheses and protein expression were purchased from Sigma-Aldrich, Thermo Fisher Scientific Inc. and Alfa Aesar, except as otherwise noted. Ultrapure water was used during all experiments. Mini-Protean TGX precast protein gels (4-15%) and 10x Tris/glycine/SDS running buffer were purchased from Bio-RAD. HiMark unstained high molecular weight protein standard (40-500 kDa), NuPAGE tris-acetate gel (3–8%), tris-acetate SDS running buffer and BenchMark protein ladder (10-220 kDa) were purchased from Thermo Fisher Scientific Inc..

### 2 Methods

**2.1 Recombinant protein design, synthesis, and purification.** We used pET21b(+) vector and golden gate cloning methods to construct the expression plasmids of cutinase and Cut-SnapTag. C-terminal 6×His Tag was introduced as metal affinity tags for protein purification.[1-3] The plasmids were transferred and expressed in Shuffle T7 express competent *E. coli* cell upon β-D-1-thiogalactopyranoside (IPTG) induction. The protein purification was carried out by IMAC using HisPur cobalt resin (Thermo Fisher Scientific). The size exclusion chromatography (SEC) was executed using AKTA FPLC (GE Healthcare) equipped with a Superdex 200 Increase 10/300 GL column. The column is suitable for the characterization and purification of proteins with molecular weights between 10 kDa and 600 kDa. The elution was performed using 1× PBS buffer (137 mM NaCl, 10 mM $Na_2HPO_4$, 1.8 mM $KH_2PO_4$, 2.7 mM KCl, pH 7.4) at a fixed flow rate of 1 mL/min. [4,5] The purified recombinant protein was characterized by SDS-PAGE and LC/MS.[6]

**2.2 Linker synthesis.** The detailed synthesis information of conjugation linkers was provided in Supporting Information. $^1$H NMR characterization was performed on the Bruker Avance III HD 500 MHZ system (TXO 5mm Prodigy probe w/ Z-Gradient). The mass spectrometry of synthetic linkers was recorded on Bruker AmaZon SL LC/MS mass

spectrometer and 5800 MALDI-TOF/TOF mass spectrometer (AB Sciex, Framingham, MA).

**2.3 Octamer I and II megamolecules syntheses and purification.** For the octamer I section, cutinase (50 μM) and linker **1** (10 μM) were incubated in PBS buffer for 3 h at ice bath and then purified by SEC to obtain the trimer of $Cut_3$-yne. And the Cut-SnapTag fusion protein (50 μM) and linker **2** (60 μM) were incubated in HEPES buffer for 3 h, and then washed by HEPES buffer using Amicon Ultra centrifugal filter unit with 10 kDa cutoff. The click reaction was carried out in HEPES buffer (1 mM), the pure trimer of $Cut_3$-yne (20 μM) and azide-tagged Cut-SnapTag fusion protein (30 μM) were mixed by Vortex Mixer. Moreover, $CuSO_4$ solution (10 mM) and sodium ascorbate solution (20 mM) were pre-mixed for 1 minute, and then added to the click reaction mixture to reach the copper ion concentration of 0.5 mM. After 12 h reaction, the mixture was washed by HEPES buffer using 30 kDa cutoff filter unit, and then purified by SEC (GE Healthcare AKTA FPLC system) to obtain $Cut_3$-Triazole-SnapTag-Cut using the same procedure as described above. The final product of octamer I megamolecule was acquired after the 6 h incubation of $Cut_3$-Triazole-SnapTag-Cut (20 μM) and linker **3** (10 μM), which was purified by SEC.

For the octamer II section, cutinase (100 μM) and linker **4** (Cut-EG7-SnapTag, 120 μM) were incubated in PBS buffer for 3 h at room temperature, and the mixture was then washed by PBS buffer using 10 kDa cutoff filter unit to remove the excess linker. The Cut-SnapTag fusion protein (50 μM) was added to reach half the ratio of cutianse-bcp, and the reaction was carried out for 3 h. After SEC purification, the pure Cut-SnapTag-Cut (50 μM) was then incubated with linker **5** (10 μM) for 3 h. The pure octamer II megamolecule of tetra(Cut-SnapTag-Cut) was obtained after SEC purification (GE Healthcare AKTA FPLC system).

**2.4 Mass spectrometry for protein analysis.** We use Agilent 6210A time-of-flight (TOF) mass spectrometer to perform liquid chromatography-mass spectrometry (LC-MS) protein analysis.[7] C18 trap column (Waters) was employed for HPLC separation on Agilent 1200

series. The mass analysis and deconvolution were completed using Agilent MassHunter Qualitative Analysis B.04.00.[8,9]

**2.5 Protein morphology analysis.** The sample was treated following the negative staining procedure.[10] Typically, 3.5 µl of sample was absorbed for 60 seconds on the grids of TEM carbon supporting film. Thereafter, the grids were washed twice by floating in distilled water and blotting with filter paper. The negative staining was followed by applying the grid on 0.75% (w/v) uranyl formate solution twice for 20 seconds, and the excess staining agent was removed by filter paper. Afterwards the grid was left open to air for complete dry (60 minutes). The octameric megamolecule was imaged using a Hitachi HT-7700 biological TEM at 100 kV with an Orius SC 1000A camera (Gatan, CA).

A 20 µL sample of octamer I megamolecule (50µg·mL$^{-1}$) was applied to a mica substrate, incubated for 60 seconds, then rinsed with Milli-Q water and dried with a stream of clean air. AFM images were obtained using a Bruker Innova atomic force microscope, operating in tapping mode and under ambient conditions.

## Recombinant Protein Construction[11-13]

**Cutinase-SnapTag sequence** (This recombinant fusion protein was obtained following the reported methods)**:**

Cutinase-$(EAAAK)_4$-SnapTag-Thrombin Sequence-6x His

(Catalytic cutinase S120 underlined in Bold)

MGLPTSNPAQELEARQLGRTTRDDLINGNSASCADVIFIYARGSTETGNLGTLGPSIASNL
ESAFGKDGVWIQGVGGAYRATLGDNALPRGTSSAAIREMLGLFQQANTKCPDATLIAG
GY**S**QGAALAAASIEDLDSAIRDKIAGTVLFGYTKNLQNRGRIPNYPADRTKVFCNTGDL
VCTGSLIVAAPHLAYGPDARGPAPEFLIEKVRAVRGSAGSEAAAKEAAAKEAAAKEAA
AKAAADKDCEMKRTTLDSPLGKLELSGCEQGLHEIIFLGKGTSAADAVEVPAPAAVLGG
PEPLMQATAWLNAYFHQPEAIEEFPVPALHHPVFQQESFTRQVLWKLLKVVKFGEVISY
SHLAALAGNPAATAAVKTALSGNPVPILIPCHRVVQGDLDVGGYEGGLAVKEWLLAHE
GHRLGKPGLGLVPRGSLEHHHHHH

Cutinase-SnapTag Formula: $C_{2014}H_{3188}N_{574}O_{608}S_{10}$ (This formula represents the mature protein with cleaved N-terminal methionine and containing 2 disulfide bonds)

Molecular weight: 45490.87 Da

$\xi_{280}$ = 35598 $M^{-1}$ $cm^{-1}$

**Cutinase sequence** (This recombinant protein was obtained following the reported sequence and methods)**:**

Cutinase-6x His (Catalytic cutinase S120 underlined in Bold)

MGLPTSNPAQELEARQLGRTTRDDLINGNSASCADVIFIYARGSTETGNLGTLGPSIASNL
ESAFGKDGVWIQGVGGAYRATLGDNALPRGTSSAAIREMLGLFQQANTKCPDATLIAG
GY**S**QGAALAAASIEDLDSAIRDKIAGTVLFGYTKNLQNRGRIPNYPADRTKVFCNTGDL
VCTGSLIVAAPHLAYGPDARGPAPEFLIEKVRAVRGSALEHHHHHH

Cutinase Formula: $C_{1018}H_{1618}N_{304}O_{319}S_5$ (This formula represents the mature protein with cleaved N-terminal methionine)

Molecular weight: 23380.19 Da

$\xi_{280} = 14690\ M^{-1}\ cm^{-1}$

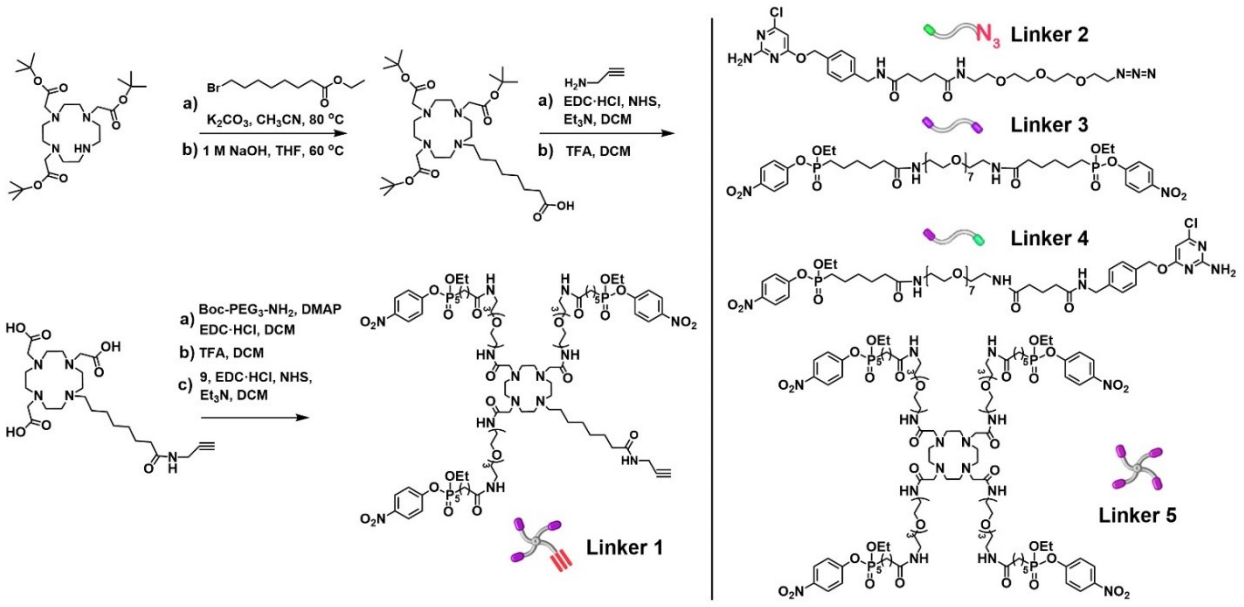



**Fig. S1** Five linkers with variable structures for megamolecules synthesis. (Left) Synthesis route of the branched linker **1** with heterogeneous ending groups of cutinase inhibitor and alkyne for click chemistry-based megamolecule construction. (Right) The molecular structure of linker **2-5** with active conjugation group for protein assembly. Linker **2** is a linear molecule with BCP and azide ending groups. Linkers (**3-5**) consist of the $EG_n$ backbone, and the homogeneous or heterogeneous end groups of pNPP and BCP. The synthetic linkers (**1-5**) initiated linker-directed protein assembly, providing hyperbranched megamolecules with a variety of structures for biomedical applications.

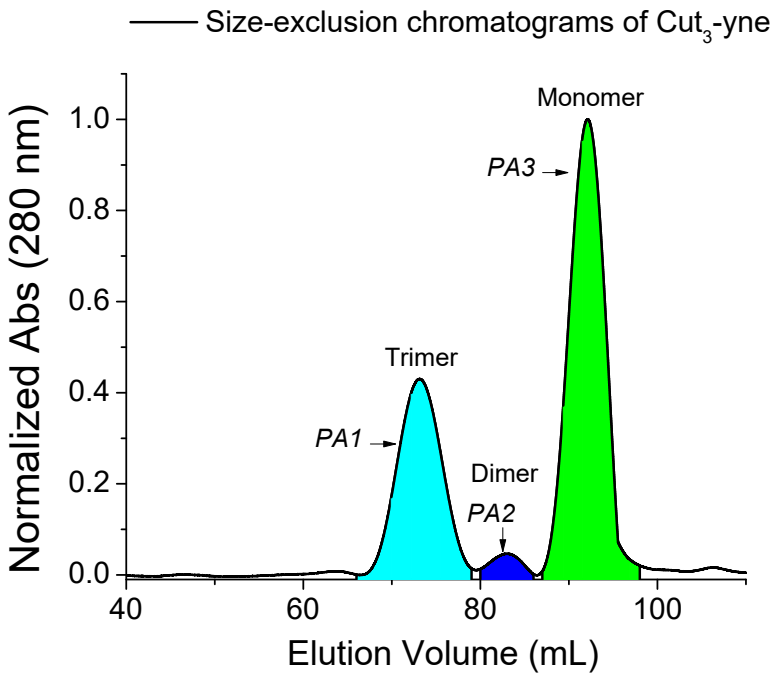



**Fig. S2** Size-exclusion chromatograms of $cut_3$-yne after the 3 h incubation of cutinase (50 μM) and linker **1** (10 μM). We utilized excess cutinase protein to promote the formation of the desired product, cutinase trimer ($Cut_3$-yne). The yield was determined using a semiquantitative approach, employing integrated area measurements based on size-exclusion chromatograms.

