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Supplementary Information

Evaluation of Bicinchoninic Acid as Ligand for Copper(I)-catalyzed Azide-Alkyne Bioconjugations

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Reagents

Unless indicated otherwise all chemicals were from Sigma (Steinheim, Germany) or Merck KGaA (Darmstadt, Germany). Azidohomoalanine (Aha)[1] and homopropargylglycine (Hpg)[2] were synthesized as described previously. 2,2′-biquinoline-4,4-dicarboxylic acid dipotassium salt (BCA; cat. no. 14338), and bathophenanthroline disulfonic acid disodium salt (BPAA; cat. no. 11890) were from Fluka (Buchs, Switzerland). Tris[(1-benzyl-1*H*-1,2,3-triazol-4-yl)methyl]amine (TBTA; cat. no. 678937) and Tris(2-carboxyethyl)phosphine hydrochloride (TCEP; cat. no. C4706) were purchased from Sigma while PEG4 carboxamide-propargyl biotin (biotin alkyne) was from Invitrogen GmbH (Karlsruhe, Germany; cat No. B10185). The copper wire was from Stawo AG (Niederlenz, Switzerland).

Cu(I)-catalyzed azide-alkyne cycloadditions (CuAAC)

The alkyne- or azide-containing proteins (4.2 µg) were incubated in a final volume of 30 µl PBS (phosphate buffered saline; 50 mM NaH_2PO_4, 150 mM NaCl, pH 8) containing 2 mM TCEP (50 mM in 500 mM $NaHCO_3$, pH 9), 1 mM $CuSO_4$, and ~0.5 cm copper wire (1 mm diameter). 0.6 µg azide- or alkyne-labeled compounds, i.e. fluorophores LM155, LM158, or the biotin PEG4 alkyne, were added together with the different copper ligands at the indicated final concentration and the samples were shaken on a thermoshaker at 1400 rpm at 37 °C for the indicated times. The reaction was stopped by adding 2 µl 0.5 M EDTA pH 9 and 4 µl SDS-loading buffer[3], mixed and flash frozen in liquid nitrogen. The samples were incubated for 5 min at 95 °C prior to analysis by SDS-PAGE.

Quantification of the coupling efficiency by gel electrophoresis

For the detection of fluorescent protein bands, proteins were separated on 16 % tricine gels[3] after the cycloaddition reaction. Fluorescence was excited by illumination of the gel immediately after electrophoresis at 254 nm on a transilluminator and images were acquired on a LAS-4000 imager (Fujifilm Europe). The gels were Coomassie stained and imaged on a LAS-4000 imager. The quantification of the bands was performed with the Multi Gauge Imager software (Fujifilm Europe) as described by the manufacturer. The intensity of the band in the P sample (protein without conjugation reagents, label or ligand) was set to 100 %. The coupling efficacy was assessed by comparing the intensities of the upper, conjugated protein band and the lower, unconjugated protein band.

Immunoblotting

Protein samples were separated on 16 % tricine gels[3] and electro-transferred to a PVDF membrane (Millipore Schwalbach, Germany, cat. no. IPV00010). The membrane was blocked with BSA (Sigma-Aldrich, St. Louis, MO) in PBS for 60 min at room temperature. After three washes for 15 min each with PBST (50 mM NaH_2PO_4, 150 mM NaCl, 0.5 % (w/v) Tween-20, pH 8), the membrane was incubated with 1:1000 diluted streptavidin-HRP (R&D Systems, Minneapolis, MN, cat no. 7164000) in PBS for 60 min at room temperature. The membrane was washed three times with PBST as described above and the biotin-containing bands were detected with Enhanced Chemiluminescent (ECL) reagent (GE Healthcare, Chalfont UK cat no. RPN2132). Images were acquired using a LAS-4000 imager (Fujifilm Europe, Düsseldorf, Germany). The direction of protein migration in the gel and blot images in this study was from top to bottom.

