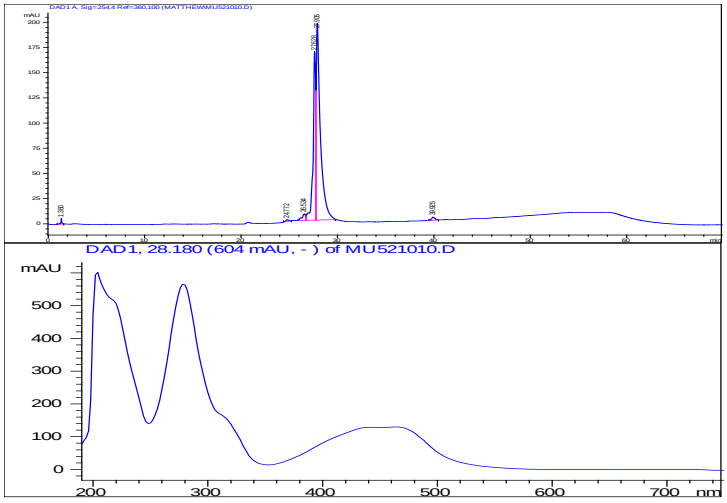
GENERAL

All reagents were purchased from commercial sources (Aldrich, Fluka, Acros, ABCR and Alfa Aesar) and used without further purification. Polyethylene glycol with molecular wt. 3000 and TentaGel S RAM resin were obtained from Rapp Polymer, Tübingen, Germany. Ru(II) bathophenanthroline complex **1** was synthesised according to the literature procedure.[1] HPLC: *Agilent-1100* with column *EC 125/4 Nucleosil 100-5 C18* PPN using gradient of Water/acetonitrile with 0.1% TFA. MPLC: *Büchi* MPLC system (fraction collector *C660*, pump module *C605*, pump manager *C615* and UV photometer *C635*) for purification of Ru-complexes; the C18 reverse-phase material was synthesised by a modified standard procedure.[2] Eppendorf centrifuge 5804 R was used for isolation of compounds after precipitation. MS: *LCQ Advantage* (ESI) mass spectrometer. MALDI-TOF mass spectra were acquired on a Reflex III mass spectrometer (Bruker Daltonik GmbH, Bremen, Germany) in the reflector mode (positively charged ions) with external calibration. Protein Desalting was performed with Protein Desalting Spin Columns from Pierce (ThermoFisher). Sample loading buffer (RotiLoad) was purchased from Roth (Germany).

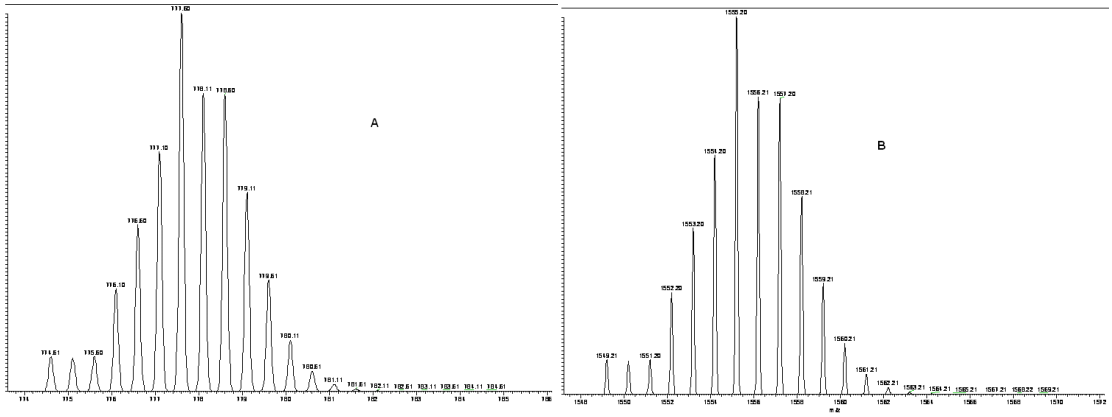
EXPERIMENTAL PROCEDURE

*Syntheses of Ru (II) bathophenanthroline-alkyne conjugate **2a***:

Ru (II) bathophenanthroline complex (**1)** (46 mg, 27.4 µmol) was treated with TBTU (13.2 mg, 41.1 µmol) and DIPEA (48 µl, 274 µmol) in 2 ml DMF for 10 min. To this propargyl amine (8.8 µl, 137 µmol) was added and the resulting mixture was stirred at room temperature for 8 h. The reaction mixture was concentrated under vacuum. Acetonitrile was added to the residue to precipitate the product. The solid isolated by filtration was purified with RP MPLC using gradient of water/acetonitrile with 0.1% TFA. Fraction containing product was lyophilized to obtain 40 mg (94%) of product.
MS analysis: base peak: 778 (M/2), M^+: 1556.2 (expected mol.wt. for protonated cation: $C_{80}H_{59}N_7O_{13}RuS_4$, 1555.21).