The calculation formula is as follows:

$$\text{Cut}_3\text{-yne yield} = \frac{PA1}{(PA1 + PA2 + PA3) \times \frac{3}{5}} = \frac{2.5}{(2.5 + 0.2 + 4.3) \times \frac{3}{5}} \approx 60\ \%$$

**Table S1. The calculated molecular weights of synthetic linkers and megamolecules**

| *Name* | *Formula* | *Molecular weight (Da)* |
|---|---|---|
| **Linker 1** | $C_{91}H_{151}N_{14}O_{31}P_3$ | 2030.20 |
| **Linker 2** | $C_{25}H_{35}ClN_8O_6$ | 579.06 |
| **Linker 3** | $C_{44}H_{72}N_4O_{19}P_2$ | 1023.02 |
| **Linker 4** | $C_{47}H_{71}ClN_7O_{16}P$ | 1056.54 |
| **Linker 5** | $C_{104}H_{172}N_{16}O_{40}P_4$ | 2410.46 |
| **Cut$_3$-yne** | $C_{3127}H_{4990}N_{923}O_{979}S_{15}P_3$ | 71752.37 |
| **Cut$_3$-SnapTag-Cut** | $C_{5162}H_{8209}N_{1502}O_{1592}S_{25}P_3$ | 117677.06 |
| **Octamer I Megamolecule** | $C_{10356}H_{16480}N_{3006}O_{3197}S_{50}P_8$ | 236098.26 |
| **Cut-SnapTag-Cut** | $C_{3069}H_{4868}N_{881}O_{939}S_{15}P$ | 69642.78 |
| **Octamer II Megamolecule of (Cut-SnapTag-Cut)$_4$-L** | $C_{12356}H_{19624}N_{3536}O_{3784}S_{60}P_8$ | 280425.13 |

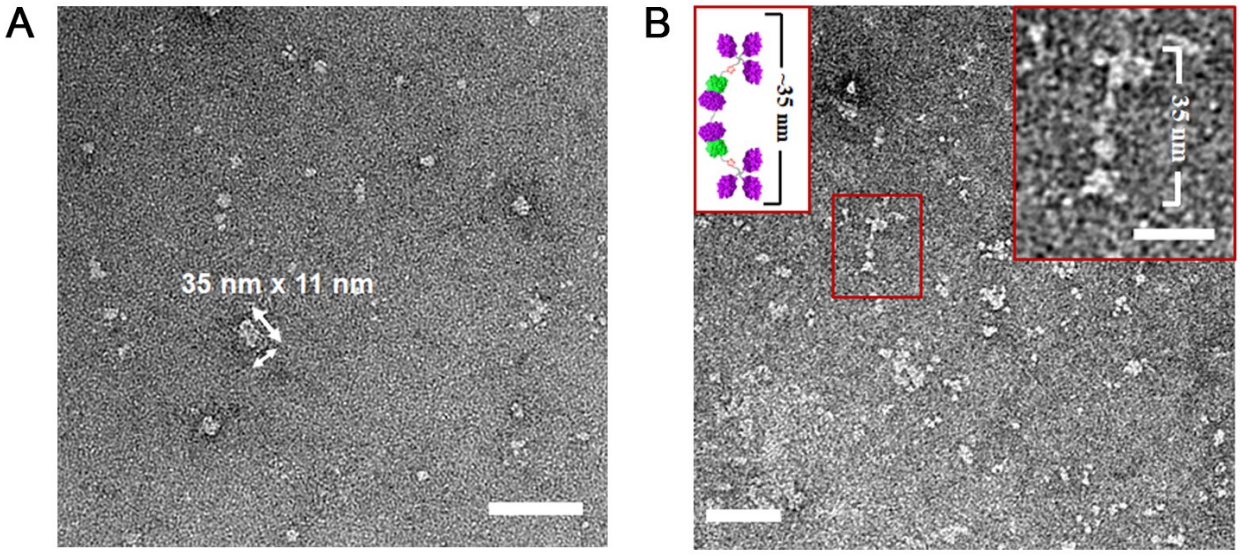



**Fig. S3** (A) The representative architecture of the octamer I is visualized through negative-stain imaging, with the scale bar indicating 100 nm for the full image. (B) The observed TEM images and simulated orientation of octamer I. The scale bars represent 50 nm for the full image and 20 nm for the inset.

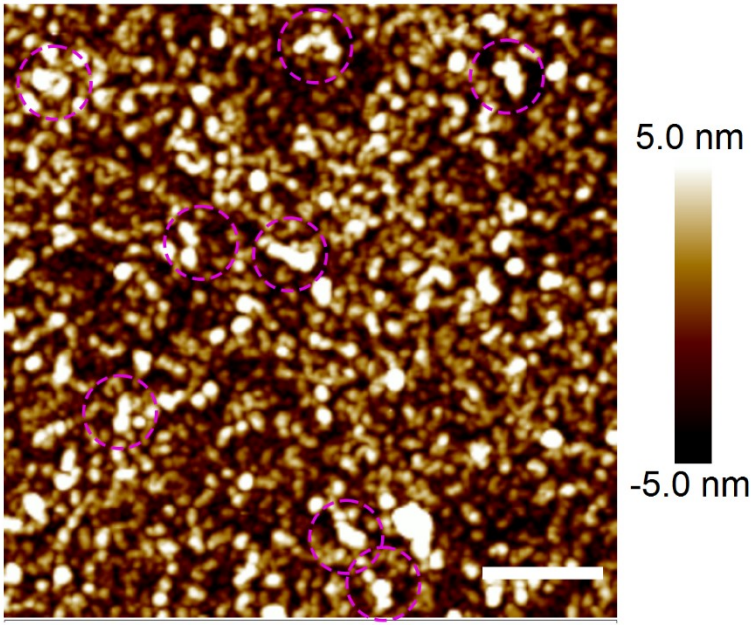



**Fig. S4** AFM image of octamer I megamolecule supported on a mica substrate. The scale bar corresponds to 100 nm. The megamolecule concentration was 50μg·mL$^{-1}$.

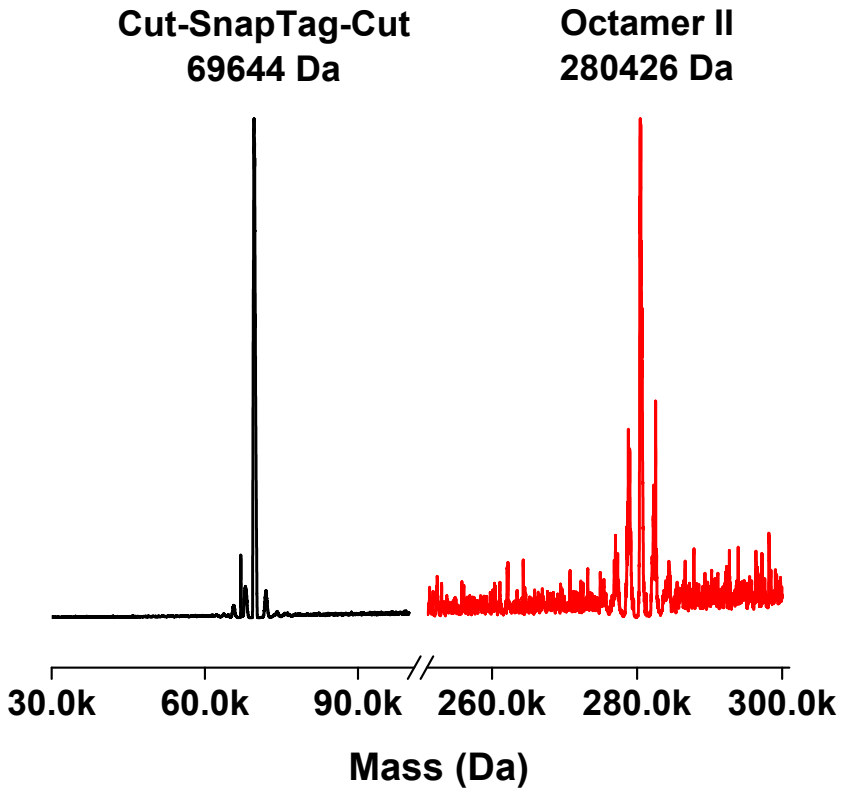



**Fig. S5** The deconvoluted ESI-MS spectra of Cut-SnapTag-Cut and octamer II megamolecules, indicating the mass of 69644 Da and 280426 Da, respectively.

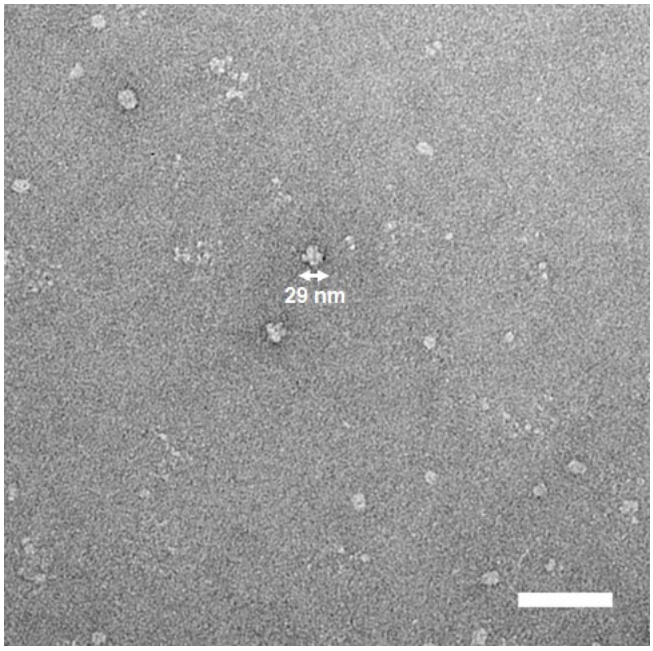
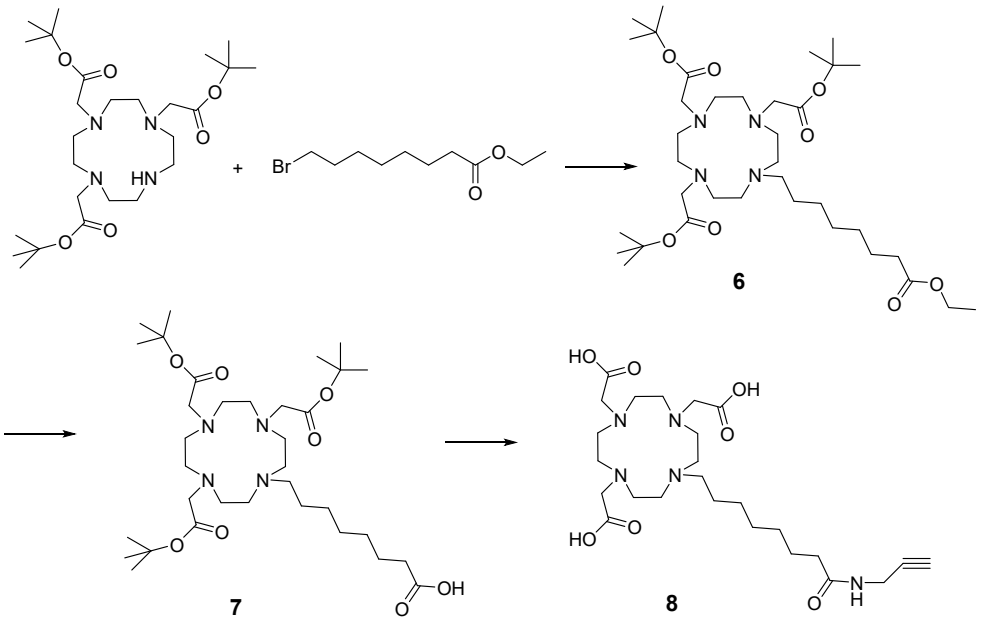


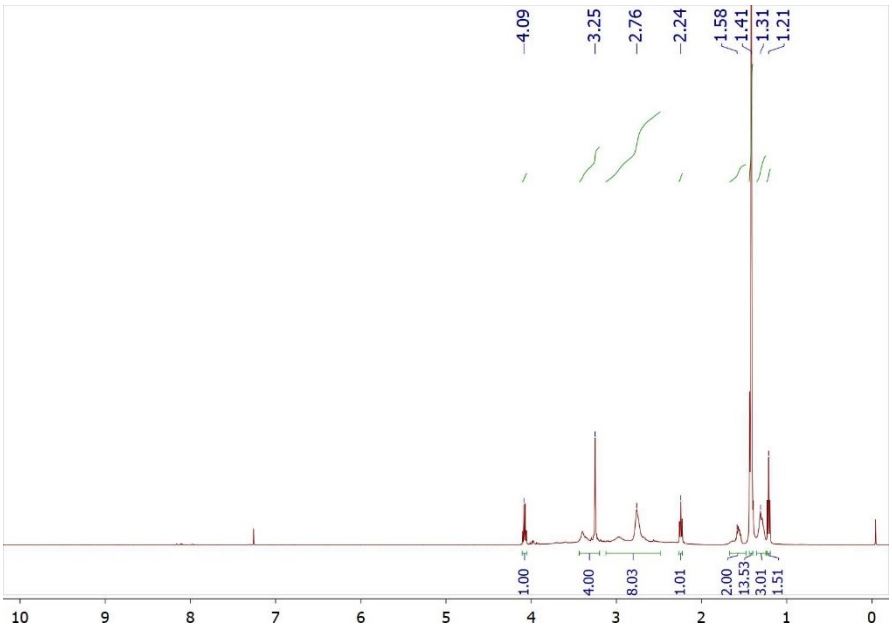

**Fig. S6** The representative architecture of octamer II observed by high-resolution TEM, and the scale bars indicated 100 nm for the full image.