Preparation of b* containing non-canonical amino acids

As model protein for the coupling studies, the P27A/C41A/C83A mutant of barstar was used. (b*, complete sequence: MKKAVINGEQIRSISDLHQTLKKELALAEYYGENLDALW DALTGWVEYPLVLEWRQFEQSKQLTENGAESVLQVFREAKAEGADITIILS). The N-terminal methionine was substituted by Aha or Hpg. Electrocompetent *E. coli* B834(DE3) cells (genotype: F^- *ompT* $hsdS_B(r_B^- m_B^-)$ *gal dcm met* (DE3); Novagen Merck Chemicals Ltd., Nottingham, UK) were transformed with the b* expression plasmid pQE80L-b*.[4] Transformants were grown in New Minimal Medium (NMM)[5] containing 45 µM L-methionine and 100 mg/L ampicillin. After depletion of methionine in the mid-log phase (OD_{600} 0.5–0.8) as indicated by a growth arrest, 100 mg/L Aha or Hpg were added and gene expression was induced by the addition of 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG; Applichem, Darmstadt, Germany). b* expression was performed by vigorous shaking for 4 h at 30 °C.

b* variants were purified as described previously.[6] Briefly, the cells were harvested by low speed centrifugation (3'000 x g, 10 min, 4 °C) and ruptured by lysozyme digestion and sonication. After centrifugation at 20'000 x g for 30 min the pellet was dissolved in 50 mM Tris/HCl, pH 8.0 containing 7.5 M urea. The suspension was centrifuged again at 7'000 x g for 30 min and the supernatant dialyzed three times against 5 L of 50 mM Tris/HCl, 0.1 M NaCl, pH 8.0. The cloudy solution was centrifuged at 20'000 x g for 30 min, and the supernatant containing the refolded protein was applied to a HiTrap Q Sepharose FF (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) anion exchange column on an FPLC system.

b*[Aha] and b*[Hpg] were eluted with a linear sodium chloride gradient at about 300 mM NaCl. Peak elution fractions were analyzed on a 20 % SDS gel[7] and the purest fractions were pooled. The buffer was exchanged for 50 mM Tris/HCl pH 8.0, the protein samples concentrated by ultrafiltration (Sartorius AG, Göttingen, Germany), and analyzed by SDS-PAGE. The purity of the protein samples prepared by the procedure described above was approximately 50 %. b*[Aha] and b*[Hpg] were obtained at 5 mg/L culture volume.

The preparation, purification, and analysis of TTL[Aha] was described earlier.[8] TTL[Aha] was >95% pure and obtained at 20 mg/L *E. coli* culture. Quantitative substitution of the 11 Met residues by Aha was confirmed by mass spectrometry.

LC-ESI-MS analysis

The incorporation of non-canonical amino acids into the proteins was analyzed by LC-ESI-MS. For this aim 20 µL aliquots of the purified proteins were pre-separated on a Discovery Bio Wide Pore C5 column (3.5 µm particle size, 100 x 2.1 mm, Supelco, Bellefonte, USA) by elution with a gradient from 80 % A to 90 % B within 15 min and 90 % B to 95 % B in 2 min (eluent A: 0.05 % (v/v) TFA in water, eluent B: 0.05 % (v/v) TFA in acetonitrile). A flow rate of 250 µL/min was used. The masses of the eluted fractions were analyzed on a MicroTOF ESI-MS (Bruker Daltonics, Bremen, Germany). Absorbance was detected at 210 nm.

MALDI-TOF analysis

Protein masses were analyzed using matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF-MS) with an Ultraflex-II TOF/TOF instrument (Bruker Daltonics, Bremen, Germany) equipped with a 200 Hz solid-state Smart beam™ laser. The mass spectrometer was operated in the positive linear mode. MS spectra were acquired over an m/z range of 4,500−12,500 and data was analysed using FlexAnalysis® software provided with the instrument. Sinapinic acid was used as the matrix and samples were spotted using the dried droplet technique.