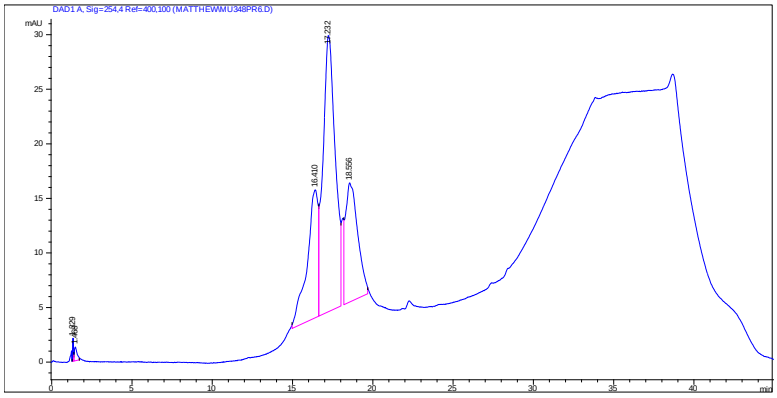
HPLC chromatogram and UV spectrum of the peak for **2a**.



Characteristic mass distribution of Ru-isotops, indicating mass of product A: M/2 and B: $M^{+\cdot}$ for **2a**.

*Ru (II) bathophenanthroline complex-alkyne conjugate tetrabutyl ammonium salt **2b:***

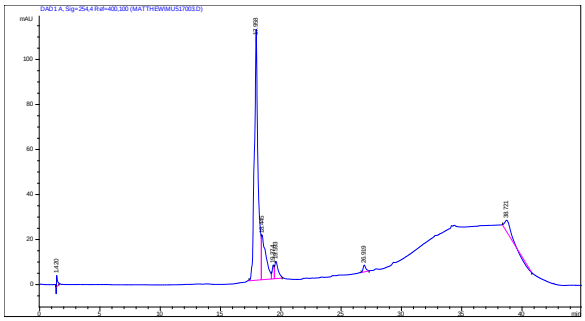
Ru (II) bathophenanthroline-alkyne conjugate **2a** (8 mg, 4.87 µmol) and tetrabutyl ammonium chloride (5.41 mg, 19.5 µmol) were dissolved in 5 ml water and stirred for 10 min. The aqueous solution was the extracted with dichloromethane (5 ml x 3) till the dichloromethane layer was obtained colourless. The organic layer was concentrated under vacuum to dryness to obtain 10.9 mg (89%) of compound **2b**.
MS analysis: 242.3 (Bu_4N^+: expected mol.wt. for cation $C_{16}H_{36}N$: 242.285) base peak: 777.7 (m/2), M^+: 1556.2 (expected mol.wt. for protonated cation: $C_{80}H_{59}N_7O_{13}RuS_4$, 1555.21)



HPLC chromatogram of **2b**.

*Synthesis of alkyl spaced alkyne derivative of Ru (II) bathophenanthroline complex **3**:*

Ru (II) bathophenanthroline complex **1** (85 mg, 48 µmol) was activated by treating 6 hr. at room temperature with TSTU (17 mg, 0.056 mmol) and DIPEA (46 µl, 0.27 mmol) in DMF. To the stirred mixture 1, 3-diamino propane (11.3 µl, 0.135 µmol) was added and the resultant mixture was stirred for 8 h. The reaction mixture was concentrated under vacuum and purified by RP MPLC using gradient of water: acetonitrile with 0.1% TFA. Fraction containing product was lyophilized to obtain 82 mg (93 %) of product.
MS analysis: base peak: 787.2 (m/2), M^+: 1574.6 (expected mol.wt. for protonated cation: $C_{80}H_{64}N_8O_{13}RuS_4$, 1574.25)