## Linker Syntheses Section

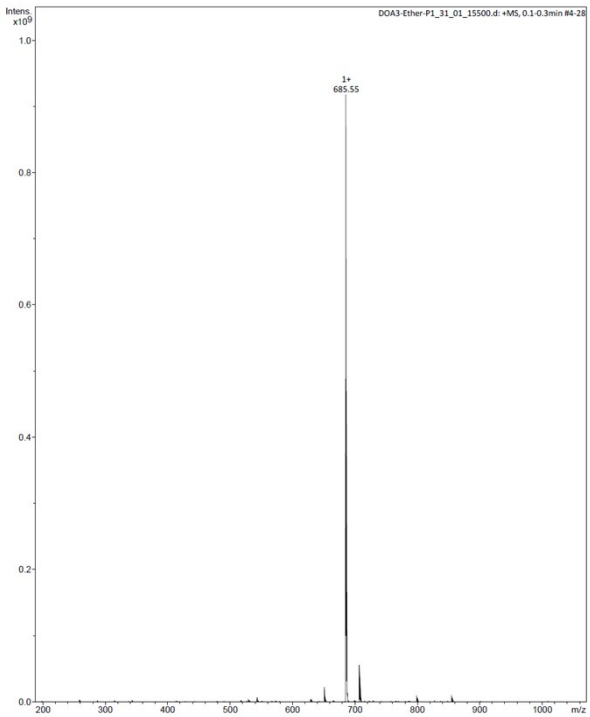



**1-(ethyl octanoate)-4,7,10-tris(*tert*-butylacetate)-1,4,7,10-tetraazacyclododecane (6)[14]**

1,4,7,10-Tetraazacyclododecane-1,4,7-tris(*tert*-butylacetate) (5.15 g, 10 mmol) was dissolved in dry acetonitrile, and then $K_2CO_3$ (6.91 g, 50 mmol) and ethyl 8-bromooctanoate (2.64 g, 10.5 mmol) was added to this solution. The suspension was stirred for 6 h at 80 °C. The $K_2CO_3$ was filtered away and the filtrate was evaporated under reduced pressure. The residue was purified by silica gel chromatography to afford **6** as a viscous liquid (6.51 g, 9.5 mmol, 95%). TLC: hexane/EtOAc, 1:1, v/v. $^1$H-NMR (500 MHz, $CDCl_3$): δ 4.09 (m, 2 H), 3.44-3.19 (m, 8H), 3.12-2.48 (m, 16H), 2.24 (t, $J$ = 7.5 Hz, 2H), 1.67-1.48 (m, 4 H), 1.44-1.40 (m, 27 H), 1.35-1.25 (m, 6 H), 1.21 (t, $J$ = 7.9 Hz, 3H). ESI-MS m/z: calcd for $[M+H]^+$ 685.51, found 685.55.



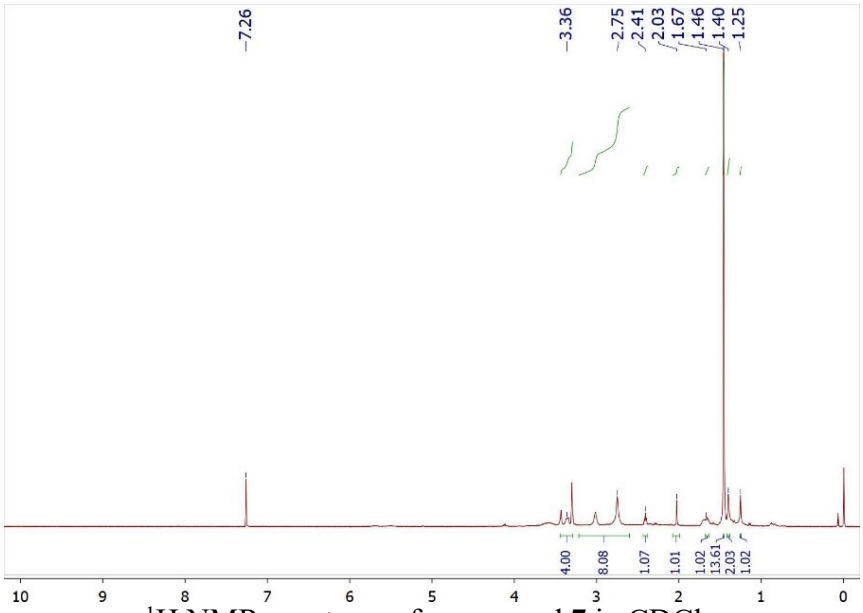

$^1$H NMR spectrum of compound **6** in $CDCl_3$.



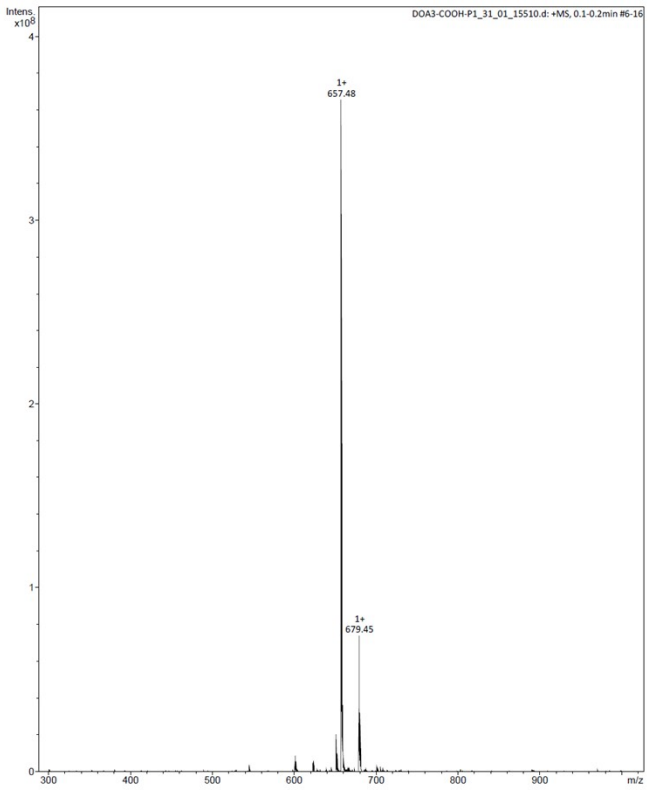

The ESI-MS spectrum of compound **6**.

**1-(octanoic acid)-4,7,10-tris(*tert*-butylacetate)-1,4,7,10-tetraazacyclododecane (7)**

Compound **6** (3.42 g, 5 mmol) was dissolved in 50 mL mixed solution of acetonitrile and 1 M NaOH at 4/1 (v/v). This solution was stirred for 6 h at room temperature. Acetonitrile was evaporated under vacuum, and 50 mL of brine solution was added. After extraction with dichloromethane (3×100 mL), the combined organic phase was dried over anhydrous $Na_2SO_4$, filtered, and evaporated under vacuum. The obtained viscous liquid was purified by silica column chromatography to afford **7** as a colorless oil (2.96 g, 4.5 mmol, 90%). TLC: hexane/EtOAc, 1:1, v/v. $^1$H-NMR (500 MHz, $CDCl_3$): δ 3.44-3.29 (m, 8 H), 3.22-2.60 (m, 16H), 2.44-2.38 (m, 2H), 2.03 (s, 2H), 1.68-1.64 (m, 2 H), 1.46 (s, 27 H), 1.40 (s, 4 H), 1.46 (s, 27 H). ESI-MS m/z: calcd for $[M+H]^+$ 657.48, found 657.48.



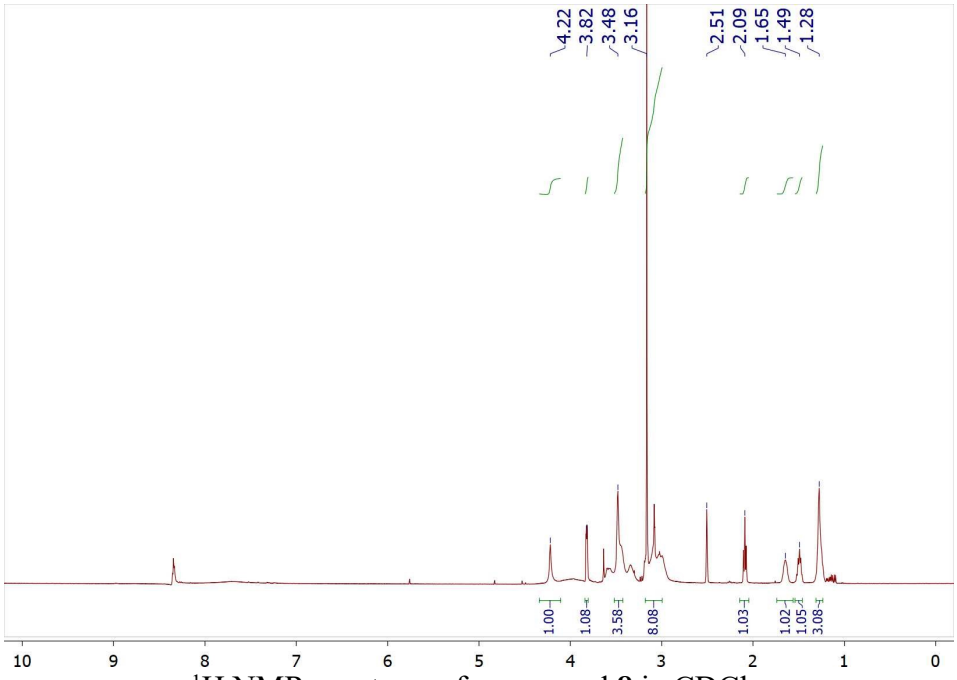
$^1$H NMR spectrum of compound **7** in $CDCl_3$.



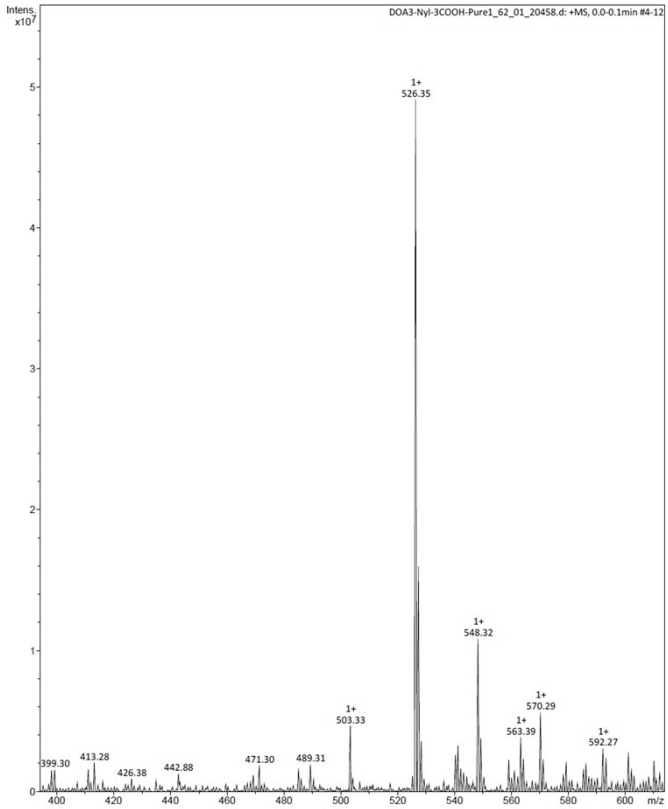
The ESI-MS spectrum of compound **7**.