Synthesis of the alkyne-functionalized dye LM155

H-Leu-Lys(Boc)-O*t*Bu (270 mg, 0.65 mol; a kind gift from Prof. Moroder, Max Planck Institute of Biochemistry, Munich, Germany) was dissolved in DMF (20 mL) at room temperature. Subsequently Fmoc-Pra-OH (200 mg, 0.6 mmol), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDCI, 230 mg, 1.2 mmol), hydroxybenzotriazole (HOBt, 162 mg, 1.2 mmol) and diisopropylethylamin (DIPEA, 206 µL, 12 mmol) were

added. The solution was stirred at room temperature and after 8 hours additional DIPEA (103 µL, 6 mmol) was added. This solution was stirred for additional 14 h. Afterwards, the solvents were removed by vacuum distillation and the crude product was dissolved in ethyl acetate (20 mL) and washed three times with 20 mL $KHSO_4$ solution (5 %), three times with 20 mL $NaHCO_3$ solution (5 %) and three times with 20 mL brine. The organic layer was dried over Na_2SO_4 and the solvent removed by vacuum distillation. The crude product was purified by silica gel chromatography (cyclohexan/ethyl acetate 20:15) and Fmoc-Pra-Leu-Lys(Boc)-O*t*Bu was obtained as a yellowish powder in 60 % yields (287 mg, 0.45 mmol). The compound was identified by ESI-MS (m/z = 633.6 (95 %) [M –Boc+ H^+], 733.8 (100 %) [M + H^+]; calculated for $C_{41}H_{56}N_4O_8$: 732.40 amu).

For the deprotection of the α-amino group and the carboxyl group of the lysine, Fmoc-Pra-Leu-Lys(Boc)-O*t*Bu (240 mg, 0.33 mmol) was dissolved in TFA (5 mL) and stirred for 1 hour at room temperature. Subsequently, TFA was removed by vacuum distillation and the crude product was dissolved in toluene. The solvent was again removed by vacuum distillation. The crude product (a white powder) was suspended in cyclohexane/methyl *tert*-butyl ether (MTBE, 1:1), filtrated and the retentate was washed with cyclohexane/MTBE (1:1) and subsequently dried in a desiccator. Fmoc-Pra-Leu-Lys-OH resulted as the corresponding trifluoroacetate in 80 % yield (180 mg, 0.26 mmol). The compound was identified by ESI-MS (m/z = 577.4 (100 %) [M + H^+]; calculated for $C_{32}H_{40}N_4O_6$: 576.29 amu).

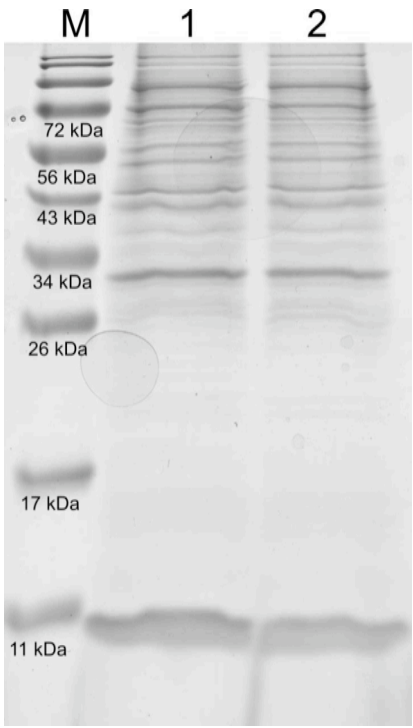
Fmoc-Pra-Leu-Lys-OH (1,5 mg, 2.1 µmol) was dissolved in dimethylformamide (DMF, 5 mL) and 5-carboxyfluorescein-*N*-succinimidylester (CFl-OSu, 1 mg, 2.1 µmol) and DIPEA (5 µL, 30 µmol) were added. The solution was stirred for 14 hours at room temperature. The solvents were removed by vacuum distillation and the crude product was dissolved in butanol/ethyl acetate (1:1, 20 mL) and washed with water (20 mL). The organic layer was dried and the solvents removed by vacuum distillation. Fmoc-Pra-Leu-Lys(CFl)-OH was obtained as a yellow oil in 90 % yield (1,8 mg, 1.9 µmol). The compound was identified by ESI-MS (m/z = 935.4 (100 %) [M + H^+]; calculated for $C_{53}H_{50}N_4O_{12}$: 934.34 amu).

