Angewandte Chemie
Eine Zeitschrift der Gesellschaft Deutscher Chemiker

Supporting Information

for

Angew. Chem. Int. Ed. Z52084

69451 Weinheim, Germany

Affinity-Based Tagging of Protein Families with Reversible Inhibitors – A Novel Concept in Functional Proteomics

Miriam C. Hagenstein[#], Jan H. Mussgnug[§], Kirsten Lotte[#], Regina Plessow[#], Andreas Brockhinke[#], Olaf Kruse[§]*, Norbert Sewald[#]*

Department of Chemistry[#] and Department of Biology[§], University of Bielefeld, Universitätstrasse 25, D-33615 Bielefeld
e-mail: norbert.sewald@uni-bielefeld.de

Supporting Information

Contents

Abbreviations

Boc: *tert*-butoxycarbonyl, **Bpa:** L-4-benzoylphenylalanine, **DBU:** 1,8-diazabicyclo[5.4.0]undec-7-ene, **DCM:** dichloromethane, **Dde:** *N*-(1-(4,4-dimethyl-2,6-dioxocyclohexylidene)ethyl), **DHB:** 2,5-dihydroxybenzoic acid, **DIPEA**: *N*-ethyldiisopropylamine, **DMF:** *N*,*N*-dimethylformamide, **EDC:** *N*-ethyl-*N'*-(3-dimethylaminopropyl)-carbodiimide hydrochloride, **Fmoc:** 9-fluorenylmethoxy-carbonyl, **HOBt:** 1-hydroxy-benzotriazole, **NHS:** *N*-hydroxysuccinimide, **NMM:** *N*-methyl-morpholine, **TBME:** *tert*-butylmethylether, **TFA:** trifluoroacetic acid.

General

Unless otherwise noted, materials were obtained from commercial suppliers and were used without further purification. DMF was distilled at 55 mbar over ninhydrine, DCM was distilled first over calcium chloride and subsequently dried over calcium hydride.

For thin-layer chromatography (TLC) silica gel plates Merck 60 F_{254} and alumina plates Macherey-Nagel ALOX N/UV_{254} were used; compounds were visualized by irradiation with UV light and/or by treatment with a solution of ninhydrine in ethanol followed by heating.
Column chromatography was performed using silica gel Merck 60 (particle size 0.040-0.063 mm) or activated basic alumina Merck 90 (particle size 0.063-0.200 mm). Preparative RP-HPLC was

carried out using a Thermo Separations Products system consisting of a UV 1000 Detector and a P4000 pump equipped with a Vydac High Performance Guard Column (Protein and Peptide C18) and a Vydac 218 TP 1022 Efficiency Protein and Peptide C18 Column; Eluents: A = $H_2O/CH_3CN/TFA$ (95/5/0.1), B = $H_2O/CH_3CN/TFA$ (5/95/0.1). ^1H NMR and ^{13}C NMR spectra were recorded at room temperature either on Bruker AC 250 P (250.1 / 62.9 MHz) or Bruker DRX 500 (500.1 / 125.8 MHz). Chemical shifts δ are given in ppm relative to tetramethylsilane (TMS) or to residual solvent peaks. Infrared spectra (IR) were recorded on a Jasco Spectrometer FT/IR-410; peaks are reported in cm^{-1}. MALDI-ToF mass spectra were recorded on a Voyager-DE instrument (PerSeptive Biosystems) with a DHB-matrix. Electrospray ionization (ESI) mass spectrometry experiments were performed on a Bruker Esquire 3000 spectrometer. Melting points were recorded on a Büchi melting point B-540 and are uncorrected.

All SPR measurements were carried out on the BIAcore 3000 instrument (Biacore 3000 Control Software 3.1.1) with research grade CM5 sensor chips (supplied by BIAcore AB).

Protein kinase A, protein kinase A catalytic subunit (both from bovine heart) and 3PGA phosphokinase were purchased from Sigma, creatine kinase was purchased from Roche.

Protein staining and detection: Proteins were stained after gel electrophoresis as described in the literature.[1] Protein fluorescence was documented using a fluoroimager (Typhoon 8600, Molecular Dynamics).

Miriam Hagenstein, Jan Mussgnug, Kirsten Lotte, Regina Plessow, Andreas Brockhinke, Olaf Kruse*, Norbert Sewald*

Synthesis

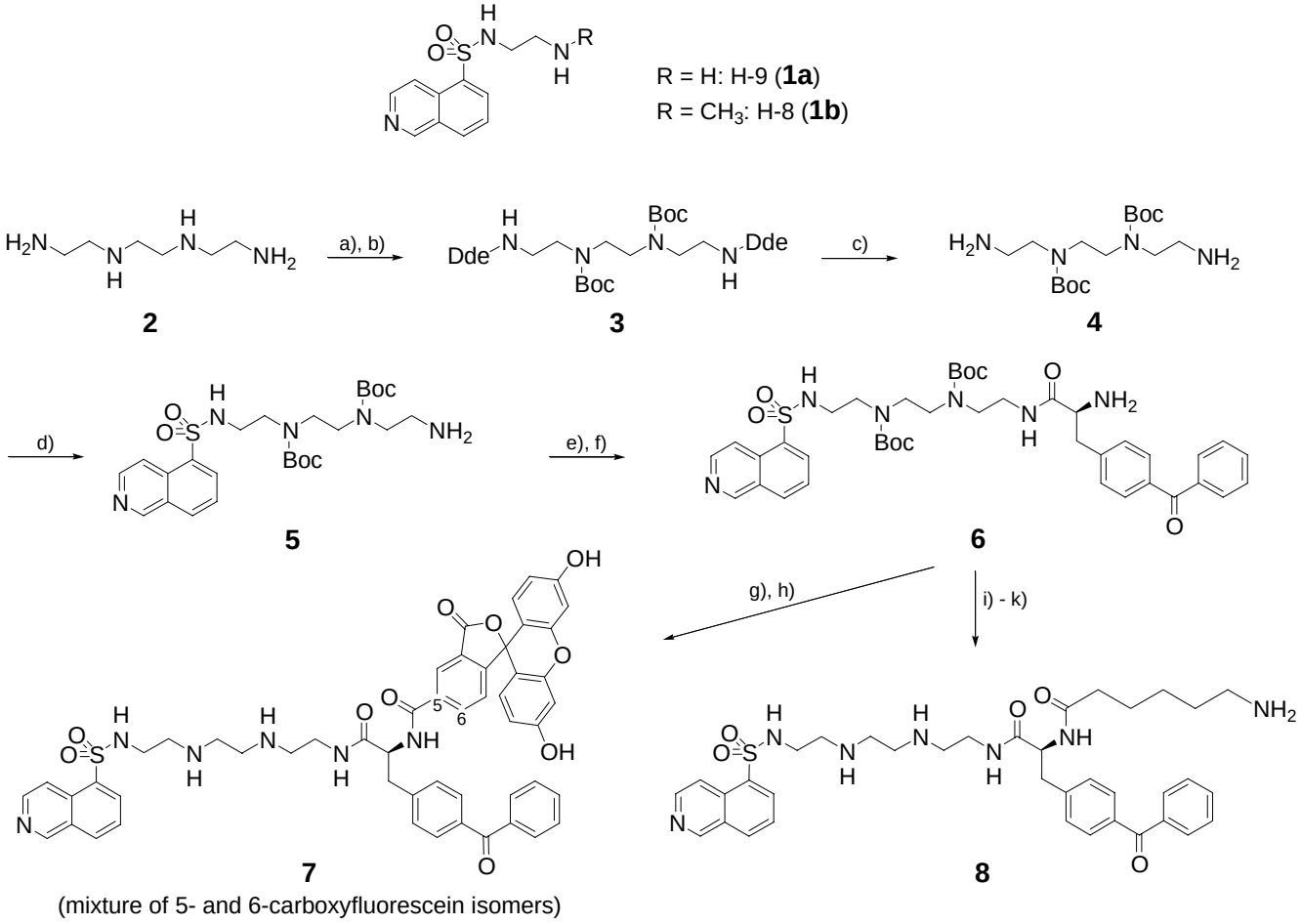


Figure S 1: Synthesis of the chemical probe **7** comprising the kinase inhibitor moiety H-9 (**1a**), the photoreactive group 4-benzoylphenylalanine, and the fluorescent reporter group 5-/6-carboxyfluorescein. Conjugate **8** is a derivative of **7** that can be immobilized to surfaces, e.g. on a surface plasmon resonance sensor chip.

H-9 (**1a**) was synthesized according to methods of Hidaka *et al*.[2]

Synthesis of 6,9-Di-(tert-butoxycarbonyl)-2,13-bis-(4,4-dimethyl-2,6-dioxocyclohexylidene)-3,6,9,12-tetra-azatetradecane (3)

2,13-Bis-(4,4-dimethyl-2,6-dioxo-cyclohexylidene)-3,6,9,12-tetraazatetradecane (2a)

A solution of 2-acetyldimedone (16.1 g, 88.4 mmol) and 1,8-diamino-3,6-diazaoctane (**2**) (4.51 mL, 30.0 mmol) in DMF (100 mL) was stirred at room temperature for 16 h. DMF was removed under

reduced pressure and the oily residue was redissolved in ethyl acetate. Crystallization at 4 °C gave the bis-Dde-protected compound **2a** as colorless crystals (10.5 g, 22.2 mmol, 74%).