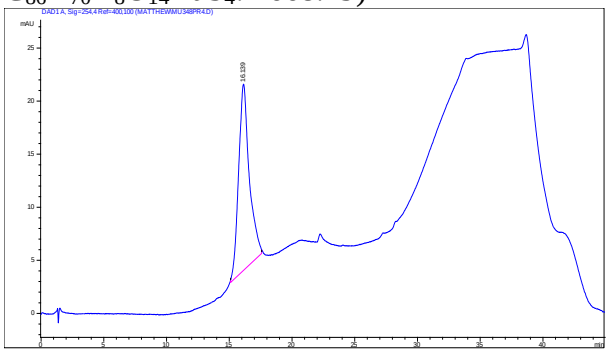


HPLC chromatogram of intermediate for compound **3**.

Attachment of 5-hexynoic acid

30 mg (17.3 µmol) of above compound was dissolved in 1 ml DMF and reacted with pre-activated 2.9 µl (26 µmol) 5-hexynoic acid by treating with 8.3 mg (26 µmol) of TBTU and 29 µl (173 µmol) of DIPEA in 1 ml DMF for 10 min. The reaction mixture was stirred at room temperature for 8 h. The mixture was then concentrated and purification was carried out using RP MPLC using gradient of water: acetonitrile with 0.1% TFA. Fraction containing product was lyophilized to yield 30 mg (94%) product.

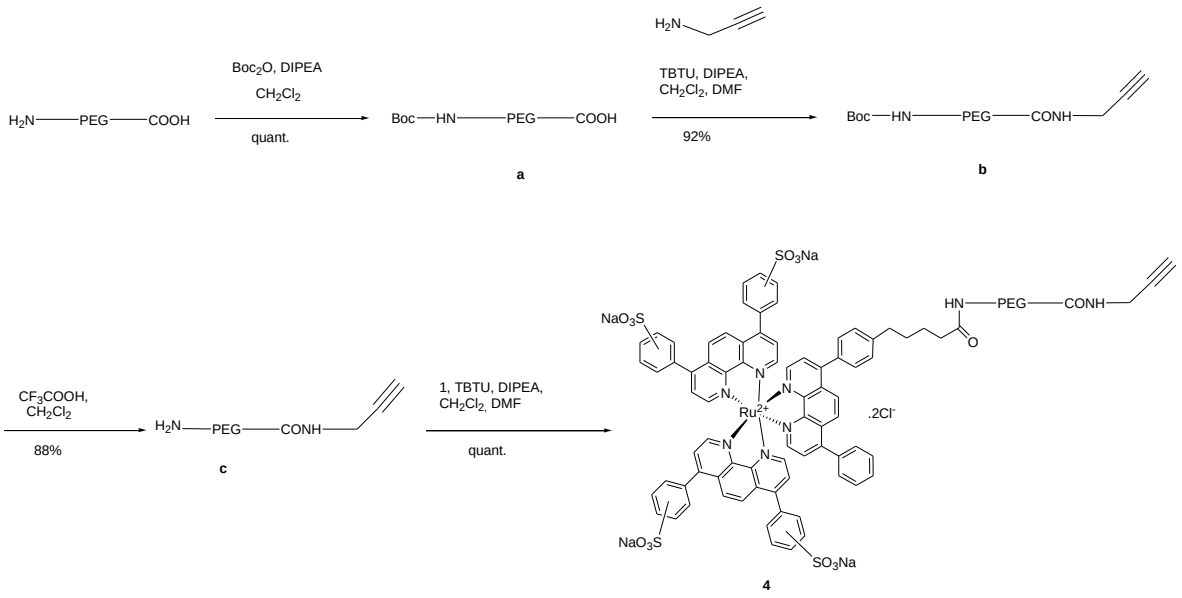
MS analysis: base peak: 834.5 (m/2), M^+: 1668.4 (expected mol. wt. for protonated cation: $C_{86}H_{70}N_8O_{14}RuS_4$: 1668.29)



HPLC chromatogram of compound **3**.

Synthesis of Ru (II) bathophenanthroline complex modified by an alkyne via a PEG spacer 4:

The synthesis was carried out using steps depicted in supporting scheme 1.



Supporting scheme 1: Synthesis of Ru (II) bathophenanthroline complex **4**.