Synthesis of the azide-functionalized dye LM158

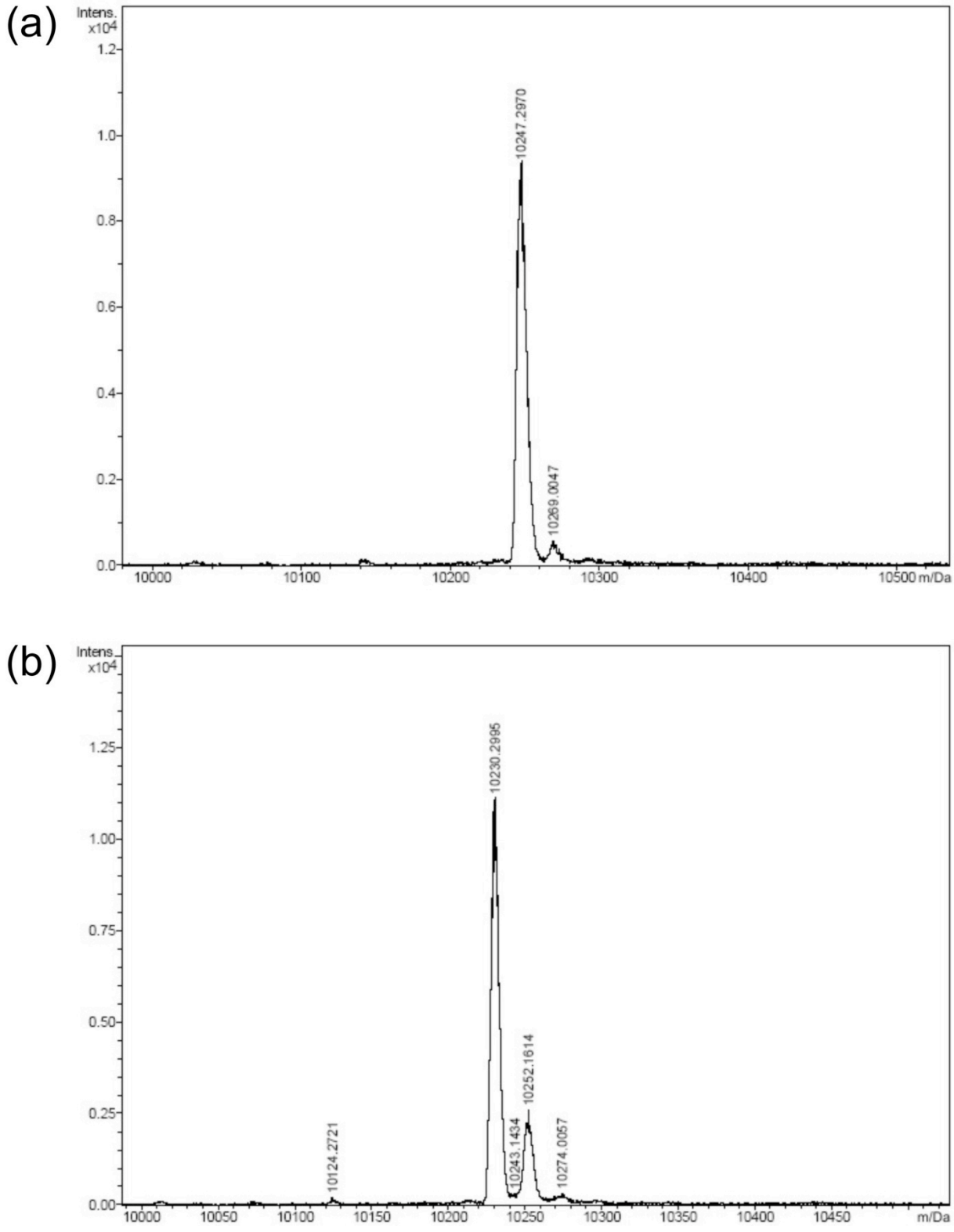
The azido dye LM158 (6-azidofluorescein) was synthesized according to the synthesis of 7-azido-4-methylcoumarin.[9] Briefly, a solution of $NaNO_2$ (25%, 1.8 mL) was added to a solution of 6-aminofluorescein (500 mg, 1.4 mmol) in concentrated aqueous HCl (20 mL) at 4 °C. Thereby, the color of the solution changed from yellow/green to orange. After stirring for 20 minutes, NaN_3 solution was added (20 %, 2.2 mL) at 0 °C. The solution was stirred for