Analytical data for **2a**: mp: 104-106 °C; IR (KBr): 3417, 3301, 2955, 2878, 2816, 1673, 1619, 1590, 1469, 1352, 1305, 1289, 1148; ^{1}H NMR (250.1 MHz, $CDCl_3$, TMS): δ = 1.02 (s, 12H), 2.25 (br. s, 2H), 2.35 (s, 8H), 2.58 (s, 6H), 2.83 (s, 4H), 2.95 (t, J = 6.2 Hz, 4H), 3.54 (dt, J = 5.1 Hz, J = 6.2 Hz, 4H), 13.44 (br. s, 2H); ^{13}C NMR (62.9 MHz, $CDCl_3$, TMS): δ = 18.2, 28.3, 30.1, 43.3, 47.7, 48.5, 53.0, 108.0, 173.3, 197.8.

6,9-Di-(*tert*-butoxycarbonyl)-2,13-bis-(4,4-dimethyl-2,6-dioxocyclohexylidene)-3,6,9,12-tetraaza-tetradecane (3)

A solution of di-*tert*-butyl dicarbonate (12.0 g, 55.0 mmol) in DCM (60 mL) was added dropwise to a solution of **2a** (10.9 g, 23.0 mmol) in DCM (30 mL). After stirring for 1 h at 45 °C the solvent was removed and the residue was recrystallized from DCM to give **3** as colorless crystals (13.8 g, 20.5 mmol, 89%).

Analytical data for **3**: mp: 153-154 °C; IR (KBr): 3433, 2999, 2961, 2931, 2899, 2873, 1698, 1633, 1576, 1458, 1406, 1364, 1338, 1235, 1160; ^{1}H NMR (250.1 MHz, $CDCl_3$, TMS): δ = 1.03 (s, 12H), 1.45 (s, 18H), 2.38 (s, 8H), 2.57 (s, 6H), 3.24-3.46 (m, 8H), 3.59 (m, 4H), 13.55 (br. s, 2H); ^{13}C NMR (62.9 MHz, $CDCl_3$, TMS): δ = 17.7, 28.2, 28.4, 30.1, 41.6, 45.9, 47.3, 52.8, 80.9, 108.0, 155.1, 174.2, 197.9.

1,8-Diamino-3,6-di-(*tert*-butoxycarbonyl)-3,6-diazaoctane (4)

A mixture of **3** (21.1 g, 31.4 mmol) and hydrazine hydrate (4.67 mL, 94.1 mmol) in DCM (200 mL) was stirred for 6 h at 50 °C. Afterwards the solution was dried over sodium sulfate and the solvent was removed. Column chromatography (basic alumina, 1. ethyl acetate, 2. methanol:water (1:1 v/v)) of the residual oil followed by crystallization from methanol/water gave **4** as a colorless solid (4.92 g, 14.2 mmol, 45%).

Analytical data for **4**: mp: 149 °C (decomposition); IR (KBr): 3433, 2977, 2932, 1684, 1578, 1481, 1421, 1367, 1249, 1168, 774; ^{1}H NMR (250.1 MHz, $CDCl_3$, TMS): δ = 1.46 (s, 18H), 2.28 (br. s, 4H), 2.84 (t, J = 6.6 Hz, 4H), 3.28 (t, J = 6.6 Hz, 4H), 3.34 (s, 4H); ^{13}C NMR (62.9 MHz, $CDCl_3$, TMS): δ = 28.5, 40.7, 45.9, 51.0, 79.9, 155.8.

_N_-(8-Amino-3,6-di-(*tert*-butoxycarbonyl)-3,6-diazaoct-1-yl)-5-isoquinolinesulfonamide (5)

A solution of 5-isoquinolinesulfonyl chloride hydrochloride (264 mg, 1.00 mmol) and triethylamine (348 µl, 2.50 mmol) in chloroform (20 mL) was added dropwise within one hour to an ice-cold solution of **4** (1.05 g, 3.03 mmol) in chloroform (15 mL). The reaction mixture was stirred 3 h at room temperature, washed twice with water, and the organic layer was dried over sodium sulfate. The solvent was removed under reduced pressure and the oily residue was purified by column chromatography (basic alumina, 5% (v/v) TBME:methanol) to give **5** as a pale yellow solid (340 mg, 0.63 mmol, 63%).

Analytical data for **5**: IR (KBr): 3436, 2977, 2931, 2876, 1681, 1568, 1480, 1421, 1367, 1325, 1248, 1161, 1042, 994, 832, 766; ^{1}H NMR (250.1 MHz, $CDCl_3$, TMS): δ = 1.44 (s, 18H), 3.02-3.43 (m, 12H), 7.68 (m, 1H), 8.19 (m, 1H), 8.42 (m, 1H), 8.48 (m, 1H), 8.68 (m, 1H), 9.34 (s, 1H); ^{13}C NMR (62.9 MHz, $CDCl_3$, TMS): δ = 28.4, 40.8, 42.6, 46.2, 46.7, 50.7, 50.9, 80.2, 117.5, 125.8, 129.1, 131.3, 132.9, 133.3, 135.0, 145.2, 153.2, 155.0, 155.8; MS (MALDI-ToF): found 538.4 $[M+H]^+$, 560.4 $[M+Na]^+$, 576.4 $[M+K]^+$, monoisotopic mass calcd for $C_{25}H_{40}N_5O_6S$ [M]: 537.26.

Synthesis of (S)-(4-Benzoylphenyl)alanine-[3,6-bis-(tert-butoxycarbonyl)-8-(5-isoquinoline-sulfonylamino)-3,6-diazaoct-1-yl]amide (6)

(S)-_N_-Fluorenylmethoxycarbonyl-(4-benzoylphenyl)alanine-[3,6-bis-(*tert*-butoxycarbonyl)-8-(5-isoquinolinesulfonylamino)-3,6-diazaoct-1-yl]amide (5a)

Fmoc-Bpa-OH (295 mg, 0.60 mmol) and HOBt·H_2O (91.9 mg, 0.60 mmol) were added to an ice-cold solution of EDC·HCl (115 mg, 0.60 mmol) and NMM (65.9 µl, 0.60 mmol) in DCM (5 mL). The mixture was stirred at 0 °C for 15 min and afterwards added dropwise to an ice-cold solution of **5** (215 mg, 0.40 mmol) in DCM (5 mL). After stirring at room temperature for 16 h the reaction solution was washed twice with 5% $NaHCO_3$ and once with saturated NaCl solution. The organic layer was dried over sodium sulfate and the solvent was removed. Purification of the residue by means of column chromatography (silica gel, ethyl acetate:petroleum ether (9:1 v/v)) afforded **5a** as a colorless solid (273 mg, 0.27 mmol, 68%).

Analytical data for **5a**: IR (KBr): 3421, 3329, 3062, 3004, 2975, 2931, 2868, 1694, 1661, 1609, 1537, 1478, 1449, 1416, 1366, 1319, 1280, 1245, 1156, 1040, 997, 925, 830, 759, 702; ^{1}H NMR

(500.1 MHz, (D_6)DMSO): δ = 1.28 (br. s, 18H), 2.96-3.30 (m, 14H), 3.97-4.13 (m, 2H), 4.14-4.32 (m, 2H), 7.18-7.40 (m, 4H), 7.41-7.50 (m, 4H), 7.55-7.73 (m, 7H), 7.73-7.92 (m, 3H), 8.04-8.34 (m, 4H), 8.40 (m, 2H), 8.66 (m, 1H), 9.46 (s, 1H); ^{13}C NMR (125.8 MHz, (D_6)DMSO): δ = 28.3, 37.5, 38.0, 41.2, 45.0, 45.2, 45.8, 46.7, 47.0, 56.3, 66.1, 79.1, 79.6, 117.6, 120.5, 125.6, 125.7, 126.8, 127.4, 128.0, 128.9, 129.1, 129.8, 130.0, 130.7, 132.7, 132.8, 133.8, 135.2, 135.4, 137.6, 141.1, 144.0, 144.1, 145.0, 153.8, 154.7, 155.0, 156.2, 171.6, 195.8; MS (MALDI-ToF): found 1012.1 $[M+H]^+$, 1034.2 $[M+Na]^+$, 1050.2 $[M+K]^+$, monoisotopic mass calcd for $C_{56}H_{62}N_6O_{10}S$ [M]: 1010.42.