*Synthesis of Boc-NH protected PEG carboxylate **a**:*

The PEG derivative (250 mg, 75.7 µmol) was dissolved in 2 ml dichloromethane and treated with DIPEA (44 µl, 454 µmol) followed by addition of di-tert-butyl dicarbonate (33 mg, 151 µmol). The reaction was stirred for 3 h. Small aliquot of reaction mixture was taken out and subjected to Kaiser test for the detection of free primary amine in the reaction.[3] The negative Kaiser test indicated completion of the reaction. The reaction mixture was concentrated to nearly its half and divided in two centrifuge tubes. To each tube 10 ml of diethyl ether was added and resulting precipitate was isolated by centrifugation at 10000 rpm at 5°C for 7 min. The supernatant was decanted and the operation was repeated to wash the product. The white solid thus obtained was dried in desiccator to yield 250 mg (quantitative) product.
MALDI TOF: A bell-shaped spectrum centered at 3150.167 Da with a family of adjacent peaks at 44 +/-1 (expected 3250 as compared to that of starting material 3150.125 Da; the Boc group might have been cleaved in acidic medium during the analysis)

*Synthesis of alkyne conjugate of Boc-NH protected PEG carboxylate **b**:*

The Compound from previous step (200 mg, 68 µmol) was dissolved in 1 ml dichloromethane and TBTU (117.1 mg, 365 µmol), DIPEA (107 µl, 1.095 mmol) and 1 ml DMF were added to this solution. To the stirred mixture propargyl amine (17.26 µl, 365 µmol) of was added. The resulting mixture was stirred over night at room temperature and was concentrated under vacuum. The residue was dissolved in 1 ml dichloromethane and the solution was added in two centrifuge tubes. 10 ml of diethyl ether was added to each tube and resulting precipitate was isolated by centrifugation at 10000 rpm at 5°C for 7 min. The supernatant was decanted

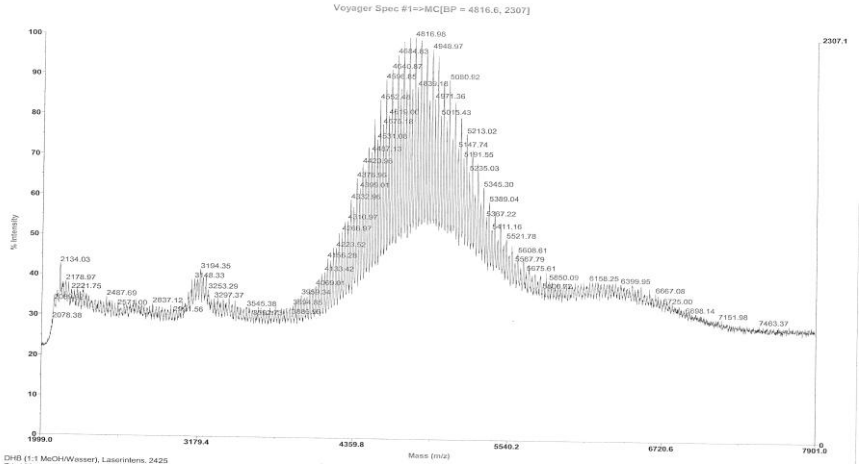
and the operation was repeated to wash the product. The off-white solid thus obtained was dried in desiccator to yield 186 mg (92%) product.
MALDI TOF: A bell-shaped spectrum centered at 3187.002 Da with a family of adjacent peaks at 44 +/-1 (expected 3287 Da as compared to analysis of starting material: 3150 Da)

*Boc-deprotection of alkyne-PEG derivative **c**:*

The solid (180 mg) obtained from earlier step was dissolved in 2 ml dichloromethane and TFA (2 ml) and TIPS (300 µl) were added to it. The reaction was continued overnight. The reaction mixture was concentrated and the residue was dissolved in dichloromethane (10 ml). The solution was washed with brine (2 x 10ml). Due to presence of PEG in brine (as indicated by Draggendorff's reagent),[4] it was extracted with dichloromethane (3 x 10 ml). The combined organic layer was dried over anhydrous sodium sulfate and concentrated to get a small volume which was then transferred to centrifuge tube and precipitated by addition of diethyl ether (10 ml each). The precipitate was isolated by centrifugation at 10000 rpm at 5°C for 7 min. The clear supernatant was decanted and the off-white solid thus obtained was dried in desiccator to yield 153mg (88%) product.
A bell-shaped spectrum centered at 3187.002 Da with a family of adjacent peaks at 44 +/-1 (expected 3187 Da as compared to that of starting material: 3150 Da)