48 h at 0 °C. The intense yellow solution was neutralized with NaOH and the color changed to red. The neutral solution was extracted three times with 40 mL portions of a mixture of butanol/ethylether (1:1). The organic layers were combined and the solvents removed by vacuum distillation. 6-azidofluorescein resulted as a red solid in 75 % yields (400 mg, 1,1 mmol).

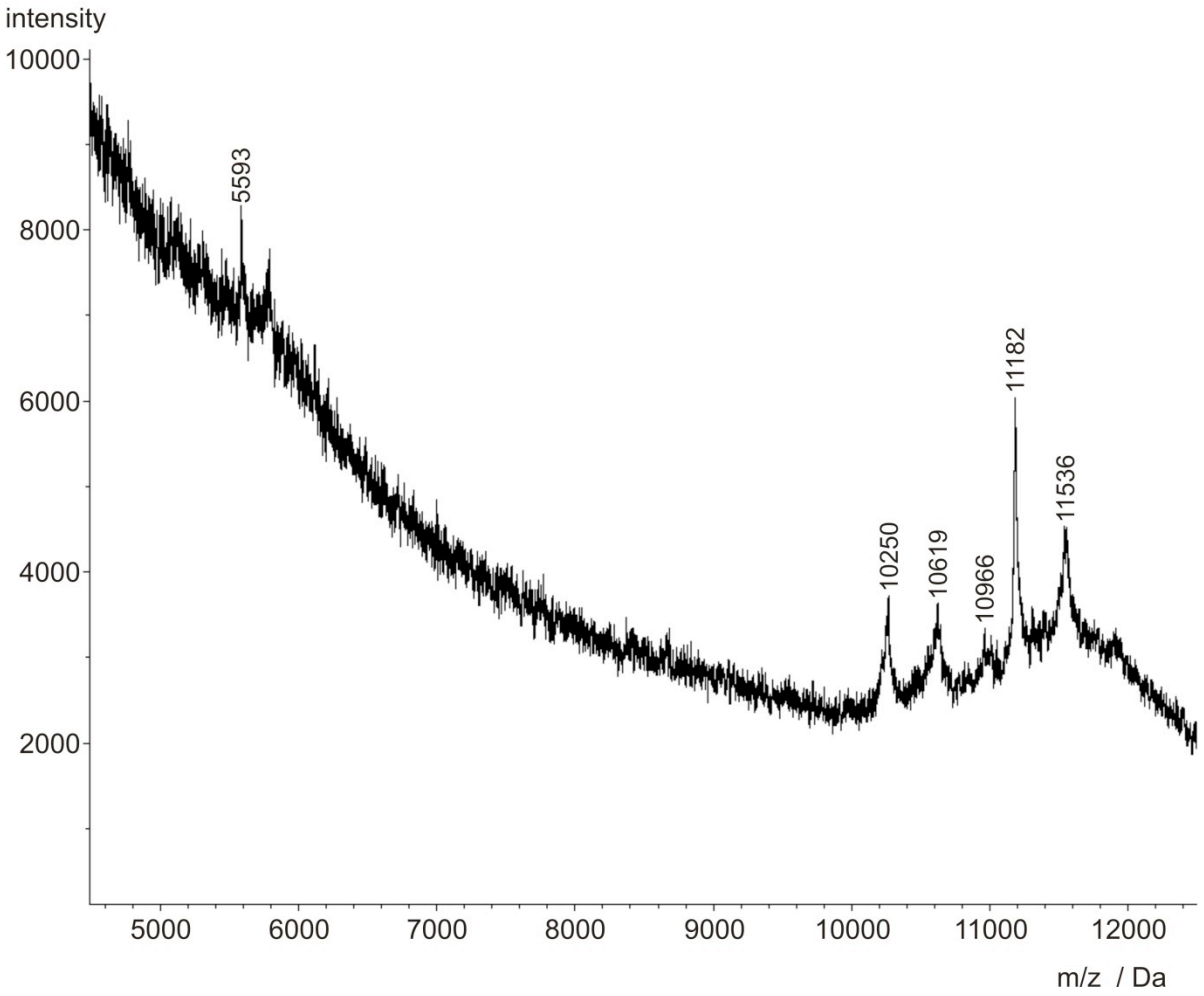
^{1}H-NMR (400 MHz, DMSO-d_6, 300 K): δ = 10.35 (s, 2H), 7.99 (d, *J* = 8.3 Hz, 1H), 7.37 (d, *J* = 8.3 Hz, 1H), 7.01 (s, 1H), 6.62 (d, *J* = 7.9 Hz, 2H), 6.59 (s, 2H), 6.52 (d, *J* = 7.9 Hz, 2H) ppm. ESI-MS: m/z = 346.2 (90 %) [M – N_2 + H^+], 374.0 (100 %) [M + H^+]; calculated for $C_{20}H_{11}N_3O_5$: 373.07 amu.