(S)-(4-Benzoylphenyl)alanine-[3,6-bis-(*tert*-butoxycarbonyl)-8-(5-isoquinolinesulfonylamino)-3,6-diazaoct-1-yl]amide (6)

A solution of **5a** (212 mg, 0.21 mmol) in DCM (5 mL) was treated with 4-(aminomethyl)piperidine (2 mL). After stirring for 30 min at room temperature the reaction mixture was washed with three 10 mL portions of phosphate buffer of pH 5.5 (prepared from 90 g of $NaH_2PO_4 \cdot H_2O$ and 32.7 g of Na_2HPO_4 in 500 mL of distilled water).[3] The organic phase was dried over sodium sulfate and concentrated *in vacuo*. The residue was purified by silica gel chromatography (using chloroform:ethanol (9:1 v/v) as eluant) to give the Fmoc-deprotected molecule **6** as a colorless solid (141 mg, 0.18 mmol, 85%).

Analytical data for **6**: IR (KBr): 3451, 3385, 3088, 3062, 2975, 2930, 2873, 1692, 1656, 1605, 1535, 1478, 1416, 1366, 1318, 1279, 1244, 1158, 991, 923, 830, 763, 703; ^{1}H NMR (250.1 MHz, (D_6)DMSO): δ = 1.29 (br. s, 18H), 2.64-3.34 (m, 16H), 3.40 (m, 1H), 7.37 (m, 2H), 7.53 (m, 2H), 7.61-7.74 (m, 5H), 7.81 (m, 1H), 7.86-7.94 (m, 2H), 8.32 (m, 1H), 8.40 (m, 1H), 8.41 (m, 1H), 8.67 (m, 1H), 9.46 (s, 1H); ^{13}C NMR (62.9 MHz, $CDCl_3$, TMS): δ = 28.3, 37.2, 37.7, 41.5, 45.0, 45.7, 46.6, 47.4, 56.5, 79.0, 79.1, 117.6, 126.8, 128.9, 129.1, 129.9, 130.0, 130.7, 132.7, 132.9, 133.8, 135.2, 135.3, 137.7, 144.8, 145.0, 153.8, 154.7, 155.0, 174.7, 195.8; MS (MALDI-ToF): found 789.7 $[M+H]^+$, 811.7 $[M+Na]^+$, 827.8 $[M+K]^+$, monoisotopic mass calcd for $C_{41}H_{52}N_6O_8S$ [M]: 788.36.

Synthesis of (S)-N-[Fluorescein-5(6)-carbonyl]-(4-benzoylphenyl)alanine-[8-(5-isoquinoline-sulfonylamino)-3,6-diazaoct-1-yl]amide (7)

(S)-*N*-[Fluorescein-5(6)-carbonyl]-(4-benzoylphenyl)alanine-[3,6-bis-(*tert*-butoxycarbonyl)-8-(5-isoquinolinesulfonylamino)-3,6-diazaoct-1-yl]amide (6a)

5(6)-Carboxyfluorescein (64.0 mg, 0.17 mmol) and HOBt·H_2O (26.0 mg, 0.17 mmol) were added to an ice-cold solution of EDC·HCl (32.6 mg, 0.17 mmol) and NMM (18.7 µl, 0.17 mmol) in DCM/DMF 1:1 (2 mL). The mixture was stirred at 0 °C for 15 min and afterwards added dropwise to an ice-cold solution of **6** (92.0 mg, 0.12 mmol) in DCM (0.5 mL). After stirring at room temperature for 16 h the solvent was removed under reduced pressure and the oily residue was purified by means of preparative RP-HPLC to give a mixture of both isomers of **6a** as a yellow solid (68.1 mg, 0.054 mmol, 45%, trifluoroacetic acid salts).

Analytical data for **6a**: RP-HPLC (gradient: A/B (92:8) → A/B (0:100) during 50 min, flow rate 10 mL/min, $\lambda = 254$ nm), t_R = 30.8 min; IR (KBr): 3435, 3079, 2978, 2931, 1660, 1605, 1542, 1457, 1418, 1394, 1369, 1317, 1281, 1246, 1174, 925, 833, 798, 705; ^{1}H NMR (500.1 MHz, (D_6)DMSO) of the isomeric mixture (ca. 1:1): $\delta = 1.28$ (br. s, 36H), 2.82-3.29 (m, 28H), 4.68 (m, 1H), 4.79 (m, 1H), 6.45-6.58 (m, 8H), 6.67 (m, 4H), 7.34 (m, 1H), 7.39 (m, 2H), 7.48-7.56 (m, 8H), 7.59-7.70 (m, 9H), 7.87 (m, 2H), 8.03-8.12 (m, 2H), 8.17 (m, 1H), 8.20-8.35 (m, 4H), 8.37 (m, 2H), 8.44-8.51 (m, 5H), 8.71 (m, 2H), 8.95 (m, 1H), 9.06 (m, 1H), 9.56 (s, 2H), 10.16 (br. s, OH); MS (ESI, CH_3CN): found 1147.5 $[M+H]^+$, 1169.4 $[M+Na]^+$, monoisotopic mass calcd for $C_{62}H_{62}N_6O_{14}S$ [M]: 1146.40.

(S)-*N*-[Fluorescein-5(6)-carbonyl]-(4-benzoylphenyl)alanine-[8-(5-isoquinolinesulfonylamino)-3,6-diazaoct-1-yl]amide (7)

A solution of **6a** (48.3 mg, 0.038 mmol) in TFA:DCM (3:7 v/v) was stirred at room temperature for 30 min. The solvent was removed under reduced pressure and the residue was lyophilised to give both regioisomers of **7** as a hygroscopic yellow solid (48.4 mg, 0.038 mmol, 99%, trifluoroacetic acid salts).

Analytical data for **7**:

MS (ESI, CH_3CN): found 947.3 $[M+H]^+$, monoisotopic mass calcd for $C_{52}H_{46}N_6O_{10}S$ [M]: 946.30.

The regioisomers of **7** with respect to the carboxyfluorescein substitution pattern may be separated by RP-HPLC (gradient: A/B (92:8) → A/B (70:30) after 5 min → A/B (30:70) after 35 min, flow rate 25 mL/min, $\lambda = 254$ nm), t_R (5-carboxyfluorescein isomer) = 14.4 min, t_R (6-carboxyfluorescein isomer) = 15.7 min.

5-carboxyfluorescein isomer: ^{1}H NMR (500.1 MHz, (D_6)DMSO): δ = 2.98-3.49 (m, 14H), 4.81 (m, 1H), 6.49-6.58 (m, 4H), 6.68 (m, 2H), 7.36 (d, J = 8.2 Hz, 1H), 7.48-7.55 (m, 4H), 7.64-7.70 (m, 5H), 7.87 (m, 1H), 8.17 (m, 1H), 8.36 (d, J = 7.2 Hz, 1H), 8.42 (d, J = 6.1 Hz, 1H), 8.45-8.53 (m, 4H), 8.73 (d, J = 6.1 Hz, 1H), 8.80-9.06 (br. s, NH), 9.14 (m, 1H), 9.52 (s, 1H), 10.20 (br. s, OH);

6-carboxyfluorescein isomer: ^{1}H NMR (500.1 MHz, (D_6)DMSO): δ = 2.93-3.39 (m, 14H), 4.71 (m, 1H), 6.45-6.59 (m, 4H), 6.68 (m, 2H), 7.38 (d, J = 8.2 Hz, 2H), 7.49-7.57 (m, 4H), 7.59-7.63 (m, 2H), 7.64-7.69 (m, 2H), 7.87 (m, 1H), 8.06-8.13 (m, 2H), 8.36 (d, J = 7.1 Hz, 1H), 8.41 (d, J = 6.1 Hz, 1H), 8.42-8.51 (m, 3H), 8.73 (d, J = 6.1 Hz, 1H), 8.75-8.96 (br. s, NH), 9.02 (m, 1H), 9.52 (s, 1H), 10.20 (br. s, OH).

Synthesis of (S)-N-[6-Aminohexanoyl]-(4-benzoylphenyl)alanine-[8-(5-isoquinolinesulfonyl-amino)-3,6-diazaoct-1-yl]amide (8)

(S)-*N*-[*N´*-Fluorenylmethoxycarbonyl-6-aminohexanoyl]-(4-benzoylphenyl)alanine-[3,6-bis-(*tert*-butoxycarbonyl)-8-(5-isoquinolinesulfonylamino)-3,6-diazaoct-1-yl]amide (11)

Fmoc-6-aminohexanoic acid (21.2 mg, 0.06 mmol) and HOBt·H_2O (14.7 mg, 0.09 mmol) were added to an ice-cold solution of EDC·HCl (11.7 mg, 0.06 mmol) and NMM (9.89 µl, 0.06 mmol) in DCM (1 mL). The mixture was stirred at 0 °C for 15 min and afterwards added dropwise to an ice-cold solution of **6** (38.0 mg, 0.05 mmol) in DCM (2 mL). After stirring at room temperature for 16 h the solvent was removed under reduced pressure and the oily residue was purified by means of preparative RP-HPLC to give **11** as a colorless solid (32 mg, 0.026 mmol, 52%, trifluoroacetic acid salt).