**1-(Propargyl octanoic amide)-4,7,10-tris(carboxy methyl)-1,4,7,10-tetraazacyclododecane (8)**

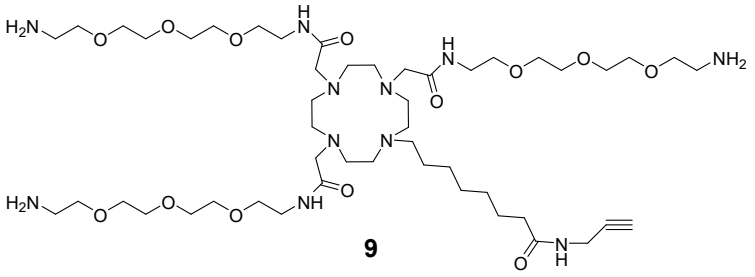
Compound **7** (1.97 g, 3 mmol), propargylamine (0,17 g, 3 mmol), N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (3 mmol, 0.575 g), N-hydroxysuccinimide (3 mmol, 0.345 g), and triethylamine (3 mmol, 0.418 mL) were dissolved in dichloromethane (50 mL), and then the solution was stirred at room temperature for 12 h. After removing the solvent, the residue was dissolved in the mixed solution (15 ml) of trifluoroacetic acid and dichloromethane at 1/2 (v/v). After stirring at room temperature for 6 h, the solvent was evaporated in vacuum. The residue was purified by silica column chromatography to obtain the product of **8** as a colorless oil (1.12 g, 2.13 mmol, 71%). TLC: hexane/EtOAc, 1:1, v/v. $^1$H-NMR (500 MHz, DMSO-d6): δ 4.22 (s, 2H), 3.82 (s, 2H), 3.52-3.42 (m, 7 H), 3.18-2.99 (m, 16H), 2.09 (t, $J$ = 8.1 Hz, 2H), 1.65 (s, 2H), 1.54-1.46 (m, 2H), 1.31-1.23 (m, 6 H). ESI-MS m/z: calcd for $[M+H]^+$ 526.32, found 526.35.




$^1$H NMR spectrum of compound **8** in $CDCl_3$.



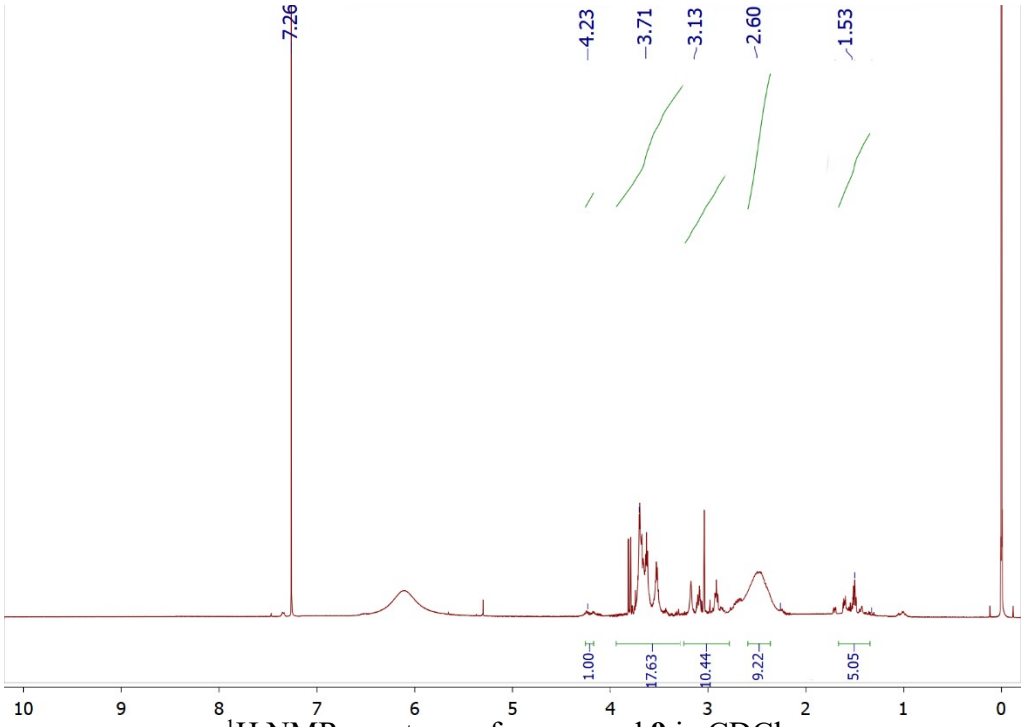

The ESI-MS spectrum of compound **8**.



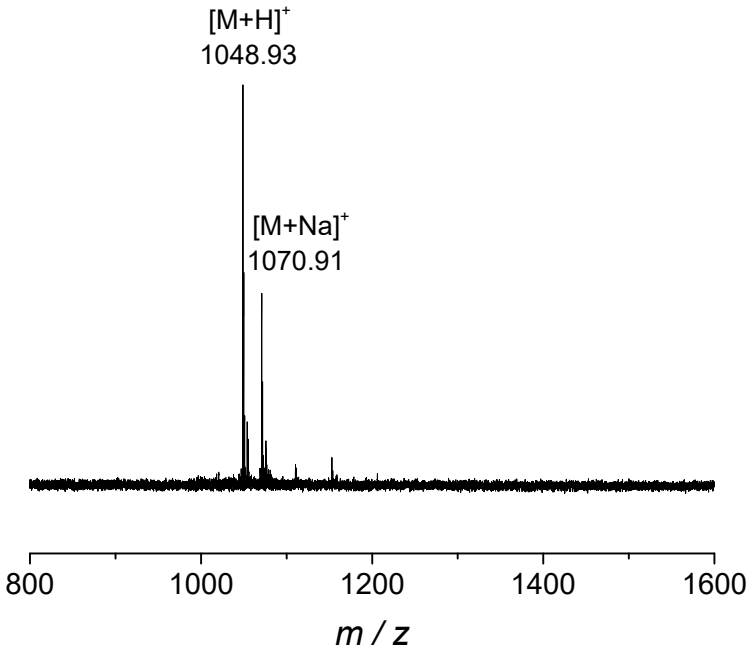
**9**

**DOTA-Tris(N-Amido-PEG3-amine)-1-(Propargyl octanoic amide) (9)**

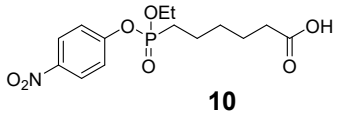
To a solution of 4-dimethylaminopyridine (6 mmol, 0.733 g), N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (6 mmol, 1.150 g) in dry dichloromethane (50 mL), was added compound **8** (0.79 g, 1.5 mmol) and BocNH-$PEG_3$-$CH_2CH_2NH_2$ (6 mmol, 1.754 g). The solution was stirred under $N_2$ atmosphere at R.T. for 12 h. Thereafter, the solvent was evaporated under reduced pressure at 40 $^oC$. A solution of dichloromethane and trifluoroacetic acid (10 mL, v/v, 1/1) was then added to the residue liquid, and was stirred for 3 h. Finally, dichloromethane was evaporated and TFA was removed under a stream of $N_2$ to get a yellow liquid. The purification was carried on a reversed phase C18 column via a Waters Delta 400 HPLC. The elution method was using a linear gradient of 75% acetonitrile in deionized ultra-filtered water (0.01% TFA) and pure water (0.01% TFA) as the mobile phase at a flow rate of 10 mL/min over 60 minutes. The fraction was lyophilized to get the product **9** (1.17 g, 1.12 mmol, 75 %). TLC: hexane/EtOAc, 1:1, v/v. $^1H$ NMR (500 MHz, $CDCl_3$): δ = 4.26-4.16(m, 2H), 3.92-3.32(m, 36H), 3.21-2.87 (m, 21 H), 2.62-2.10 (m, 18 H), 1.67-1.21 (m, 10H). MALDI-TOF MS: calcd for $[M+H]^+$ *m/z* 1048.73, found 1048.93.




$^1$H NMR spectrum of compound **9** in $CDCl_3$.



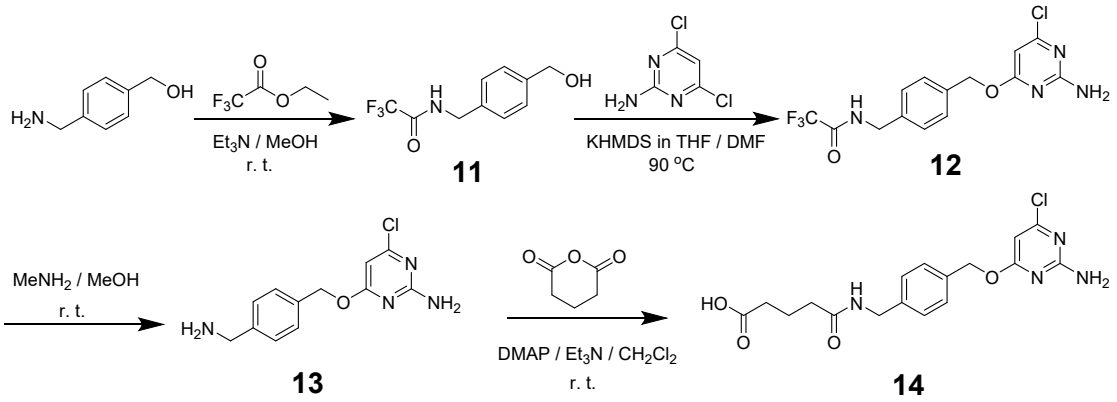

MALDI-TOF spectrum of compound **9**.



**10**

**Ethyl-4-nitrophenyl-(6-carboxyhexyl)-1-phosphonate (10)**

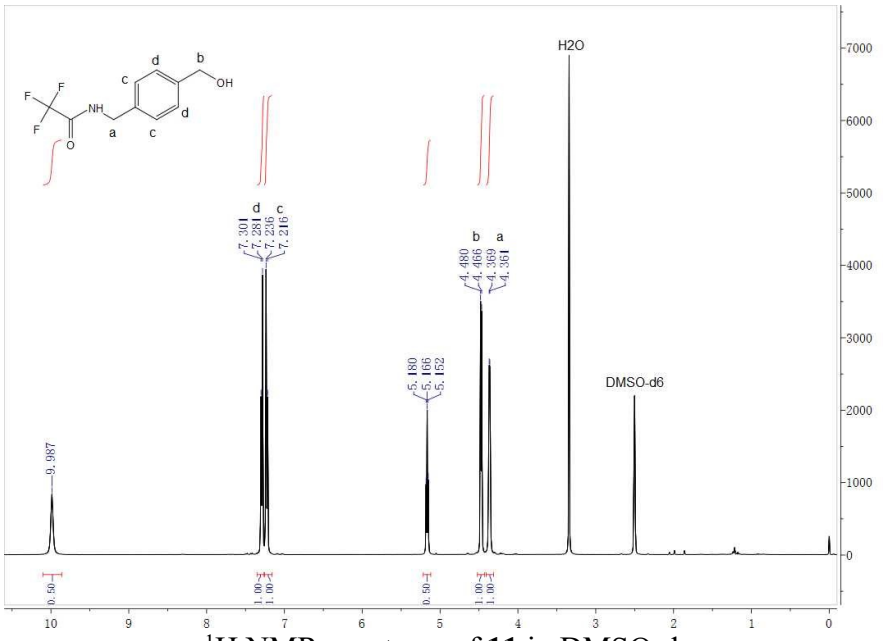
Compound **10** was obtained according to the previously reported method.[15]




**11** **12** **13** **14**

***N*-(4-(hydroxymethyl)benzyl)-2,2,2-trifluoroacetamide (11).**

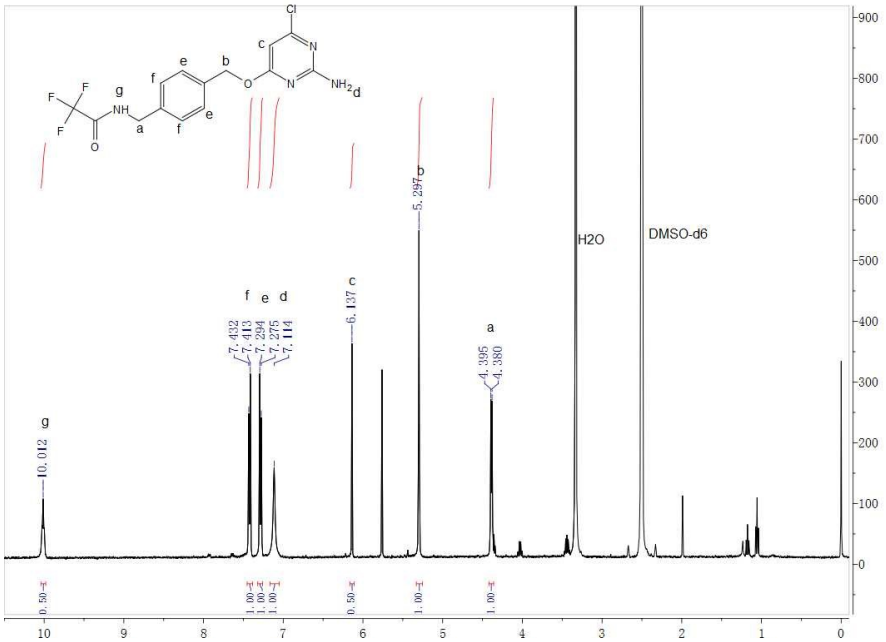
Compound (4-(aminomethyl)phenyl)methanol (3 g, 21.86 mmol) was dissolved in methanol (100 mL), and to this solution were added triethylamine (6 mL, 43.72 mmol) and ethyl trifluoroacetate (3.12 mL, 26.23 mmol). The mixture was stirred at rt for 6 h in a dry environment. After that, the solvent was firstly evaporated and then 100 mL of EtOAc was added. The organic phase was washed with brine, separated and dried over $Na_2SO_4$. Finally, the solvent was evaporated to get compound **11** as a white solid without further purification (4.58 g, 90%). $^1$H-NMR (400 MHz, DMSO-d6): δ 9.99 (br s, 1H), 7.29 (d, *J* = 7.8 Hz, 2H), 7.23 (d, *J* = 7.8 Hz, 2H), 5.17 (t, *J* = 5.7 Hz, 1H), 4.47 (d, *J* = 5.6 Hz, 2H), 4.36 (d, *J* = 3.5 Hz, 2H).