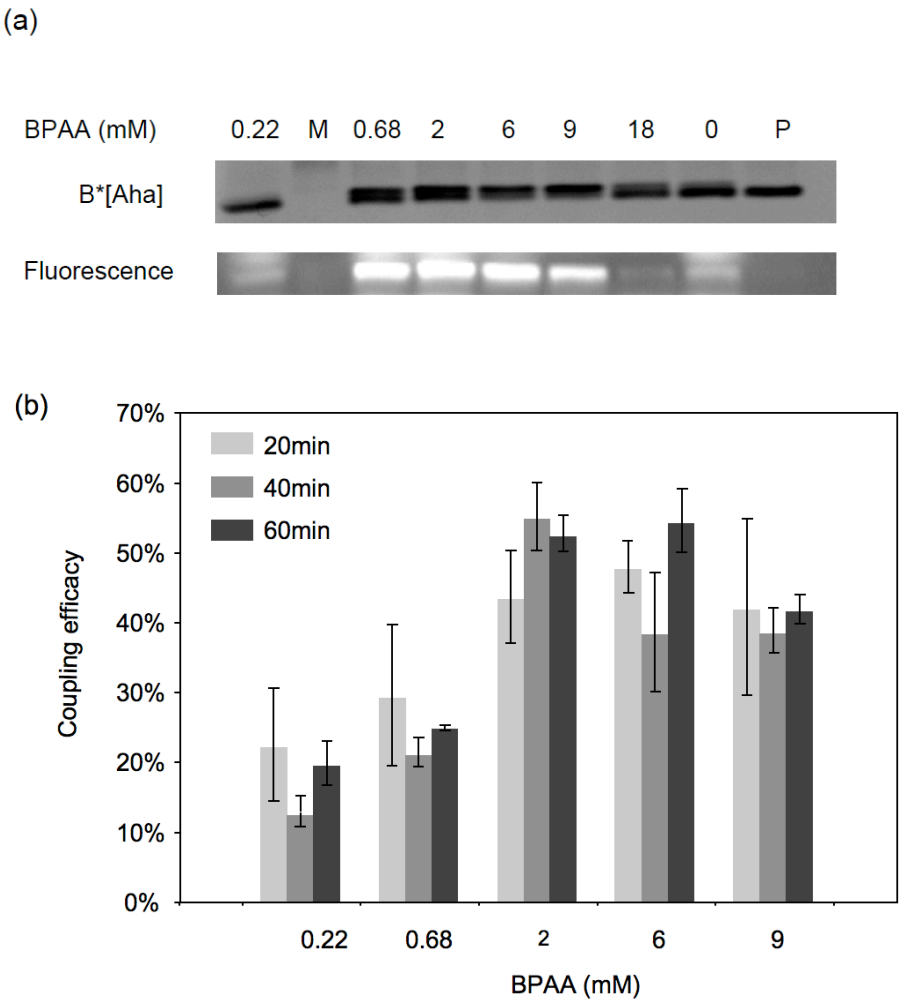
Supplementary Figure 1. Gel electrophoresis analysis of the b* variants. b*[Aha] (lane 1) and b*[Hpg] (lane 2) samples were resolved by SDS-PAGE and visualized by Coomassie staining.