Analytical data for **11**: RP-HPLC (gradient: A/B (92:8) → A/B (55:45) after 5 min → A/B (0:100) after 30 min, flow rate 10 mL/min, λ = 254 nm), t_R = 19.8 min; ^{1}H NMR (500.1 MHz, CD_2Cl_2, TMS): δ = 1.02 (m, 2H), 1.39 (br. s, 18H), 1.43-1.58 (m, 6H), 2.18 (m, 2H), 2.99-3.13 (m, 4H), 3.17-3.42 (m, 10H), 4.18 (t, J = 6.5 Hz, 1H), 4.33 (d, J = 6.5 Hz, 2H), 4.79 (m, 1H), 6.65 (br. s, NH), 7.25-7.35 (m, 4H), 7.38 (m, 2H), 7.47 (m, 2H), 7.54-7.63 (m, 3H), 7.68 (m, 2H), 7.71-7.80 (m, 4H), 7.94 (m, 1H), 8.12 (br. s, NH), 8.47 (m, 1H), 8.67 (m, 2H), 9.04 (m, 1H), 9.71 (s, 1H); ^{13}C NMR (125.8 MHz, CD_2Cl_2): δ = 25.5, 26.3, 28.5, 29.8, 36.4, 38.9, 39.5, 41.0, 42.6, 42.9, 46.9, 47.7,

48.7, 49.8, 54.6, 66.9, 81.2, 81.3, 120.3, 125.4, 125.5, 127.4, 128.0, 128.7, 129.6, 129.7, 130.3, 130.5, 130.6, 130.7, 132.8, 132.9, 134.2, 135.3, 136.6, 137.3, 137.9, 141.7, 142.1, 144.4, 144.5, 156.6, 156.7, 156.9, 171.8, 174.2, 196.6; MS (MALDI-ToF): found 1124.5 $[M+H]^+$, 1146.5 $[M+Na]^+$, 1162.5 $[M+K]^+$, monoisotopic mass calcd for $C_{62}H_{73}N_7O_{11}S$ [M]: 1123.5.

(S)-*N*-[6-Aminohexanoyl]-(4-benzoylphenyl)alanine-[3,6-bis-(*tert*-butoxycarbonyl)-8-(5-isoquinolinesulfonylamino)-3,6-diazaoct-1-yl]amide (12)

A solution of **11** (32 mg, 0.026 mmol) in DCM (1.5 mL) was treated with 4-(aminomethyl)piperidine (0.25 mL). After stirring for 1 h at room temperature the solvent was removed under reduced pressure. The oily residue was purified by means of preparative RP-HPLC to give the Fmoc-deprotected molecule **12** as a colorless solid (26.9 mg, 0.24 mmol, 92%, trifluoroacetic acid salt).

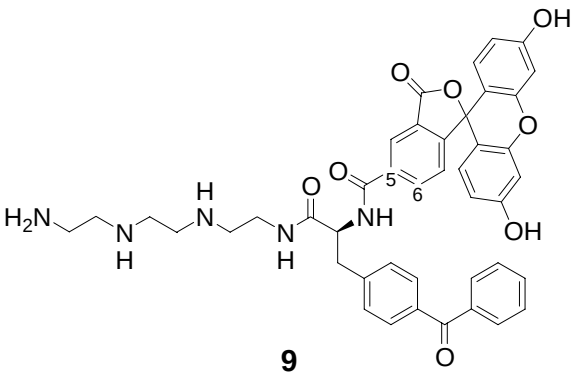
Analytical data for **12**: RP-HPLC (gradient: A/B (92:8) → A/B (20:80) after 40 min, flow rate 10 mL/min, λ = 254 nm), t_R = 20.7 min; ^{1}H NMR (500.1 MHz, CD_3OD): δ = 1.19-1.27 (m, 2H), 1.41 (br. s, 18H), 1.46-1.62 (m, 6H), 1.99 (m, 2H), 2.20 (m, 2H), 2.85 (m, 2H), 2.90 (m, 2H), 2.93-3.08 (m, 4H), 3.25 (br. s, NH), 3.30 (m, 2H), 3.44 (m, 2H), 4.67 (m, 1H), 7.41 (m, 2H), 7.51 (m, 2H), 7.63 (m, 1H), 7.69 (m, 2H), 7.73 (m, 2H), 7.93 (m, 1H), 8.18 (br.s, NH), 8.51 (m, 1H), 8.57 (m, 1H), 8.66-8.87 (m, 2H), 9.66 (s, 1H); ^{13}C NMR (125.8 MHz, CD_3OD): δ =26.1, 26.8, 27.2, 28.2, 28.8, 33.5, 39.1, 40.5, 42.4, 44.4, 44.9, 46.8, 47.4, 55.7, 81.4, 81.5, 129.0, 129.1, 129.5, 130.5, 130.9, 131.2, 133.9, 135.5, 135.6, 136.0, 136.2, 137.1, 137.3, 138.8, 144.1, 144.2, 152.6, 157.0, 157.1, 173.5, 175.5, 198.3; MS (MALDI-ToF): found 902.5 $[M+H]^+$, 924.5 $[M+Na]^+$, 940.5 $[M+K]^+$, monoisotopic mass calcd for $C_{47}H_{63}N_7O_9S$ [M]: 901.4.

(S)-*N*-[6-Aminohexanoyl]-(4-benzoylphenyl)alanine-[8-(5-isoquinolinesulfonylamino)-3,6-diazaoct-1-yl]amide (8)

A solution of **12** (5 mg, 0.004 mmol) in TFA:DCM (3:7 v/v) was stirred at room temperature for 30 min. The solvent was removed under reduced pressure and the residue was lyophilised to give **8** as a hygroscopic colorless solid (5 mg, 0.004 mmol, 98%, trifluoroacetic acid salt).

Analytical data for **8**: RP-HPLC (gradient: A/B (92:8) → A/B (20:80) after 40 min, flow rate 10 mL/min, λ = 254 nm), t_R = 14.1 min; ^{1}H NMR (500.1 MHz, CD_3OD): δ = 1.18-1.27 (m, 2H), 1.47-1.61 (m, 4H), 2.23 (m, 2H), 2.84 (m, 2H), 3.03 (m, 1H), 3.15-3.21 (m, 3H), 3.23-3.28 (m, 5H),

3.35 (m, 2H), 3.44 (m, 1H), 3.47 (br. s, NH), 3.57-3.65 (2H), 4.64 (m, 1H), 7.43 (m, 2H), 7.52 (m, 2H), 7.65 (m, 1H), 7.70 (m, 2H), 7.75 (m, 2H), 7.86 (m, 1H), 8.47 (m, 1H), 8.52 (m, 1H), 8.59 (m, 1H), 8.65 (m, 1H), 9.47 (s, 1H); MS (MALDI-ToF): found 702.8 $[M+H]^+$, 724.9 $[M+Na]^+$, 740.9 $[M+K]^+$, monoisotopic mass calcd for $C_{37}H_{47}N_7O_5S$ [M]: 701.3.



9

Figure S 2: Synthesis of conjugate **9** for photoaffinity control experiments

Synthesis of (S)-N-[Fluorescein-5(6)-carbonyl]-(4-benzoylphenyl)alanine-[8-amino-3,6-diaza-oct-1-yl]amide (9)

(S)-*N*-[Fluorescein-5(6)-carbonyl]-(4-benzoylphenyl)alanine (13)

A mixture of Fmoc-Bpa-OH (80 mg, 0.16 mmol), scavenger resin *N*-(2-mercaptoethyl)-aminoethyl polystyrene (3 eq.) and DBU (47.8 µl, 0.32 mmol) in THF (5 mL) was agitated at room temperature for 1.5 h. The resin was filtered and washed with THF, and the H-Bpa-OH containing filtrate was concentrated to 5-10 mL. A solution of NHS-activated 5(6)-carboxyfluorescein (70 mg, 0.15 mmol) and DBU (23.9 µl, 0.16 mmol) in THF (3 mL) was added dropweise to the filtrate. After stirring at room temperature for 2 h the solvent was removed under reduced pressure and the oily reddish residue was purified by means of preparative RP-HPLC to give a mixture of both isomers of **13** as an orange solid (42 mg, 0.067 mmol, 42%).

Analytical data for **13**: RP-HPLC (gradient: A/B (92:8) → A/B (0:100) after 50 min, flow rate 10 mL/min, λ = 254 nm), t_R = 39.6 min; ^{1}H NMR (500.1 MHz, CD_3OD): δ = 3.42 (m, 2H), 4.92 (m, 1H), 6.66 (m, 2H), 6.78 (m, 2H), 6.82 (m, 2H), 7.36 (m, 1H), 7.46 (m, 2H), 7.50 (m, 2H), 7.57 (m, 1H), 7.61 (m, 1H), 7.65 (m, 2H), 7.72 (m, 2H), 8.13 (m, 1H), 8.41 (br. s, OH); MS (MALDI-ToF):

found 628.5 $[M+H]^+$, 650.4 $[M+Na]^+$, 666.5 $[M+K]^+$, monoisotopic mass calcd for $C_{37}H_{25}NO_9$ [M]: 627.15.