$^1$H NMR spectrum of **11** in DMSO-$d_6$.

**N-(4-((2-amino-6-chloropyrimidin-4-yloxy)methyl)benzyl)-2,2,2-trifluoroacetamide (12).**

A solution of compound **11** (4.58 g, 19.65 mmol) dissolved in potassium bis(trimethylsilyl)amide (KHMDS, 22%, 1 M in THF, 40 mL) was stirred at rt for 15 min under nitrogen atmosphere. To this solution was added dropwise a solution of 2-amino-4,6-dichloropyrimidine (3.20 g, 19.65 mmol) in anhydrous DMF (20 mL) over a 5 min period. The reaction was allowed to stir at 90 °C for 12 h. After this period, the reaction was cooled to rt, quenched with slow addition of cold water (50 mL) and extracted with EtOAc (100 mL×3). The organic solution was dried over anhydrous $Na_2SO_4$ and concentrated on a rotary evaporator. The residue was purified by flash column chromatography to give compound **12** as a white solid (1.85 g, 26 %). TLC: hexane/EtOAc, 1:1, v/v. $^1$H-NMR (400 MHz, DMSO-d6): δ 10.01 (br s, 1H), 7.42 (d, $J$ = 7.8 Hz, 2H), 7.28 (d, $J$ = 7.8 Hz, 2H), 7.11 (br s, 2H), 6.14 (s, 1H), 5.30 (s, 2H), 4.39 (d, $J$ = 5.9 Hz, 2H).




$^1$H NMR spectrum of **12** in DMSO-$d_6$.

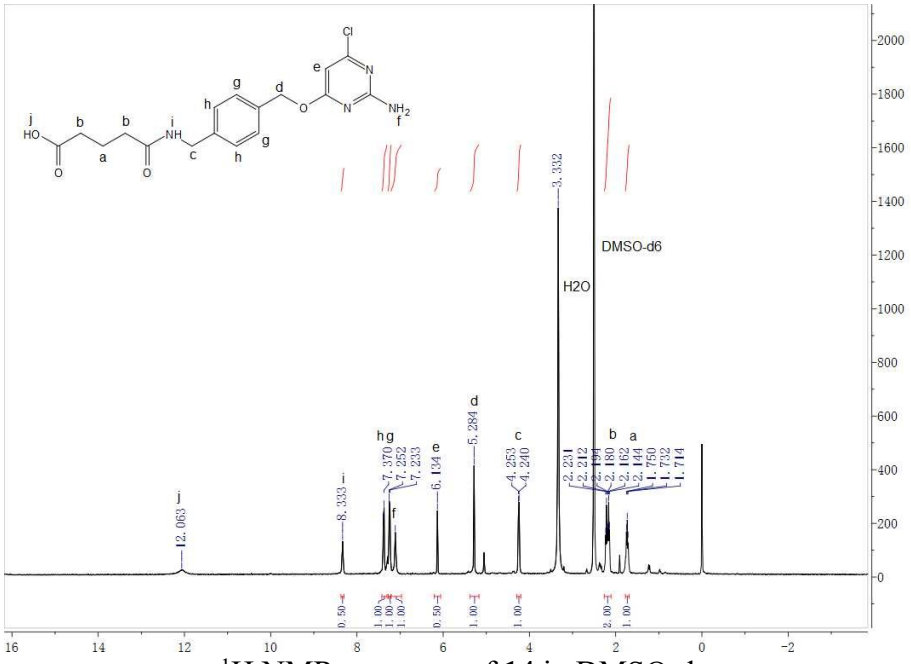
**N-(4-((2-amino-6-chloropyrimidin-4-yloxy)methyl)benzyl)-amine (13).**

Methylamine in EtOH (33%, 20 mL) was added to a solution of Compound **21** (1.85g, 5.15 mmol) in MeOH (30 mL) at rt. The solution was stirred for 12 h and concentrated on rotary evaporator to get the compound **22** as a white solid. The product was dried in vacuum and could be used directly to next step without further purification.

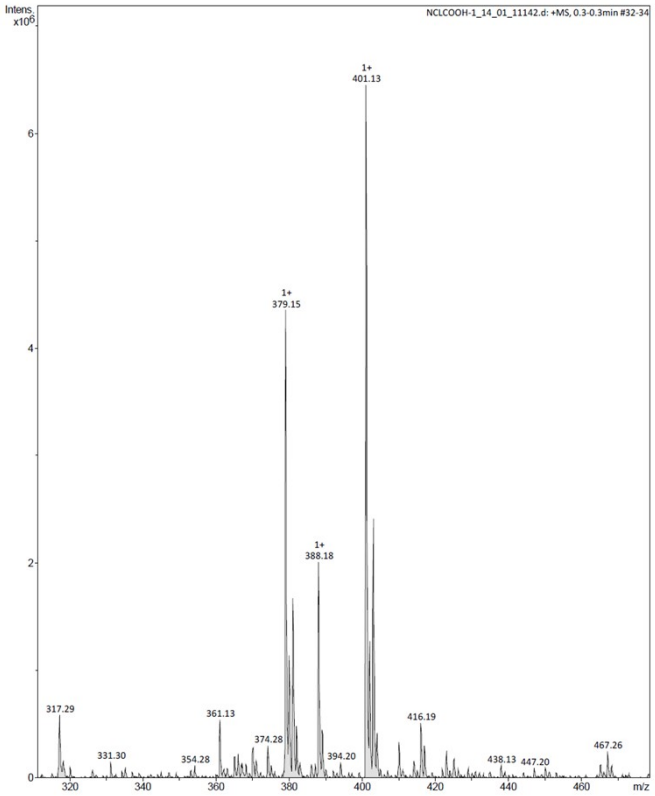
**N-(4-((2-amino-6-chloropyrimidin-4-yloxy)methyl)benzyl)-amino-5-oxo-pentanoic acid (14).**

Compound **22** (1.36 g, 5.15 mmol) was dissolved in anhydrous $CH_2Cl_2$ (50 mL), and to this solution was added glutaric anhydride (0.59 g, 5.15 mmol), $Et_3N$ (1.5 mL, 10.80 mmol) and DMAP (0.63 g, 5.15 mmol). The reaction was refluxed at 35 $^o$C for 12 h. The solvent was removed by evaporator and the mixture was purified by column chromatography. The compound **23** (0.60 g, 31%) was obtained as a white power after drying under vacuum. TLC: MeOH/$CH_2Cl_2$/AcOH, 1:10:0.1, v/v/v. $^1$H-NMR (400 MHz, DMSO-d6): δ 12.06 (br s, 1H), 8.33 (br s, 1H), 7.38 (d, $J$ = 7.6 Hz, 2H), 7.24 (d, $J$ = 7.6 Hz, 2H), 7.11 (br s, 2H), 6.13 (s, 1H), 5.28 (s, 2H), 4.25 (d, $J$ = 5.4

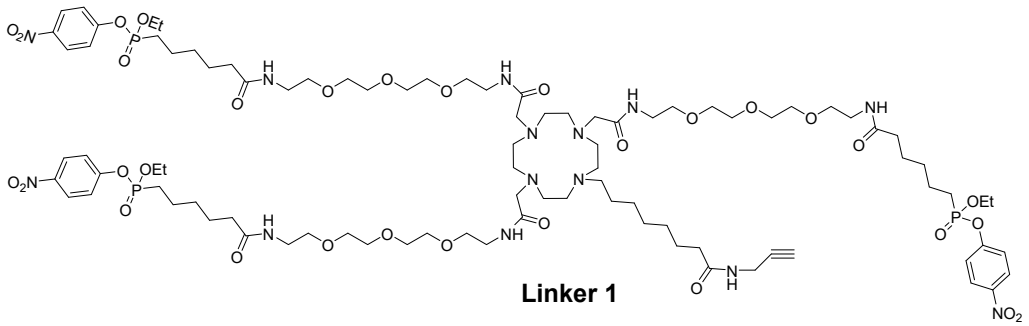
Hz, 2H), 2.19 (m, 4H), 1.73 (m, 2H). ESI-MS m/z: calcd for $C_{17}H_{19}ClN_4O_4$ $[M+H]^+$ 379.1, found 379.1.




$^1$H NMR spectrum of **14** in DMSO-$d_6$.

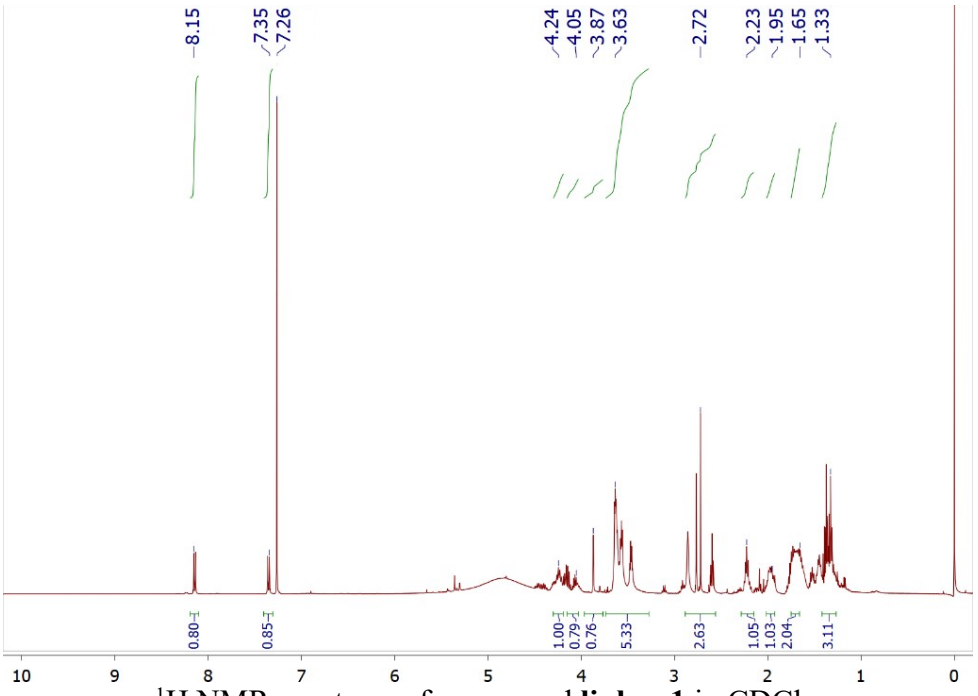



The ESI-MS spectrum of **14**.

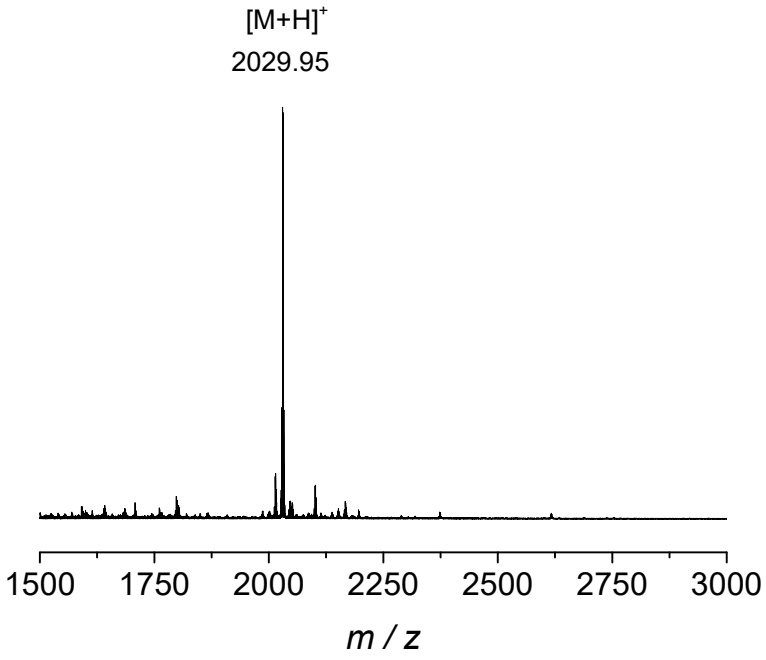



**DOTA-Tris(N-Amido-PEG3-Cutinase)-1-(Propargyl octanoic amide) (Linker 1)[16]**

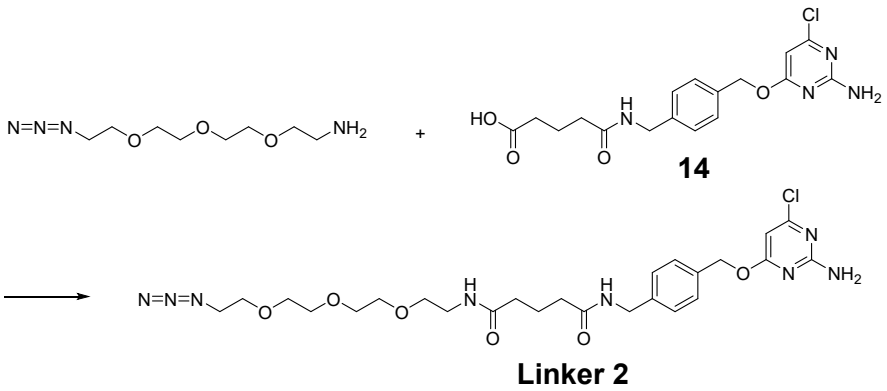
A solution of N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (3 mmol, 0.575 g), N-hydroxysuccinimide (3 mmol, 0.345 g), compound **10** (3 mmol, 1.036 g) and triethylamine (3 mmol, 0.418 mL) in 50 mL dichloromethane was stirred for 6 h at room temperature. Thereafter, compound **9** (0.8 mmol, 0.84 g) was added and the reaction was stirred for 12 h. The solvent was removed by evaporation and the residue was purified on a reversed phase C18 column via a Waters Delta 400 HPLC. The elution method was the same as described above. The fraction was lyophilized to get the **linker 1** (0.83 g, 0.41 mmol, 51 %). $^1$H NMR (500 MHz, $CDCl_3$): δ = 8.25-8.06(m, 6H), 7.39-7.28(m, 6H), 4.32-4.20 (m, 8H), 4.15-3.76 (m, 12H), 3.71-3.26 (m, 42H), 2.99-2.51 (m, 19H), 2.30-2.13 (m, 8H), 2.02-1.93 (m, 6H), 1.77-1.63 (m, 14H), 1.44-1.26 (m, 23H). MALDI-TOF MS: calcd for $[M+H]^+$ *m/z* 2030.00, found 2029.95.