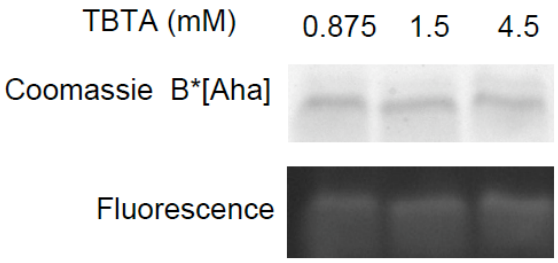
Supplementary Figure 2. ESI-mass spectra of b* variants. **(a)** Spectrum of b*[Aha] (calculated mass, 10247.4 Da). **(b)** Spectrum of b*[Hpg] (calculated mass, 10230.4 Da).



Supplementary Figure 3. MALDI-TOF analysis of the reaction product of b*[Aha] with LM155 (calculated mass 11181 Da). The signal at 10250 Da corresponds to b* parent protein containing methionine instead of azidohomoalanine (calculated mass 10252 Da). The signal at 10966 Da might correspond to the reaction product after Fmoc-deprotection (calculated mass 10960 Da). Whereas the signal at 5593 Da might correspond to the double protonated reaction product (calculated 5591,5 Da) the signals at 10619 and 11536 Da could not be assigned.



Supplementary Figure 4. Evaluation of BPAA as chelator for coupling b*[Aha] to the fluorescent dye LM155 **(a)** LM155 was coupled to b*[Aha] in the presence of increasing BPAA concentrations (reaction time: 40 min). The reaction products were resolved on SDS-PAGE and visualized by fluorescence imaging (lower panel) and Coomassie staining (upper panel). P, b*[Aha] without dye and coupling reagents; M, molecular size marker **(b)** Effect of BPAA concentration and reaction time on the coupling efficacy between LM155 and b*[Aha]. LM155 was coupled to b*[Aha] in the presence of the indicated BPAA concentrations for 20, 40 or 60 min. The reaction products were resolved on SDS-PAGE and the coupling efficacy was calculated from the intensities of the Coomassie-stained protein bands corresponding to the coupled or uncoupled products. All experiments were performed in triplicates.



Supplementary Figure 5. Evaluation of TBTA as chelator for coupling b*[Aha] to the fluorescent dye LM155. LM155 was coupled to b*[Aha] in the presence of increasing TBTA concentrations (reaction time: 40 min). The reaction products were resolved on SDS-PAGE and visualized by fluorescence imaging (lower panel) and Coomassie staining (upper panel).

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