(S)-*N*-[Fluorescein-5(6)-carbonyl]-(4-benzoylphenyl)alanine-[3,6-bis-(*tert*-butoxycarbonyl)-8-amino-3,6-diazaoct-1-yl]amide (14)

A solution of **13** (12 mg, 0.019 mmol) in CH_2Cl_2 (2 mL) was added to an ice-cold solution of EDC·HCl (3.64 mg, 0.019 mmol), NMM (2.09 µl, 0.019 mmol) and HOBt·H_2O (2.9 mg, 0.019 mmol) in DCM (1 mL). The mixture was stirred at 0 °C for 15 min and afterwards added dropwise to an ice-cold solution of **4** (32.9 mg, 0.095 mmol) in DCM (1 mL). After stirring at room temperature for 16 h the solvent was removed under reduced pressure and the oily residue was purified by means of preparative RP-HPLC to give **14** as a yellow solid (3.6 mg, 0.003 mmol, 18%, trifluoroacetic acid salt).

Analytical data for **15**: RP-HPLC (gradient: A/B (92:8) → A/B (30:70) after 40 min, flow rate 10 mL/min, λ = 254 nm), t_R = 27.2 min; MS (MALDI-ToF): found 956.6 $[M+H]^+$, 978.6 $[M+Na]^+$, 994.6 $[M+K]^+$, monoisotopic mass calcd for $C_{53}H_{57}N_5O_{12}$ [M]: 955.4.

(S)-*N*-[Fluorescein-5(6)-carbonyl]-(4-benzoylphenyl)alanine-[8-amino-3,6-diaza-oct-1-yl]amide (9)

A solution of **14** (3.6 mg, 0.003 mmol) in TFA:DCM (3:7 v/v) was stirred at room temperature for 30 min. The solvent was removed under reduced pressure and the residue was lyophilised to give **10** as a hygroscopic orange solid (3.4 mg, 0.003 mmol, 92%, trifluoroacetic acid salt).

Analytical data for **9**:

^{1}H NMR (500.1 MHz, CD_3OD): δ = 2.98 (m, 2H), 3.10-3.21 (m, 6H), 3.23-3.28 (m, 4H), 3.46 (m, 2H), 4.82 (m, 1H), 6.22 (m, 2H), 6.35 (m, 2H), 6.45 (m, 2H), 6.98 (m, 2H), 7.03 (m, 1H), 7.10 (m, 2H), 7.18 (m, 3H), 7.23 (m, 2H), 7.48 (m, 1H), 8.01 (m, 1H); MS (MALDI-ToF): found 756.6 $[M+H]^+$, 778.3 $[M+Na]^+$, 794.6 $[M+K]^+$, monoisotopic mass calcd for $C_{43}H_{41}N_5O_8$ [M]: 755.3.

Miriam Hagenstein, Jan Mussgnug, Kirsten Lotte, Regina Plessow, Andreas Brockhinke, Olaf Kruse*, Norbert Sewald*

NMR Spectra

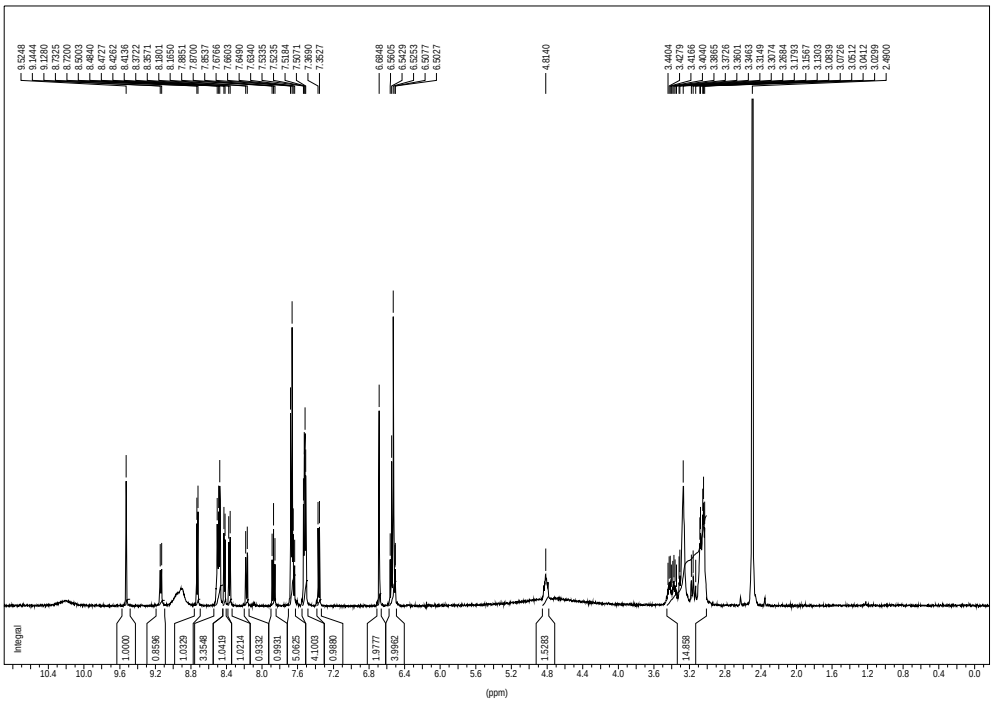


Figure S 3: ^{1}H NMR (500.1 MHz, D_6-DMSO): 5-carboxyfluorescein isomer of **7**

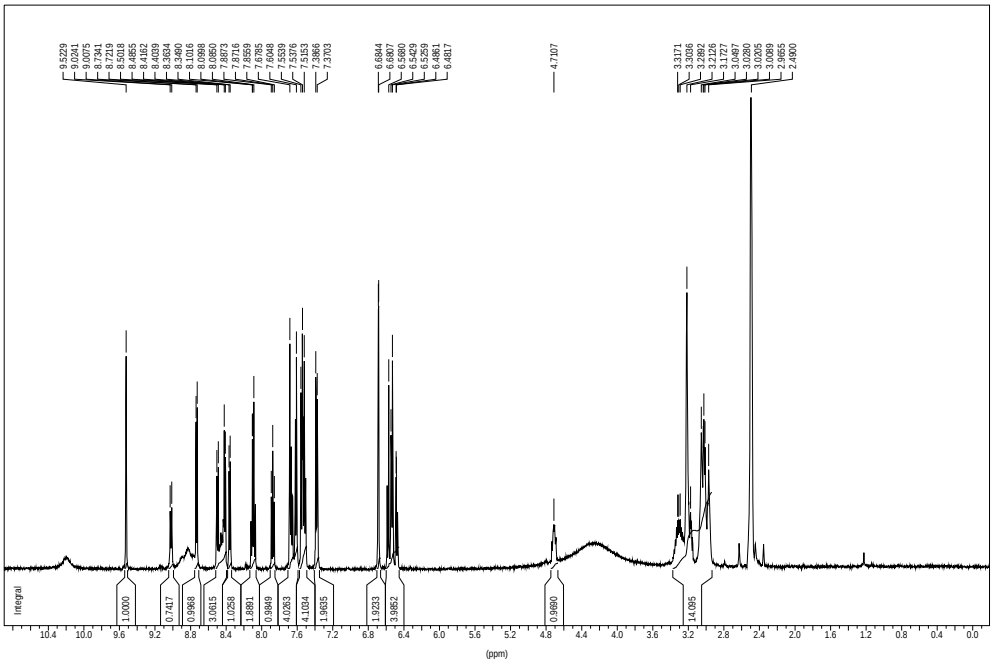


Figure S 4: ^{1}H NMR (500.1 MHz, D_6-DMSO): 6-carboxyfluorescein isomer of **7**

UV Spectroscopic Studies

A potential problem during the design of these photoactivable probes is that they contain three fluorescent groups: the inhibitor, the photoreactive group and the reporter group. In order to study effects of potential radiationless fluorescence resonance energy transfer (FRET) in the designed probe, particularly between the benzophenone triplet state and the neighboring fluorescein, the excitation-emission spectroscopy (EES) approach was used. It allows for simultaneous acquisition of all excitation and emission spectra of a given substance in a two-dimensional matrix and has been shown to be a suitable method to determine quantum efficiencies and to identify spectral changes due to FRET [4]. If FRET becomes the dominant loss channel from the excited benzophenone moiety, the probe would become useless, since it would no longer be possible to trigger the photoactivation group.

Figure S 5 shows an excitation-emission spectrum of 4-benzoylphenylalanine in acetonitrile. Besides emissions due to scattering processes, two emission bands can be seen: a strong band at short wavelengths (maximum at λ_{ex}= 260 nm), and a weaker band in the region of 300 $\leq\lambda_{ex} \leq$ 370 nm that has only 20% of the intensity of the stronger band. Emission spectra are different for these two bands as well as can be seen by the normalized emission spectra of 4-benzoylphenylalanine at three excitation wavelengths shown in Figure S 6. Interestingly, the emission of the band at λ_{ex}= 340 nm is shifted towards shorter wavelengths in comparison with the band at lower excitation wavelengths. This is in contrast to the fluorescence behavior for most other large molecules, which exhibit a shift to the red with increasing excitation wavelength.