$^1$H NMR spectrum of compound **linker 1** in $CDCl_3$.

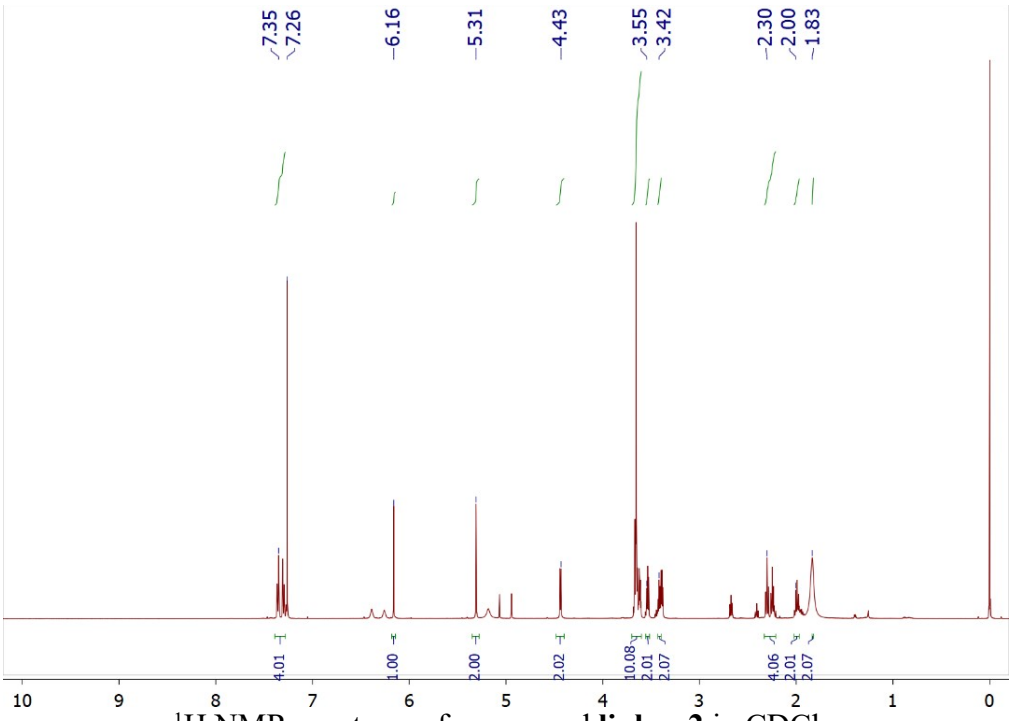



MALDI-TOF spectrum of compound **linker 1**.

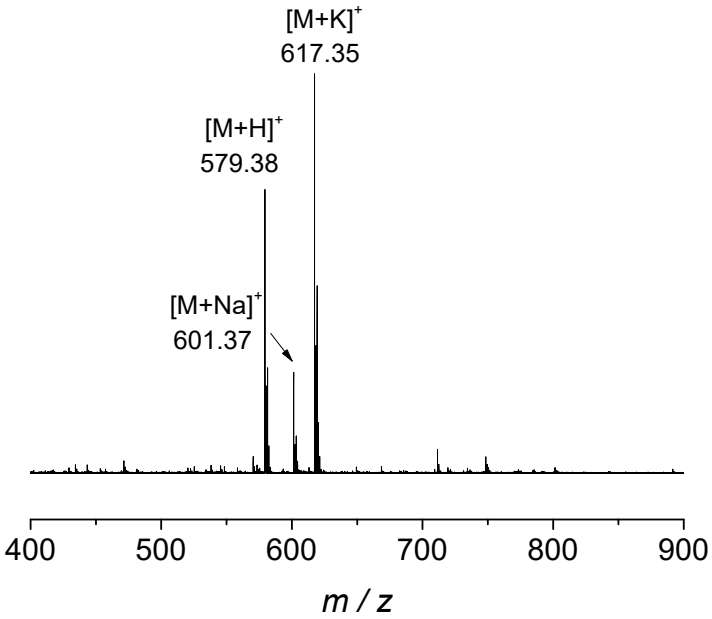



**Azido-PEG3-SNAPTag (Linker 2)**

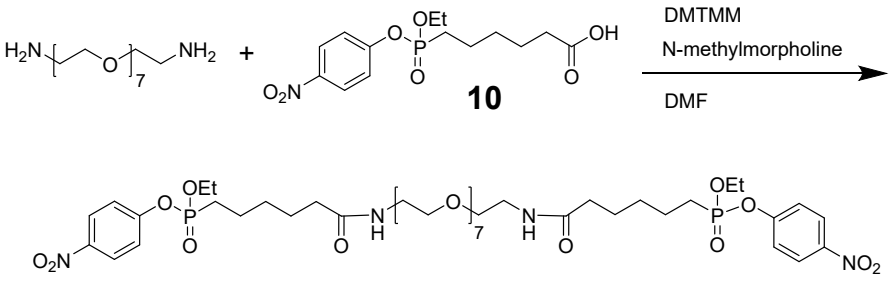
Azido-PEG3-amine (3 mmol, 0.65 g) and compound **14** (3 mmol, 1.14 g) were dissolved in 50 mL dichloromethane, and then N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (3 mmol, 0.575 g), N-hydroxysuccinimide (3 mmol, 0.345 g) and triethylamine (3 mmol, 0.418 mL) were added. After stirring under $N_2$ atmosphere for 12 h, the solvent was removed by evaporation. The residue was purified by column chromatography over silica to afford **linker 2** (1.6 g, 2.76 mmol, 92%) as yellow viscous liquid. $^1$H NMR (500 MHz, $CDCl_3$): δ = 7.39-7.28(m, 4H), 6.16(s, 1H), 5.31(s, sH), 4.43 (d, J = 5.9 Hz, 2H) 3.71-3.60(m, 10H), 3.56-3.52 (m, 2 H), 3.56-3.52 (m, 2 H), 3.43-3.39 (m, 2 H), 2.33-2.21 (m, 4H), 2.02-1.97 (m, 2H), 1.84-1.82 (m, 2H). MALDI-TOF MS: calcd for $[M+H]^+$ *m/z* 579.24, found 579.38.




$^1$H NMR spectrum of compound **linker 2** in $CDCl_3$.

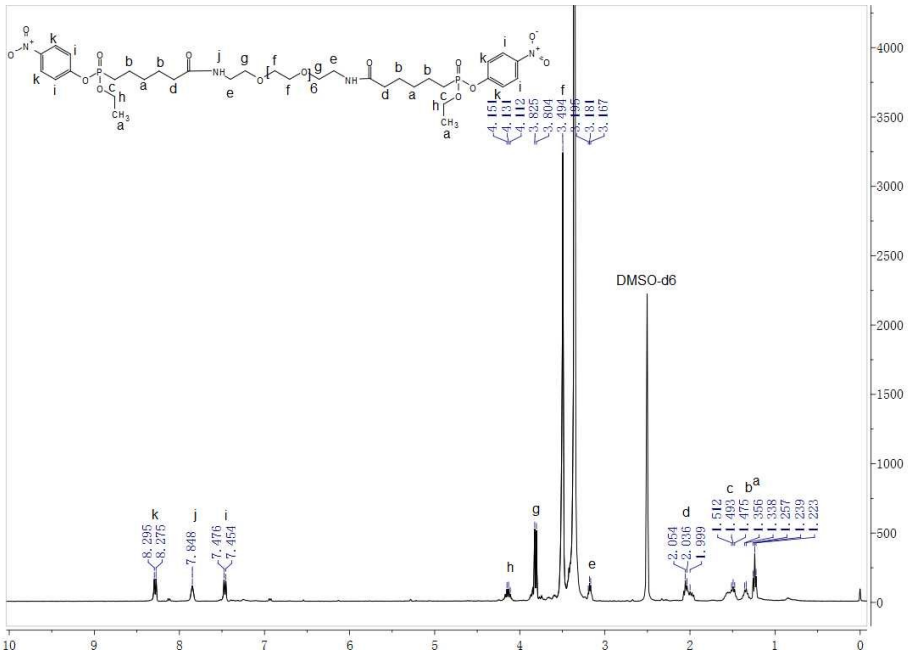



MALDI-TOF spectrum of **linker 2**.