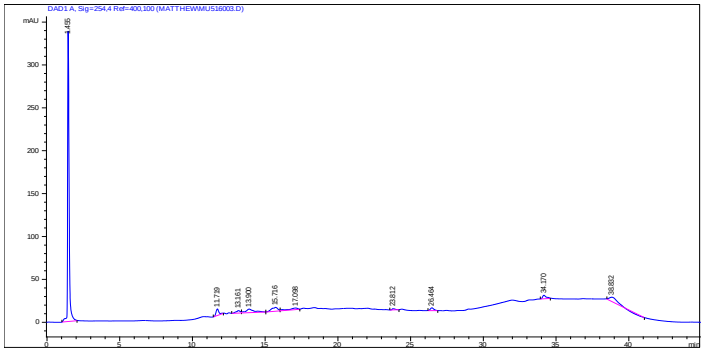
*Synthesis of alkyne derivative of PEG-3000-Ru (II) bathophenanthroline conjugate **4**:*

Ru (II) bathophenanthroline complex **1** (30 mg, 0.018 mmol) was treated with TBTU (9 mg, 0.027 mmol) and DIPEA (18.5 µl, 0.108 mmol) of in 1 ml DMF. To this mixture, solution of PEG derivative synthesised as described above (40 mg, 0.015 mmol) in 1 ml dichloromethane was added. The reaction was stirred over night at room temperature. The mixture was then concentrated under vacuum and the residue was again dissolved in 2 ml dichloromethane. The solution was divided in two equal aliquots and transferred into the centrifuge tubes. The precipitation of the product was effected by addition of 10 ml diethyl ether to each tube. The isolation of the precipitate was done by centrifugation at 10000 rpm at 5°C for 7 min. The orange solid thus obtained was re-suspended in diethyl ether and isolated by centrifugation. Solid thus obtained was purified by size exclusion chromatography using Sephadex G-25 resin as stationary phase and water as eluent. The fraction containing product was lyophilized to obtaine 64 mg (98%) product.
MALDI TOF: A bell-shaped spectrum centered at 4778.96 Da with a family of adjacent peaks at 44 +/-1 (expected 4779 Da as compared to that of starting material **3**: 3150 Da)



*Solid phase synthesis of azide functionalized peptide **5**:*

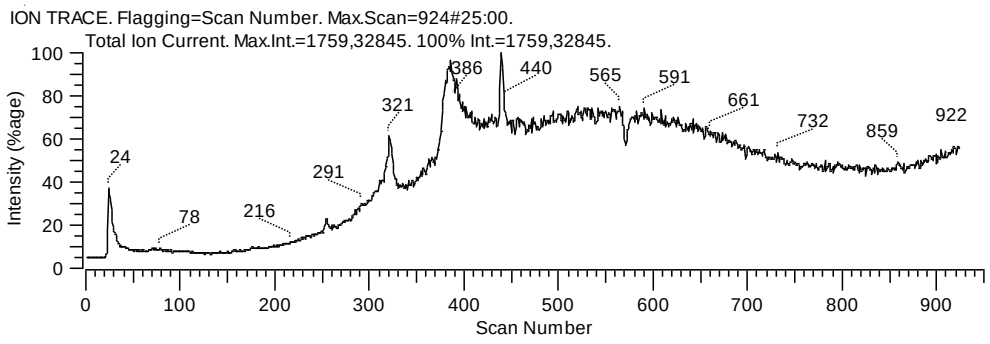
The peptide synthesis was carried out on a 0.03 mmol scale using Fmoc-protocol and TentaGel S RAM resin (loading 0.24 mmol/g) employing TBTU and DIPEA for coupling reaction. Standard Boc and *t*-Bu side-chain-protected amino acids were employed. After the addition of last amino acid and its Fmoc deprotection the amino function was converted to azide. For this NaN_3 (39.8 mg, 617 µmol) and Tf_2O (85 µl, 505 µmol) in 4 ml of pyridine were stirred for 2 h at 0°C. This mixture was then added to the protected peptide on the solid support along with copper sulphate (2.5 mg) and methanol (0.1 ml). The reaction was shaken overnight at room temperature. The resin was then washed with DMF, 0.5% DIPEA in DMF, 0.05M of diethyl dithiocarbamate sodium salt in DMF and finally with DMF followed by dichloromethane. The deprotection and cleavage of peptide from resin was effected by the treatment of 5 ml mixture of TFA: H_2O: TIPS (95: 2.5: 2.5). The solution containing peptide was concentrated to 25% of its volume and peptide was precipitated by addition of 6 ml diethyl ether. The solid peptide was isolated by centrifugation at 7000 rpm for 2 min at 4°C. The solid obtained by decantation of clear supernatant was dissolved in water and lyophilized to obtain 27 mg (93%) white peptide.
MS analysis: base peak: 989.3 (expected mol. wt. $C_{42}H_{56}N_{18}O_{11}$: 988.43)