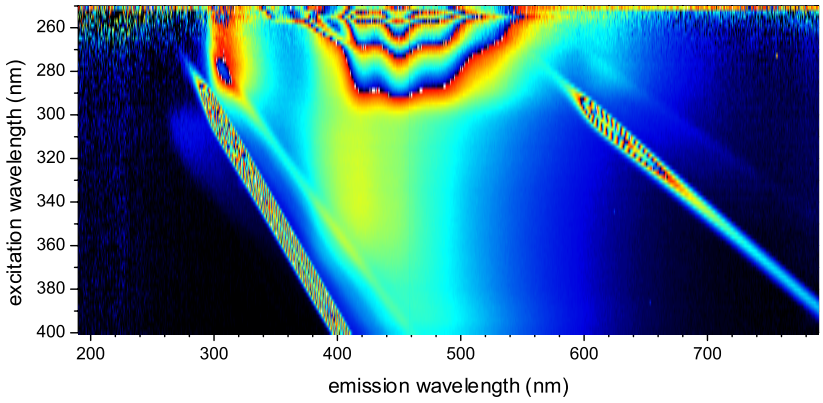


Figure S 5: Excitation – emission spectrum of 4-benzoylphenylalanine in acetonitrile.

For 4-benzoylphenylalanine dissolved in water, the band at λ_{ex}= 260 nm retains its strength. In contrast, the band at longer excitation wavelengths is more than on order of magnitude weaker. We estimate a quantum yield for fluorescence around $\Phi \approx 10^{-4}$ λ_{ex}= 350 nm. Bound to the polar spacer,

fluorescence emissions of the benzophenone moiety are even closer to the detection limit. Similar experiments have been carried out to determine the quantum efficiency of the H-9 inhibitor. It was found to be low as well ($\Phi \approx 10^{-4}$).

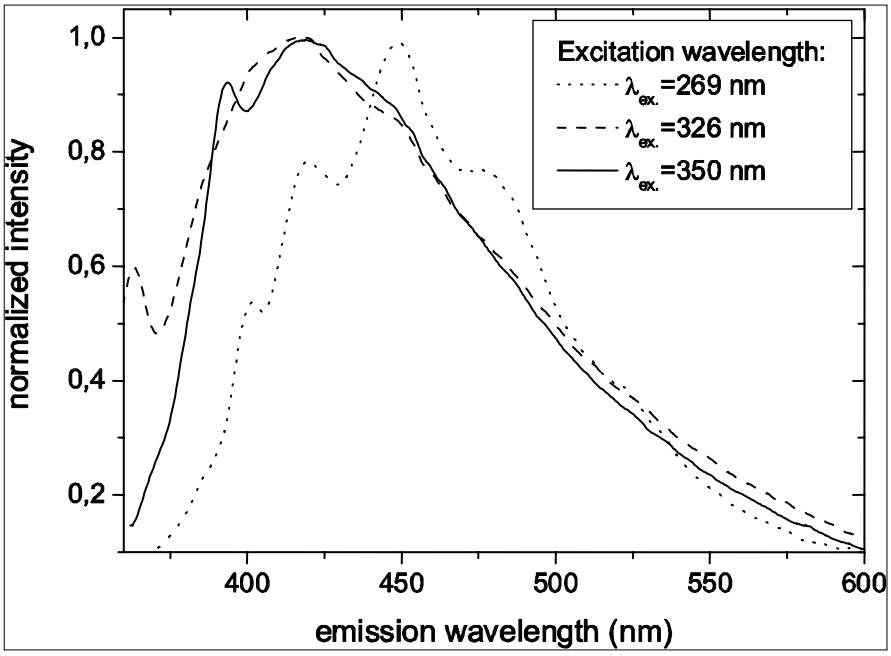


Figure S 6: Normalized emission spectra of 4-benzoylphenylalanine in acetonitrile at three different excitation wavelengths

Since there is an overlap between the emission spectrum of benzophenone (λ^{max}_{em} = 420 nm at λ_{ex} = 350 nm) and the excitation spectrum of fluorescein (λ^{max}_{ex} = 490 nm) one might speculate that energy transfer can occur. However, by comparing normalized emission spectra of **7**, the complete probe (H-9, benzophenone, fluorescein) with those of the compound **9** without reactive group, no significant FRET could be found at λ_{ex} = 350 nm ($\Phi_{FRET} \leq 10^{-2}$, cf. Figure S 7[1]). Therefore, quenching and chemical reactions are the dominant loss channels after photoactivation at this wavelength. At shorter excitation wavelengths, the emission spectrum of benzophenone shifts towards the red (λ^{max}_{em} = 450 nm) and some resonance energy transfer is observed (15% increase of acceptor fluorescence). Energy transfer between H-9 and fluorescein was found to be negligible in the complete wavelength range ($\Phi_{FRET} \leq 10^{-4}$).

In our study we found the quantum yield for fluorescence to be very low for both **1a** and 4-benzoylphenylalanine ($\phi_{Fl} \approx 10^{-4}$). When bound to the polar spacer, the quantum yield of 4-benzoylphenylalanine is even closer to the detection limit.

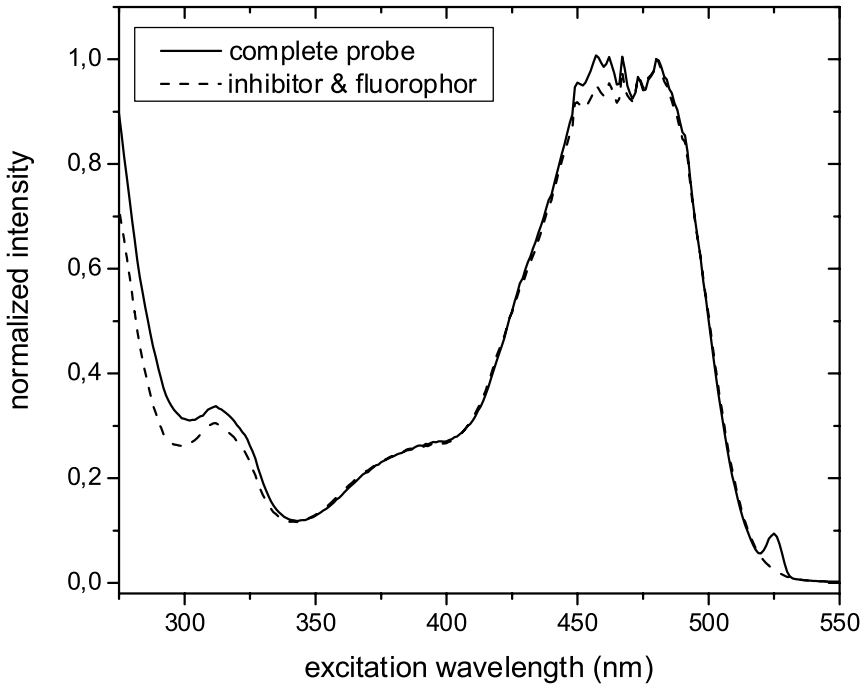


Figure S 7: Excitation spectra detected at emission wavelength λ_{em} = 527 nm (maximum of the fluorescein emission). Solid line: complete probe **7** (H-9 – linker – 4-benzoylphenylalanine – carboxyfluorescein). Dashed line: probe **9** (inhibitor H-9 and carboxyfluorescein only).

[1] The somewhat jagged form of the spectra at wavelengths between 450 and 475 nm is due to the Xe-lamp used in EES experiments. In this range, it emits a strong line spectrum superimposed on the usual broadband emission, which complicates normalization of the excitation spectra.

Biacore analysis

Surface plasmon resonance measurements:

Running buffers were sterile filtered (pore size 0.22 μm) and degassed prior to use.

a) Coupling of **8** to a CM5 sensor chip: During the coupling procedure, HBS-EP buffer (0.01 M 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (pH 7.4), 0.15 M NaCl, 3 mM ethylenediamine tetraacetate, 0.005 % (v/v) surfactant P20) was used as running buffer. The carboxymethylated dextran (CM-dextran) matrix on the sensor chip surface was activated according to the recommended protocol: equal volumes of 0.1 M *N*-hydroxysuccinimide (NHS) and 0.4 M *N*-ethyl-*N'*-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC) prepared in ultra-pure water were mixed and the mixture was injected over the surface of both the analytical flow cell and the reference cell for 7 min at a flow rate of 5 μL min^{-1}. A solution of **8** in HBS-EP buffer (concentration 170 μM for protein kinase A, 860 μM for creatine kinase studies) was injected into the analytical flow cell during 7 min at a flow rate of 5 μL min^{-1}. Up to 160 RU (protein kinase A) and 380 RU (creatine kinase) loading of the carboxy groups with **8** was achieved. Unreacted hydroxysuccinimidyl groups on the surface of both flow cells were subsequently deactivated by a 7 min pulse of 1 M ethanolamine hydrochloride, pH 8.5.

b) SPR experiments: Following the immobilization of **8**, the whole system was rinsed thoroughly with Tris-HCl buffer (Tris[hydroxymethyl] aminomethane hydrochloride (pH 7.0), 10 mM $MgCl_2$).