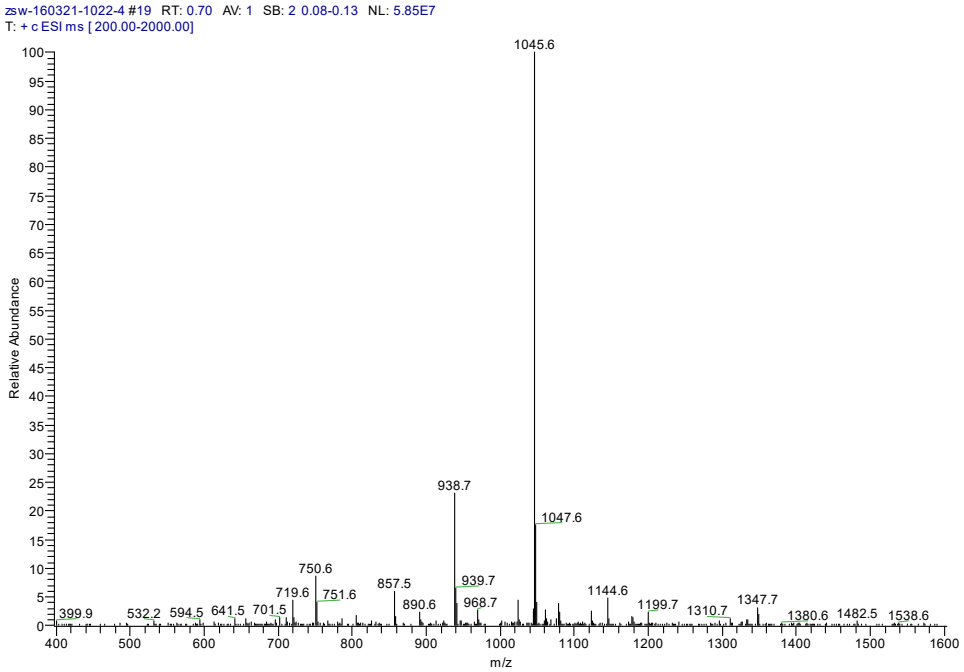



**Cutinase-PEG7-Cutinase (Linker 3)**

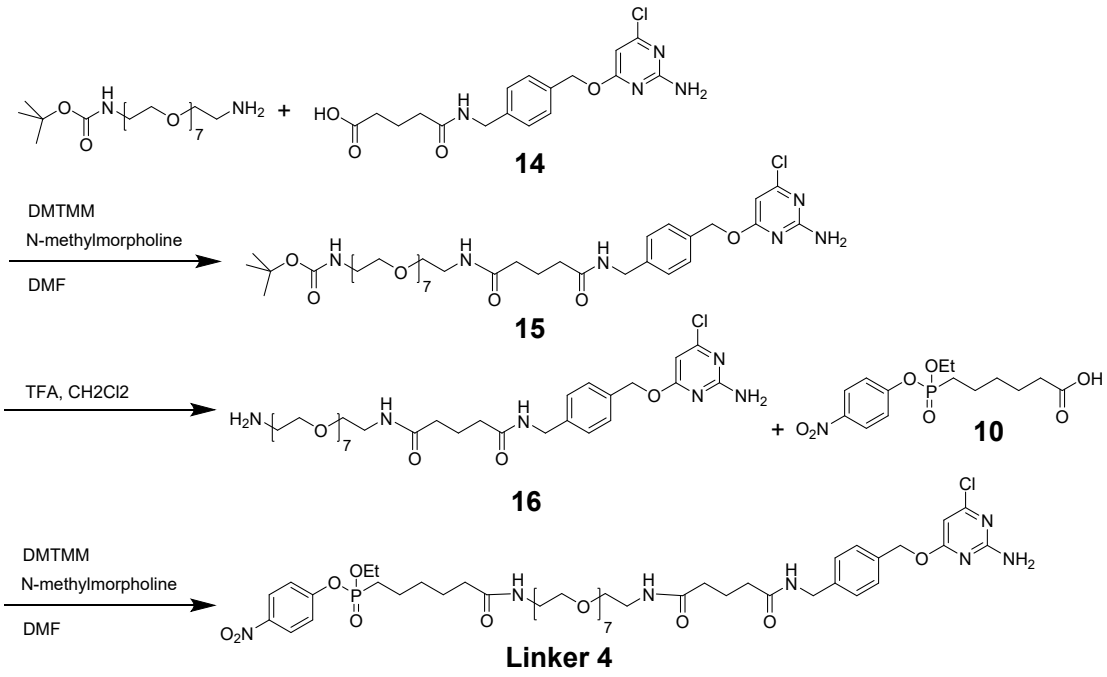
Compound **10** (0.69 g, 2 mmol) was dissolved in DMF (200 mL) followed by 4-(4,6-Dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (DMTMM, 0.59 g, 2 mmol) and N-Methylmorpholine (NMM, 0.44 mL, 4 mmol). The mixture was stirred at rt under nitrogen to obtain a clear solution. To this solution was added dropwise a solution of $H_2N$-$PEG_7$-$CH_2CH_2NH_2$ (0.37 g, 1 mmol) in dry DMF. The solution was stirred at rt for 12 h under the condition away from light. The solvent was evaporated under high vacuum and the residue submitted to column chromatography to give compound **linker 3** as a viscous oil (0.26 g, 26 %). TLC: EtOAc/MeOH, 5:1, v/v. $^1$H-NMR (400 MHz, DMSO-d6): 8.28 (d, $J$ = 8.2 Hz, 4H), 7.85 (s, 1H), 7.46 (d, $J$ = 8.7 Hz, 4H), 4.13 (m, 4 H), 3.81 (d, $J$ = 8.3 Hz, 4H), 3.49 (s, 24H), 3.18 (m, 4 H), 2.03 (m, 4 H), 1.49 (m, 4 H), 1.35 (m, 8 H), 1.24 (m, 10 H). ESI-MS m/z: calcd for $C_{44}H_{72}N_4O_{19}P_2$ $[M+Na]^+$ 1045.4, found 1045.6.




$^1$H NMR spectrum of **linker 3** in DMSO-$d_6$.

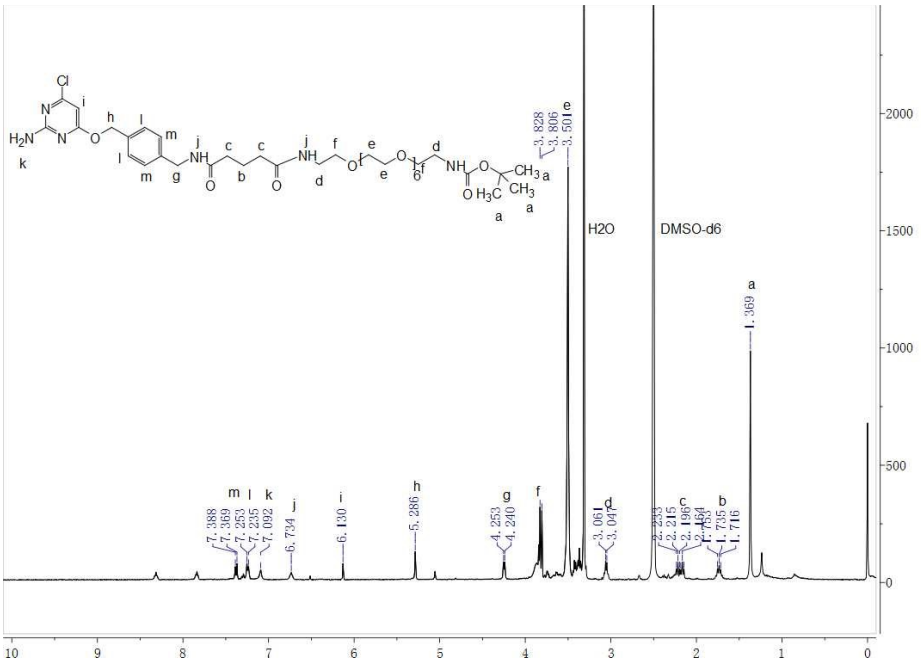



The ESI-MS spectrum of **linker 3**.

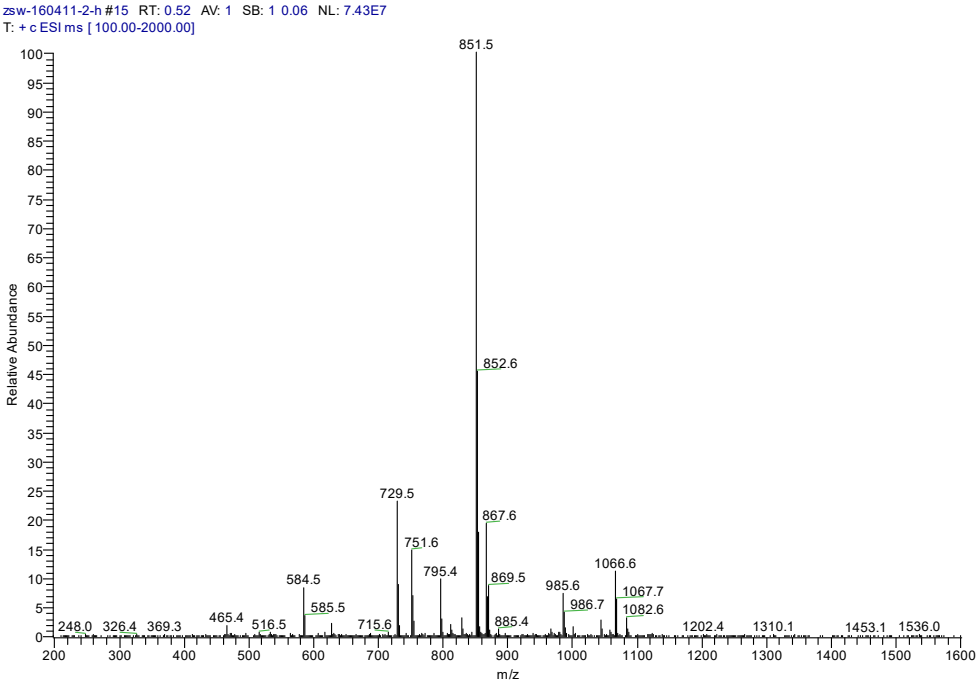



**SnapTag-PEG7-NH-Boc (15)**

Compound **14** (0.76 g, 2 mmol), DMTMM (0.59 g, 2 mmol) and NMM (0.44 mL, 4 mmol) were dissolved in dry DMF (200 mL) and stirred under nitrogen to get a clear solution. To this mixture was added dropwise a solution of BocNH-$PEG_7$-$CH_2CH_2NH_2$ (0.94 g, 2 mmol) in 10 mL of dry DMF, the reaction allowed to stir at rt for 12 h. After this period, the mixture was concentrated on a rotary evaporator and then purified by column chromatography to yield compound **15** as a a viscous oil (1.33 g, 80 %). TLC: EtOAc/MeOH, 5:1, v/v. $^1$H-NMR (400 MHz, DMSO-d6): 7.38 (d, *J* = 7.7 Hz, 2H), 7.24 (d, *J* = 7.2 Hz, 2H), 7.09 (s, 2H), 6.73 (s, 2H), 6.13 (s, 1H), 5.29 (s, 2H), 4.25 (s, 2H), 4.25 (d, *J* = 5.5 Hz, 2H), 3.82 (d, *J* = 8.7 Hz, 4H), 3.50 (s, 24H), 3.05 (m, 4H), 2.19 (m, 4H), 1.73 (m, 2H), 1.37 (m, 9 H). ESI-MS m/z: calcd for $C_{38}H_{61}N_6O_{12}Cl$ $[M+Na]^+$ 851.4, found 851.5.



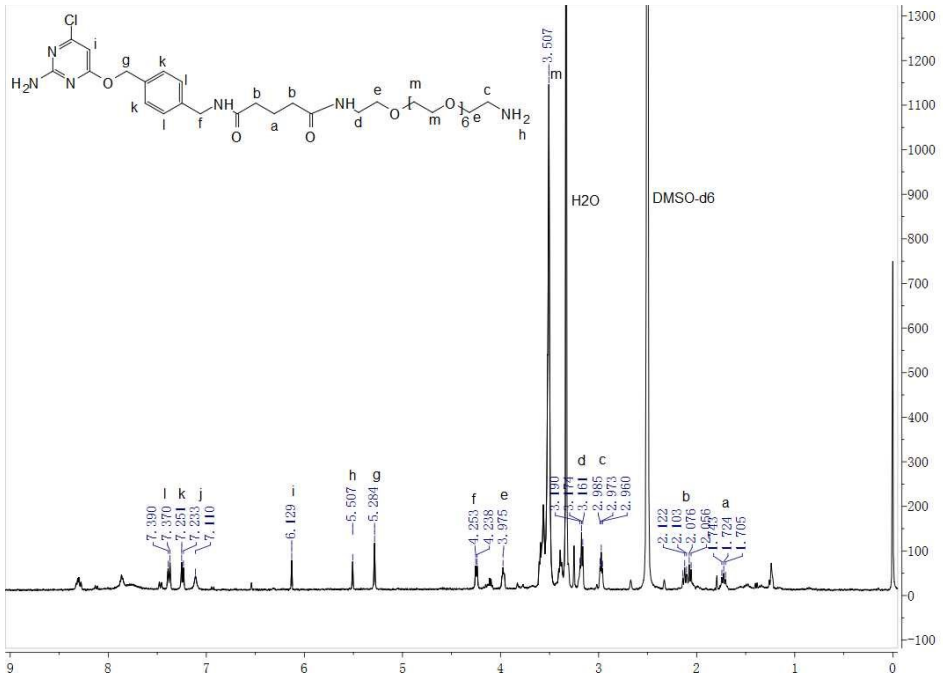

$^1$H NMR spectrum of **15** in DMSO-$d_6$.



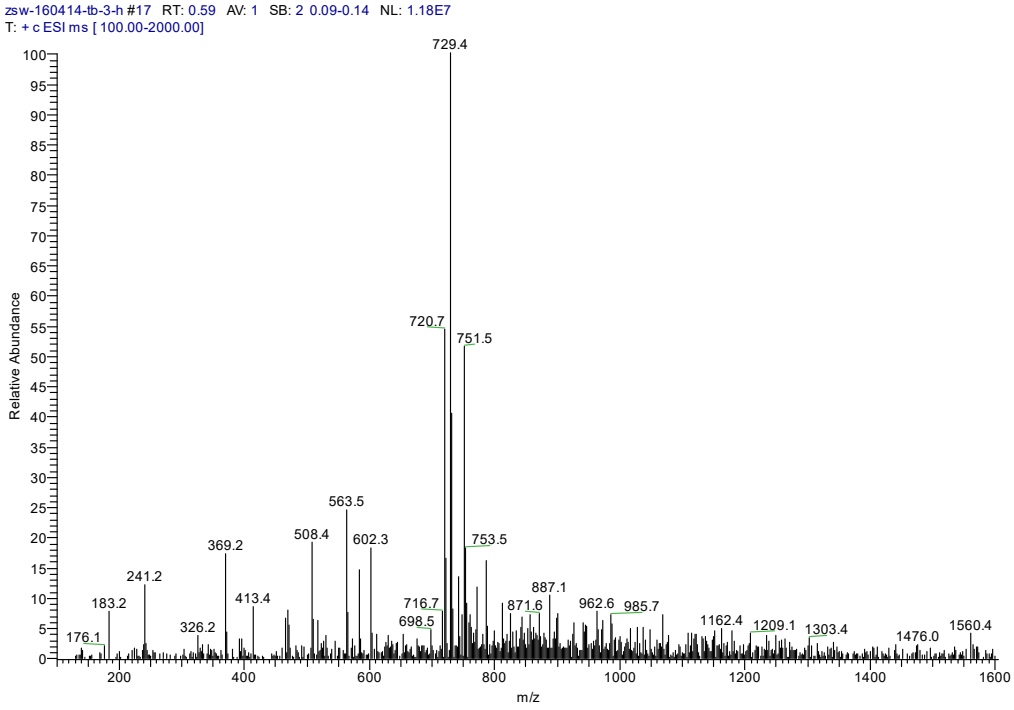

The ESI-MS spectrum of **15**.