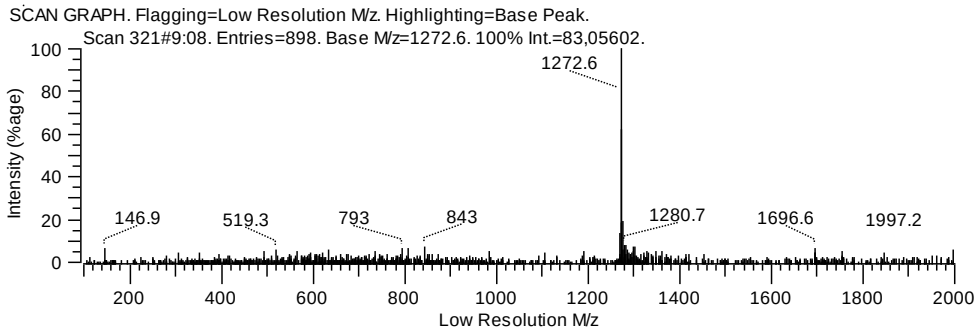


HPLC chromatogram of peptide **5**.

Labelling of peptide with Ru (II) bathophenanthroline-alkyne conjugate with click reaction:

Ru (II) bathophenanthroline-alkyne conjugate **2a** (1.2 mg, 0.9 µmol), azide functionalized peptide **5** (0.2 mg, 0.2 µmol), copper sulphate (0.8 mg, 3.24 µmol) and L(+) ascorbic acid (1.1 mg, 6.25 µmol) and DIPEA (1.1 µl, 6.48 µmol) were mixed together in 200 µl mixture of water and DMSO (1:1). The mixture was shaken over night at room temperature. LCMS analysis of a small aliquot of reaction mixture indicated the complete conversion of peptide to the labeled peptide **6**.
LCMS: base peak (m/2): 1272.6 (expected mol. wt. $C_{122}H_{115}N_{25}O_{24}RuS_4$: 2543.647)





LCMS analysis of the reaction mixture: scan no. 321: product, scan no. 386: compound **2a**.

Synthesis of proteins with site-specifically incorporated azido amino acid:

Protein synthesis was performed using an E. coli derived cell-free translation system depleted from termination factor RF1[5] and supplemented with enriched fractions of orthogonal *amber* suppressor tRNA and *p*-azidophenylalanyl-tRNA synthetase[6] specific for *p*-azidophenylalanine. The system contained p-azidophenylalanine (Bachem, Switzerland) in addition to all 20 natural amino acids. ^{14}C-labelled leucine was added for protein quantitation by hot TCA precipitation and in-gel detection by radioactive imaging. In additon, single chain antibodies were expressed in the presence of PDI and a mixture of GSSG and GSH to enable the formation of disulfide bonds. Additional chaperone DnaK was added to support folding into active molecules.
Each protein gene contained an amber stop codon (UAG) for site-specific unnatural amino acid incorporation at a defined position (see table 1). Yield of azido proteins was between 150 and 300 µg per ml reaction as estimated by hot TCA precipitation of the ^{14}C-leucine labelled proteins.

	protein	**description**	**MW [dalton]**	**site of AzF incorporation**
1	$SecB_{AzF156}His$	E.coli secretory protein B	18,260	aa 156, C-terminus folllowed by HisTag
2	$EfTu\text{-}His_{AzF}$	E.coli elongation factor Tu	44,350	C-terminus
3	$hLFAR_{AzF}$	human liver fatty acid binding protein	14,210	C-terminus
4	scFv Anti-$EC5218_{AzF}$	single chain antibody anti cyclophilin A	28,280	C-terminus
5	scFv Anti-$EC5218_{AzF2}$	single chain antibody anti cyclophilin A	28,890	aa2