1) Protein kinase A catalytic subunit (PKA): Protein kinase A catalytic subunit was reconstituted as recommended by Sigma to a concentration of 0.05 mg protein/mL (1.195 μM). The enzyme was diluted in running buffer to various concentrations (7.5 nM - 120 nM) and injected for 3 min at a flow rate of 30 μL min^{-1}. For the competition experiment with H-9, a 24 nM PKA solution was incubated for 15 min with different concentrations of the inhibitor (19 nM - 1.85 μM) prior to injection (2 min at a flow rate of 20 μL min^{-1}). The inhibitory efficiencies of H-9 and some derivatives (**7** - **9**) were compared by preincubating a 30 nM PKA solution with 100 equivalents of the respective inhibitor for 15 min and injecting the mixture into the analytical flow cell (2 min at a flow rate of 20 μL min^{-1}). Due to solubility problems the inhibitors had to be dissolved in dimethylsulfoxide (DMSO). Therefore, the enzyme-inhibitor-mixtures contained 2.5% (v/v) DMSO (a corresponding amount DMSO was added to the pure protein sample). Regeneration of the sensor chip surface was accomplished with one-minute pulses of 50 mM NaOH.

2) Creatine kinase: Creatine kinase was reconstituted as recommended to a concentration of 4 μM. The enzyme was diluted in Tris-HCl buffer (running buffer) to various concentrations (12.5 nM - 400 nM) and injected over the immobilized **8** for 5 min at a flow rate of 20 μL min^{-1}. Regeneration of the sensor chip surface was accomplished with one-minute pulses of 50 mM NaOH and 10 mM glycine-HCl, pH 1.5 (from BIAcore AB).

c) Data analysis: Binding response (in response units, RU) was recorded continuously and presented as a sensorgram. All sensorgrams were corrected by subtraction of the reference cell signal.

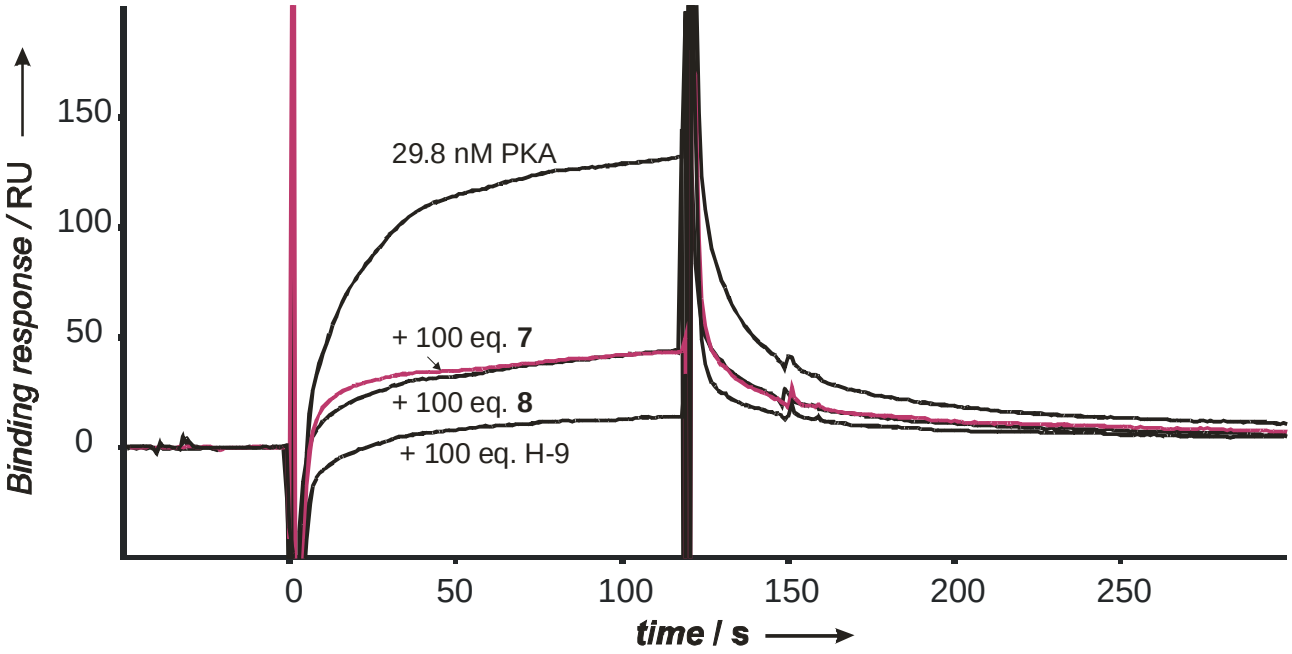


Figure S 8: Surface plasmon resonance sensorgram showing competition of immobilized **8** with H-9 and soluble derivatives **7** and **8** for binding to protein kinase A.

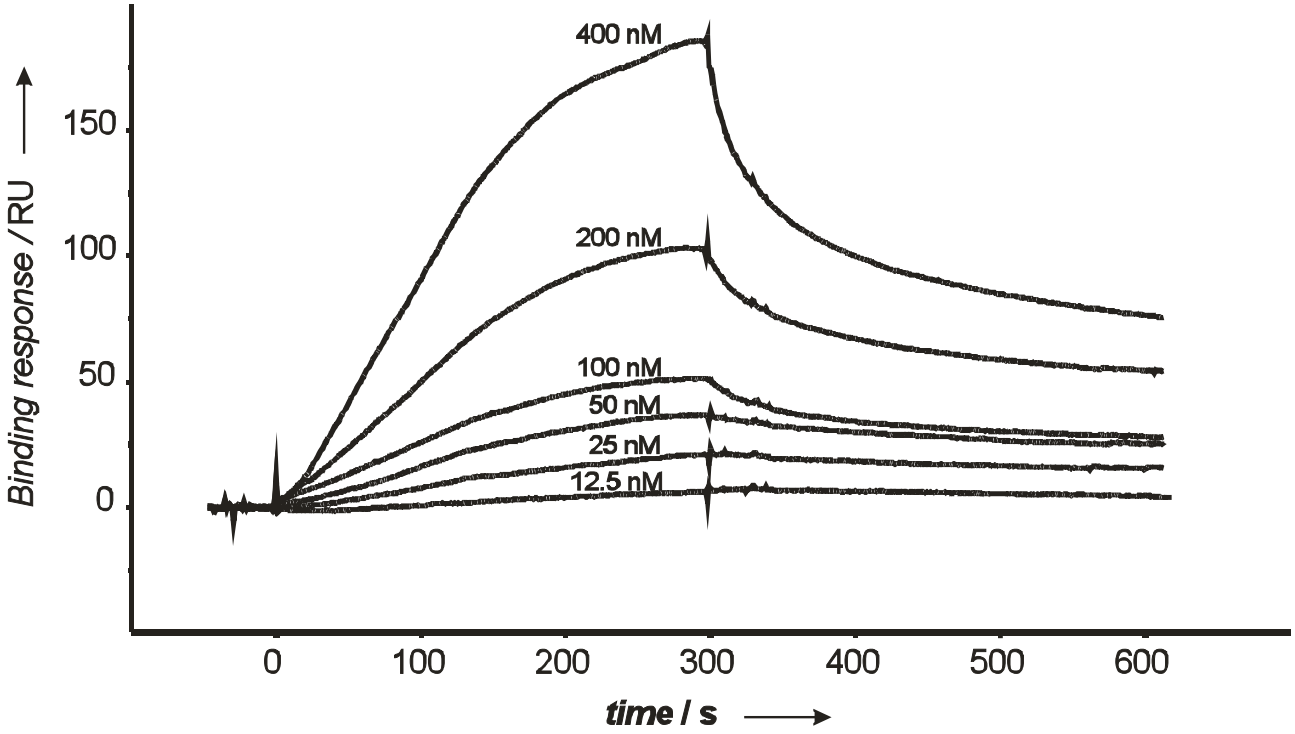


Figure S 9: Surface plasmon resonance sensorgram showing concentration-dependent binding of creatine kinase to immobilized **8.**

Photoaffinity labelling

The proteins or protein mixtures were incubated with the indicated concentrations of derivative **7** in buffer A (10 mM HEPES, pH= 7,3; 10 mM $MgCl_2$; 50 nM PefaBlock (Merck)). Covalent cross-linking of **7** was subsequently achieved by immediate irradiation of cooled samples in borosilicate glass tubes for 30 min in a continuous photoreaction with UV light (Rayonet Photochemical Reactor, Southern N.E., Ultraviolet Co., equipped with 16 RPR-3500 lamps, λ = 350 nm, 5 cm distance). Protein samples were separated on denaturating TRIS-Tricine SDS-PAGE in 10% acrylamide as described by Kruse *et al*.[5]

Photoaffinity labelling of different kinases with the H-9 derivative 7 (Fig. 3):

The different kinases (hexokinase, creatine kinase, 3PGA phosphokinase or cAMP dependent protein kinase A) were diluted to a concentration of 200 µg/mL. For the photoaffinity labelling experiments, 100 µL of protein solution were mixed with 10 µL of a 2 mM solution of derivative **7** (~ fiftyfold excess).