**SnapTag-PEG7-Amine (16)**

Compound **15** (1.33 g, 1.61 mmol) was deprotected from Boc as follows, the product was dissolved in a solution of trifluoaroacetic acid (2.5 mL, 3.36 mmol/) in $CH_2Cl_2$ (10 mL) under nitrogen away form light. After stirring for 6 h, the solvent and TFA was removed under vacuum, then the residue was basified and purified with column chromatography to get compound **16** as a a viscous oil (0.76 g, 65 %). TLC: EtOAc/MeOH, 5:1, v/v. $^1$H-NMR (400 MHz, DMSO-d6): 7.38 (d, *J* = 7.7 Hz, 2H), 7.24 (d, *J* = 7.3 Hz, 2H), 7.11 (s, 2H), 6.13 (s, 1H), 5.51 (s, 2H), 5.28 (s, 2H), 4.25 (d, *J* = 5.8 Hz, 2H), 3.97 (s, 4 H), 3.51 (s, 24H), 3.18 (t, *J* = 5.7 Hz, 2H), 2.97 (t, *J* = 5.0 Hz, 2H), 2.10 (m, 4H), 1.72 (m, 2H). ESI-MS m/z: calcd for $C_{33}H_{53}N_6O_{10}Cl$ $[M+H]^+$ 729.4, found 729.4.



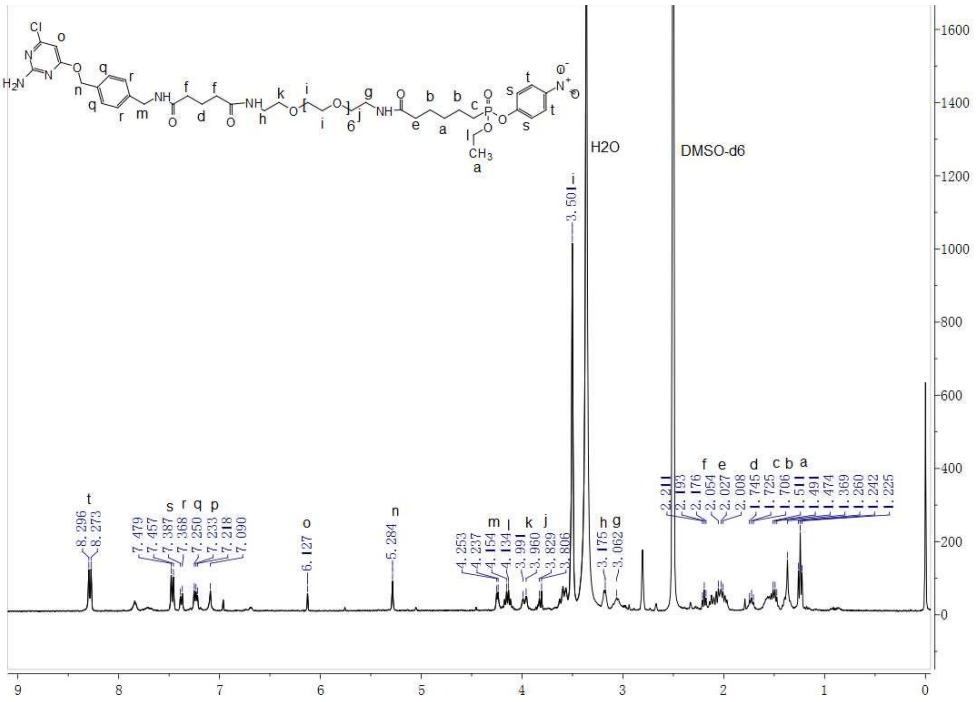

$^1$H NMR spectrum of **16** in DMSO-$d_6$.



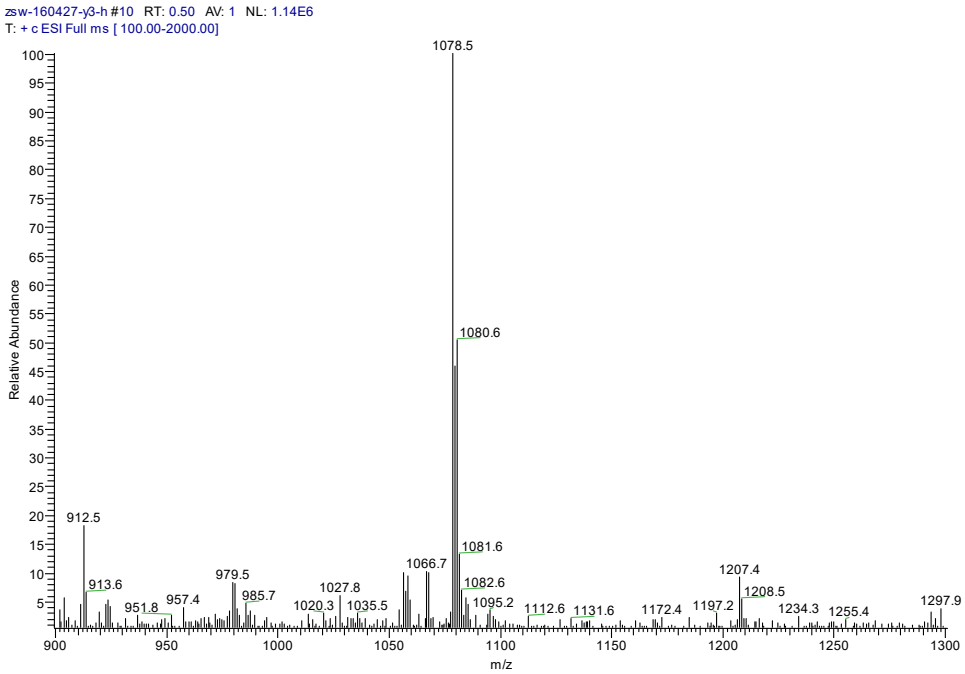

The ESI-MS spectrum of **16**.

**SnapTag-PEG7-Cutinase (Linker 4)**

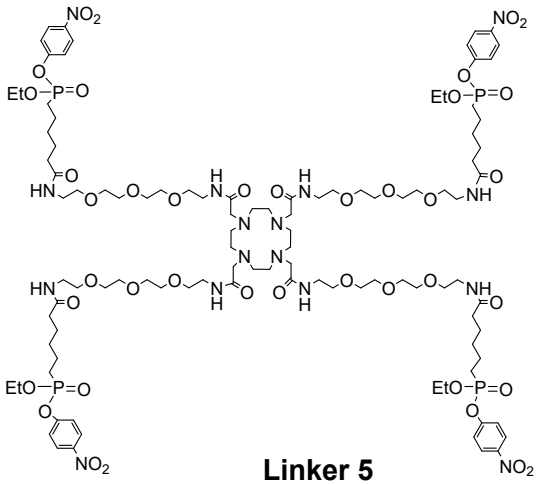
A mixture of compound **10** (0.35 g, 1 mmol), DMTMM (0.29 g, 1 mmol) and NMM (0.22 mL, 2 mmol) in dry DMF (100 mL) was stirred to get a clear solution. To this was added dropwise a solution of compound **16** (0.73 g, 1 mmol) in DMF (10 mL) and the reaction was stirred in the dark for 12 h. The reaction mixture was then concentrated in high vacuum and purified with column chromatography to obtain compound **linker 4 (**SnapTag-EG8-Cutinase Linker**)** as a a viscous oil (0.21 g, 20 %). TLC: EtOAc/MeOH, 5:1, v/v. $^{1}$H-NMR (400 MHz, DMSO-d6): 8.28 (d, $J$ = 9.0 Hz, 2H), 7.47 (d, $J$ = 9.0 Hz, 2H), 7.38 (d, $J$ = 7.5 Hz, 2H), 7.23 (m, 2H), 7.09 (s, 2H), 6.13 (s, 1H), 5.28 (s, 2H), 4.24 (d, $J$ = 6.1 Hz, 2H), 4.14 (m, 2H), 3.98 (d, $J$ = 12.5 Hz, 2H), 3.82 (d, $J$ = 8.9 Hz, 2H), 3.50 (s, 24H), 3.18 (m, 2H), 3.06 (m, 2H), 2.19 (m, 4 H), 2.03 (m, 2H), 1.73 (m, 2H), 1.49 (m, 2H), 1.37 (m, 4H), 1.24 (m, 5H). ESI-MS m/z: calcd for $C_{47}H_{71}N_7O_{16}PCl$ $[M+Na]^+$ 1078.4, found 1078.5.




$^1$H NMR spectrum of **linker 4** in DMSO-$d_6$.



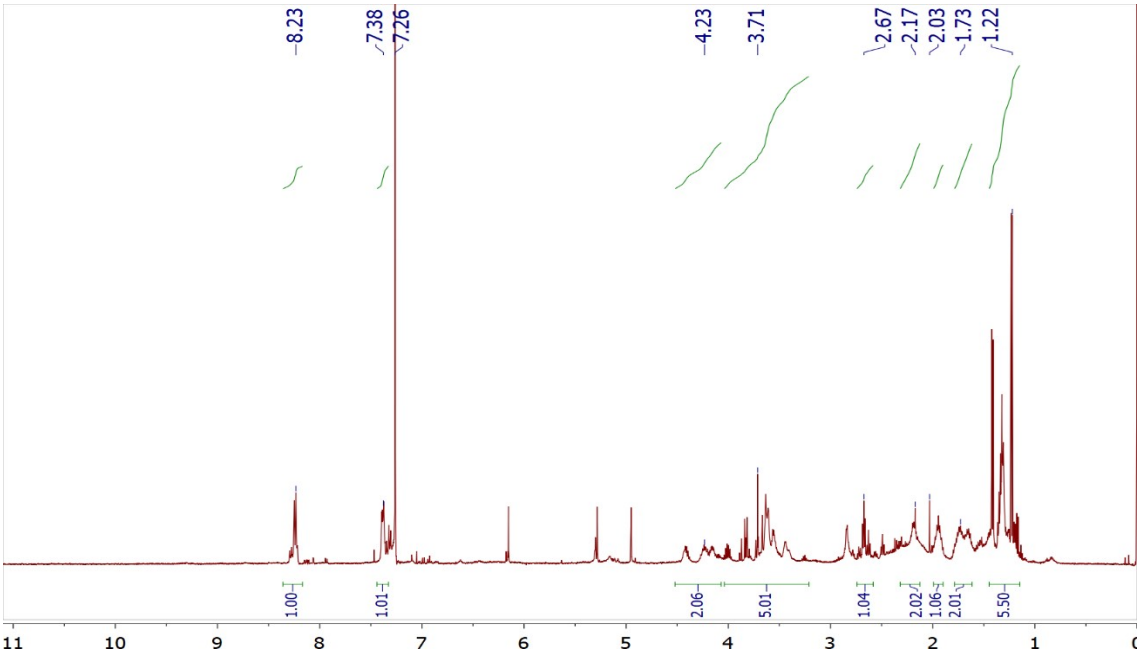

The ESI-MS spectrum of **linker 4**.



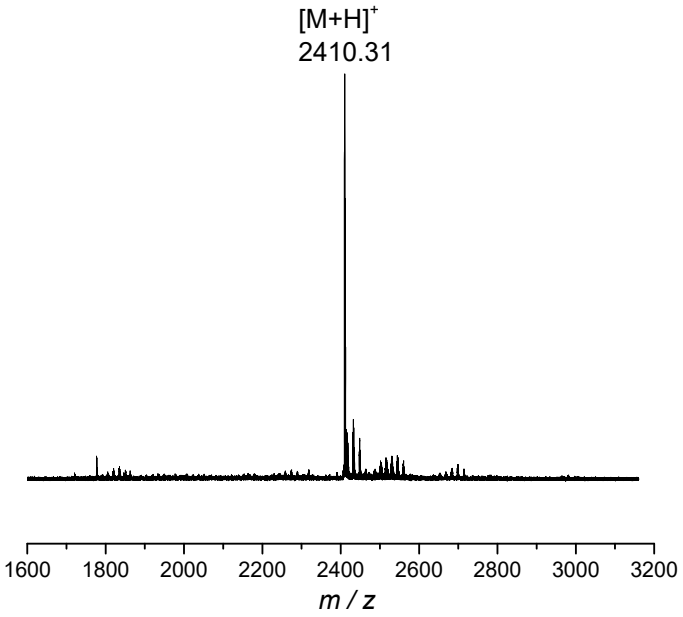
**Linker 5**

**DOTA-Tetra(N-Amido-PEG3-Cutinase) (Linker 5)**

Linker 5 was obtained according to the previously reported method. $^1$H NMR (500 MHz, $CDCl_3$): δ = 8.36-8.17(m, 8 H), 7.44-7.33(m, 8H), 4.52-4.07(m, 16H), 4.04-3.21(m, 40 H), 2.74-2.58(m, 8H), 2.32-2.13(m, 16 H), 1.99-1.90(m, 8H), 1.79-1.61(m, 16H), 1.45-1.15(m, 44H). MALDI-TOF MS: calcd for $C_{104}H_{173}N_{16}O_{40}P_4$ $[M+H]^+$ *m/z* 2410.09, found 2410.31.




$^{1}$H NMR spectrum of compound **linker 5** in $CDCl_3$.




MALDI-TOF spectrum of compound **linker 5**.

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