Supporting table 1: protein data

Modification of azido poteins and analysis:

After protein synthesis, the translation reactions were desalted to remove unincorporated amino acids and the reaction buffer was exchanged to a buffer containing 50 mM Tris-HCl (pH 7.5), 70 mM KCl and 30 mM NH_4Cl using spin desalting columns (Pierce, ThermoFisher, USA). Alternatively PBS (phosphate buffered saline, pH 8.0) was used. Stock solutions of 5 mM alkynes in 50% DMSO (water/DMSO, 1:1) were used. Stock solutions of aqueous $CuSO_4$ (50 mM) and aqueous Na ascorbate (20 mM) were prepared freshly.

Click reactions (40 µl each) with alkyne-derivatives of PEG and ruthenium dyes were performed using a mixture of 10 µl protein synthesis reaction in exchanged buffer, 0.25 mM

alkyne, 1.3 mM $CuSO_4$, 2.5 mM Na ascorbate, 2.5 mM TBTA, 1.3 mM DIPEA and 50% DMSO. This resulted in a final concentration of azido protein of approx. 2.5 µM. Reactions were incubated at 25°C and 300 rpm over night using a thermomixer comfort (Eppendorf, Germany).

After reactions the respective mixtures were desalted, diluted with 100 µl water and precipitated with 3 volumes of ice cold acetone. Precipitates were collected via centrifugation (12000 rpm, 10 min), dried and dissolved in 1*x* SDS gel loading buffer (RotiLoad, Roth, Germany). Protein samples were denatured for 3 min at 90°C prior to analysis on 15% SDS gels. After electrophoresis the gels were washed with 50% methanol and detected at 302 nm excitation on the UV table of a chemImager (Alpha Innotech Corporation). After detection of ruthenium dye labelled proteins the gels were stained with coomassie blue and dried. Radioactive proteins were detected using a PhosphorImager system (Molecular Dynamics, USA).

$SecB_{AzF156}His$: The Escherichia coli soluble protein SecB (the product of the secB gene) is involved in the export of periplasmic and outer membrane proteins.[7] The isolation of secB mutations permitted the demonstration that SecB is required for rapid and efficient export of certain proteins. Consistent with the results of these genetic studies, purified SecB has been shown to stimulate protein translocation across E. coli inner membrane vesicles in vitro. This protein is modified by amber suppressor codon at AA position 156 to incorporate *p*-azido phenylalanine. The C-terminus is modified by 6x His-tag.

a) DNA and amino acid sequence of the p-azidophenylalanine containing protein $SecB_{AzF156}His$:

```
  1 atgtcagaacaaaacaacactgaaatgactttccagatccaacgt
  1  M  S  E  Q  N  N  T  E  M  T  F  Q  I  Q  R

 46 atttataccaaggatatctctttcgaagcgccgaacgcgccgcac
 16  I  Y  T  K  D  I  S  F  E  A  P  N  A  P  H

 91 gttttccagaaagattggcaaccagaagttaaacttgatctggat
 31  V  F  Q  K  D  W  Q  P  E  V  K  L  D  L  D

136 acggcatcttcccaactggcagatgacgtatacgaagtggtactg
 46  T  A  S  S  Q  L  A  D  D  V  Y  E  V  V  L

181 cgtgttaccgtaacggcctctttgggcgaagaaaccgcgttcctg
 61  R  V  T  V  T  A  S  L  G  E  E  T  A  F  L

226 tgtgaagttcagcagggcggtattttctccatcgcgggtatcgaa
 76  C  E  V  Q  Q  G  G  I  F  S  I  A  G  I  E

271 ggcacccagatggcgcattgcctgggagcatactgcccgaacatt
 91  G  T  Q  M  A  H  C  L  G  A  Y  C  P  N  I

316 ctgttcccgtatgctcgtgagtgcatcaccagcatggtatcccgc
106  L  F  P  Y  A  R  E  C  I  T  S  M  V  S  R

361 ggtacattcccgcaactgaaccttgcgccggttaacttcgatgcg
121  G  T  F  P  Q  L  N  L  A  P  V  N  F  D  A

406 ctgttcatgaactatttgcagcagcaggctggcgaaggtactgaa
136  L  F  M  N  Y  L  Q  Q  Q  A  G  E  G  T  E

451 gaacatcaggatgcctagggtcaccaccatcaccatcactaa
```

```
151  E  H  Q  D  A AzF G  H  H  H  H  H  H  *
```

p-azido-phenylalanine (AzF) residue and corresponding amber codon within the SecB amino acid and DNA sequences are underlined.