Comparison of photoaffinity labelling efficiency of native creatine kinase with labelling of SDS-denatured creatine kinase (Fig. 4 A):

Two samples were prepared: both contained 6 µL creatine kinase solution (concentration 2 mg/mL) and 15 µL of a 40 µM solution of derivative **7** (two equivalents); to one sample were added 36 µL 20% SDS. Both samples were diluted with buffer A to a final volume of 120 µL.

Retrieval of added creatine kinase from thylakoid membrane preparations (Fig. 4 B):

Thylakoid membranes from the green unicellular algae *Chlamydomonas reinhardtii* strain CC125 were isolated and purified by a slightly modified literature procedure.[6]

6 µl creatine kinase solution (concentration 100 ng/µL) were added to 6 µL of a thylakoid protein preparation (concentration 10 µg/µL) and the protein mixture was incubated with derivative **7** (1.5 µL of a 2 mM solution, 200 equivalents of creatine kinase) in a final volume of 30 µL prior to labelling and gel electrophoresis. MALDI-ToF fingerprint analysis was performed with the extracted kinase band following a procedure of the W.M. Keck Foundation Biotechnology Resource Laboratory.[7]

exp. mass	obs. mass	peptide pos.	peptide seq.	missed cleav.
906,79	907,8	308-314	FEEILTR	0
x	1201,1	x	x	x
1230,79	1231,8	87-96	DLFDPIIQDR	0
1507,19	1508,2	157-170	LSVEALNSLTGEFK	0
1529,89	1530,9	117-130	GGDDLDPHYVLSSR	0
1643,09	1644,1	224-236	SFLVWVNEEDHLR	0
x	1676,2	x	x	x
1785,39	1786,4	342-358	LGSSEVEQVQLVVDGVK	0
2008,29	2009,3	321-341	GTGGVDTAAVGSVFDISNADR	0
2164,49	2165,5	320-341	RGTGGVDTAAVGSVFDISNADR	1
x	2211,6	x	x	x

```
  1 MPFGNTHNKY KLNYKSEEEY PDLSKHNNHM AKVLTPDLYK KLRDKETPSG
 51 FTLDDVIQTG VDNPGHPFIM TVGCVAGDEE SYTVFKDLFD PIIQDRHGGF
101 KPTDKHKTDL NHENLKGGDD LDPHYVLSSR VRTGRSIKGY TLPPHCSRGE
151 RRAVEKLSVE ALNSLTGEFK GKYYPLKSMT EQEQQQLIDD HFLFDKPVSP
201 LLLASGMARD WPDARGIWHN DNKSFLVWVN EEDHLRVISM EKGGNMKEVF
251 RRFCVGLQKI EEIFKKAGHP FMWNEHLGYV LTCPSNLGTG LRGGVHVKLA
301 HLSKHPKFEE ILTRLRLQKR GTGGVDTAAV GSVFDISNAD RLGSSEVEQV
351 QLVVDGVKLM VEMEKKLEKG QSIDDMIPAQ K
```

Control experiments with respect to mechanism-based tagging of creatine kinase:

5 µL of creatine kinase solution (concentration 1 mg/mL) and 12.5 µL of a 2 mM solution of derivatives **7**, **9** or 5(6)-carboxyfluorescein (200 equivalents) were mixed and diluted with buffer A to a final volume of 150 µL prior to the photoreaction.

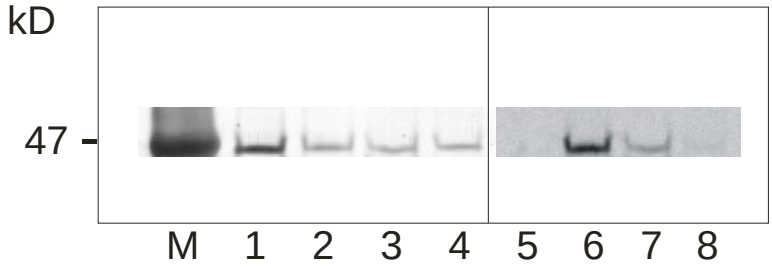


Figure S 10: Control experiments with respect to mechanism-based tagging of creatine kinase Lanes 1/5: silver stain/fluorescence image, creatine kinase; lanes 2/6: silver stain/fluorescence image, tagging of creatine kinase with the complete conjugate **7**; lanes 3/7: silver stain/fluorescence image, tagging of creatine kinase with the conjugate **9** lacking the inhibitor moiety; lanes 4/8: silver stain/fluorescence image, irradiation of creatine kinase in the presence of 5(6)-carboxyfluorescein; M: protein marker.

Figure S 10 displays the results of these control experiments. All samples of creatine kinase, with or without the probes **7**, **9** or 5(6)-carboxyfluorescein, were exposed to the same conditions (irradiation at λ = 350 nm, ultrafiltration). The fluorescent band in lane 6 proves efficient mechanism-based labelling of creatine kinase with **7**. Unspecific tagging with **9**, containing no inhibitor moiety, is shown to be sufficiently low (lane 7). Adducts between creatine kinase and carboxyfluorescein are not found (lane 8).

Biological Studies

The inhibitory activity of **1a** and **7** was further demonstrated in *in vitro* phosphorylation experiments using thylakoid preparations isolated from the unicellular green alga *Chlamydomonas reinhardtii*. In a parallel approach dark-adapted cells were incubated prior to illumination in phosphorylation medium containing 100 µM **1a** or **7**, respectively. The reduced levels of protein phosphorylation obtained after incubation with **1a** and **7**, resp., demonstrate the efficiency of the inhibitor H-9 **1a** and its conjugate **7**. The inhibitory capacity was calculated densitometrically to be approx. 60 %, which is similar to that described earlier for the efficiency of commercially available **1a**.[8]

Thylakoid protein kinase activity and H-9 inhibition capacity

The inhibitory activity of H-9 (**1a)** and **7** was demonstrated in *in vitro* phosphorylation experiments using thylakoid preparations. Thylakoid membranes containing mainly non-phosphorylated proteins were isolated from CC125 wild type cells by adaptation to darkness for 16 h (Fig. S 11, lane a). Subsequently, protein phosphorylation was induced by light activation (white light for 15 min) of the serine/threonine kinases (Fig. S 11, lane d). Protein samples were separated on denaturating SDS-PAGE and transferred to a nitrocellulose membrane by electro blotting as described earlier. Development of the membrane was carried out by a slightly modified literature procedure.

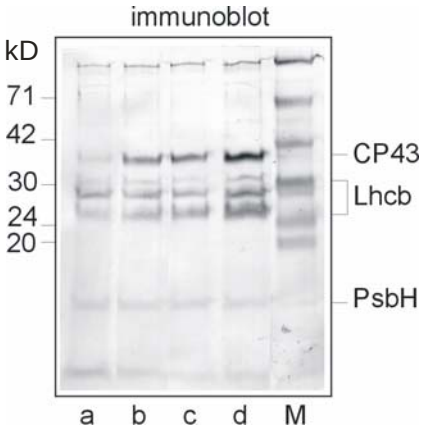


Figure S 11: Phosphorylation of thylakoid preparations from *Chlamydomonas reinhardtii* (Western Blot with polyclonal anti-phosphothreonine) (a) dark adapted, (b) in the presence of H-9 (**1a**), (c) in the presence of the H-9 conjugate **7**, and (d) without inhibitor. M: Protein marker.

References

[1] O. Kruse, D. Zheleva, J. Barber, *FEBS Lett.* **1997**, *408*, 276.

[2] H. Hidaka, M. Inagaki, S. Kawamoto, Y. Sasaki, *Biochemistry* **1984**, *23*, 5036.

[3] M. Beyermann, M. Bienert, H. Niedrich, *J. Org. Chem.* **1990**, *55*, 721.

[4] A. Brockhinke, R. Plessow, P. Dittrich, K. Kohse-Höinghaus, *Appl. Phys. B* **2000**, *71*, 755-763.

[5] O. Kruse, P.J. Nixon, G.H. Schmid, C.W. Mullineaux, *Photosynth. Res.* **1999**, *61*, 43.

[6] B.A. Diner, F.A. Wollman, *Eur. J. Biochem.*, **1980**, *110*, 521.

[7] http://info.med.yale.edu/wmkeck/prochem/geldig3.htm

[8] Z. Ishak, I. R. White, P. A. Millner, *Biochem. Soc. Trans.* **1992,** *20*, 8S.