b) DNA and amino acid sequence of the protein SecB that was used as the negative control for click reaction:

```
  1 atgtcagaacaaaacaacactgaaatgactttccagatccaacgt
  1  M  S  E  Q  N  N  T  E  M  T  F  Q  I  Q  R

 46 atttataccaaggatatctctttcgaagcgccgaacgcgccgcac
 16  I  Y  T  K  D  I  S  F  E  A  P  N  A  P  H

 91 gttttccagaaagattggcaaccagaagttaaacttgatctggat
 31  V  F  Q  K  D  W  Q  P  E  V  K  L  D  L  D

136 acggcatcttcccaactggcagatgacgtatacgaagtggtactg
 46  T  A  S  S  Q  L  A  D  D  V  Y  E  V  V  L

181 cgtgttaccgtaacggcctctttgggcgaagaaaccgcgttcctg
 61  R  V  T  V  T  A  S  L  G  E  E  T  A  F  L

226 tgtgaagttcagcagggcggtattttctccatcgcgggtatcgaa
 76  C  E  V  Q  Q  G  G  I  F  S  I  A  G  I  E

271 ggcacccagatggcgcattgcctgggagcatactgcccgaacatt
 91  G  T  Q  M  A  H  C  L  G  A  Y  C  P  N  I

316 ctgttcccgtatgctcgtgagtgcatcaccagcatggtatcccgc
106  L  F  P  Y  A  R  E  C  I  T  S  M  V  S  R

361 ggtacattcccgcaactgaaccttgcgccggttaacttcgatgcg
121  G  T  F  P  Q  L  N  L  A  P  V  N  F  D  A

406 ctgttcatgaactatttgcagcagcaggctggcgaaggtactgaa
136  L  F  M  N  Y  L  Q  Q  Q  A  G  E  G  T  E

451 gaacatcaggatgcctagggtcaccaccatcaccatcactaa
151  E  H  Q  D  A  *
```

The protein was synthesized in the absence of orthogonal amber suppressor tRNA and *p*-azidophenylalanyl tRNA synthetase that are necessary to incorporate the azido amino acid.

EF-Tu (**elongation factor thermo unstable**): is one of the prokaryotic elongation factors. The elongation factor Tu (EF-Tu) plays an essential role in the elongation cycle of prokaryotic protein synthesis.[8] It mediates the entry of the aminoacyl-tRNA into a free site of the ribosome. EF-Tu functions by binding an aminoacylated, or charged, tRNA molecule in the cytoplasm. This complex transiently enters the ribosome, with the tRNA anticodon domain associating with the mRNA codon in the ribosomal A site. If the codon-anticodon pairing is correct, EF-Tu hydrolyzes guanosine triphosphate (GTP) into guanosine diphosphate (GDP) and inorganic phosphate, and changes in conformation to dissociate from the tRNA molecule. The aminoacyl-tRNA then fully enters the A site, where its amino acid is brought near the P site's polypeptide and the ribosome catalyzes the covalent transfer of the amino acid onto the polypeptide. This protein is modified by amber suppressor codon at C-terminus to incorporate *p*-azido phenylalanine. It is also modified to carry 6x His-tag at C-terminus.

HLFAR$_{AzF}$: Liver fatty acid-binding protein is a small protein (~15 kD) involved in the intracellular transport of long-chain fatty acids in the liver. It is regarded as a sensitive marker for liver cell damage.[9] This protein is modified by amber suppressor codon at final amino acid position at C-terminus to incorporate *p*-azido phenylalanine.

scFv Anti-EC5218: It is a anti-cyclophilin A antibody. The single-chain variable fragment (scFv) is a fusion protein of the variable regions of the heavy (V_H) and light chains (V_L) of immunoglobulins, connected with a short linker peptide of ten to about 25 amino acids.

scFv Anti-EC5218$_{AzF1}$: This protein is modified by amber suppressor codon at final amino acid position at C-terminus to incorporate *p*-azido phenylalanine.

scFv Anti-EC5218$_{AzF2}$ This protein is modified by amber suppressor codon at amino acid position 2 to incorporate *p*-azido phenylalanine.

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