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Supporting Information

A Fast, Visible-Light-Sensitive Azobenzene for Bioorthogonal Ligation

Claudia Poloni, Wiktor Szymański, Lili Hou, Wesley R. Browne,* and Ben L. Feringa*[a]

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1. General Remarks

All chemicals for synthesis were obtained from commercial sources and used as received unless stated otherwise. Solvents were reagent grade. Thin-layer chromatography (TLC) was performed using commercial Kieselgel 60, F254 silica gel plates, and components were visualized with $KMnO_4$ or phosphomolybdic acid reagent. Flash chromatography was performed on silica gel (Silicycle Siliaflash P60, 40-63 m, 230-400 mesh). Drying of solutions was performed with $MgSO_4$ or Na_2SO_4 and solvents were removed with a rotary evaporator. Chemical shifts for NMR measurements were determined relative to the residual solvent peaks ($CHCl_3$, δ = 7.26 ppm for hydrogen atoms, δ = 77.0 for carbon atoms). The following abbreviations are used to indicate signal multiplicity: s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; br s, broad signal. HRMS (ESI) spectra were obtained on a Thermo scientific LTQ Orbitrap XL. Melting points were recorded using a Buchi melting point B-545 apparatus.

Room temperature UV-Vis absorption spectra were recorded on an Agilent 8453 UV-Visible Spectrophotometer using Uvasol grade solvents. Irradiation experiments were performed with a spectroline ENB-280C/FE UV lamp (365 nm) and Thor Labs OSL1-EC Fiber Illuminator (white light).

The low temperature UV-Vis absorption and kinetics are recorded on a Hewlett-Packard HP 8452 FT spectrophotometer, and the irradiation is performed with a 200W Oriel Hg-lamp using a 400 nm long pass filter.

The laser flash photolysis measurements were obtained using a home-built system. The pump beam is from an Innolas 400 Nd:YAG laser (excitation at 532 nm, 10 Hz, 8 mJ/pulse), and a Si-diode photodiode is used as a trigger sensor. A Xenon acr Lamp is used as probe light source. The transient signal is detected on a Zolix PMTH-S1-CR131 PMT detector equipped with a Zolix Omni-λ 300 monochromator, and recorded on a Tektronix DPO 4032 Digital Phosphor Oscilloscope. The software of controlling the devices, acquiring the data and preliminary data analysis is programmed based on LabVIEW 8.2.

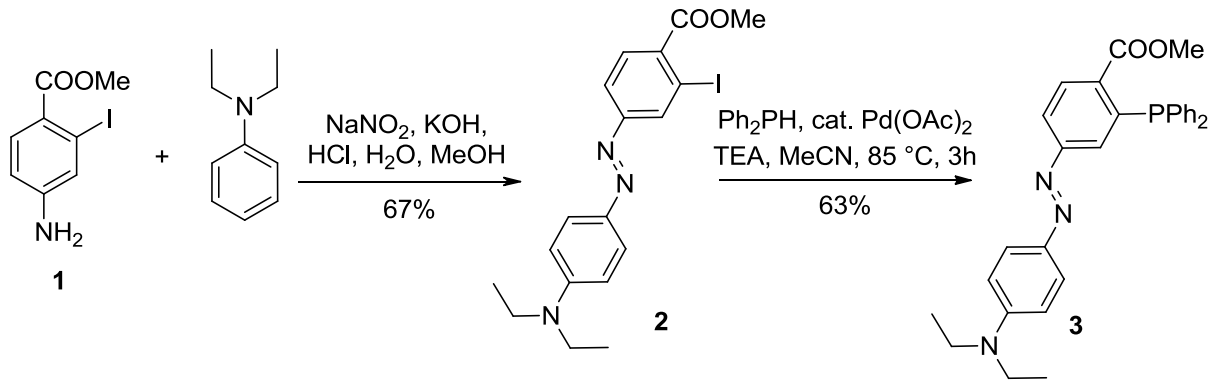
Contact angles were measurured on a Data Physics contact angle goniometer. The contact angle was calculated using software provided by the company. The contact angle was measured at three different locations on each surface and the results averaged.

Palladium(II) acetate was purchased at Sigma Aldrich. Diphenylphosphine was purchased at Acros. Methyl 4-amino-2-iodobenzoate (compound **1**) was prepared according to published procedures from 2-iodo-4-nitrotoluene by subsequent oxidation to 2-iodo-4-nitrobenzoic acid,[1] transformation of the acid into the methyl ester[2] and reduction of the nitro group.[2] Compounds 4 and **7** was prepared

[1] J. Protiva, V. Krecek, B. Maca, J. Urban, M. Budesinsky, M. Prochazka. *Collect. Czech. Chem. Commun.* **1989**, *54*, 1012–1018.
[2] J. M. Cary, J. S. Moore, *Org. Lett.*, **2002**, *4*, 4663-4666.

according to a previously-published procedure.[3] Compound **5** was prepared according to a published procedure.[4]

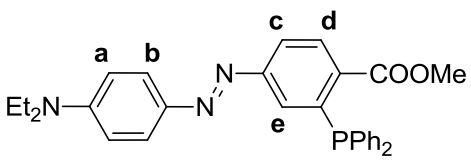
2. Synthesis of compound 3

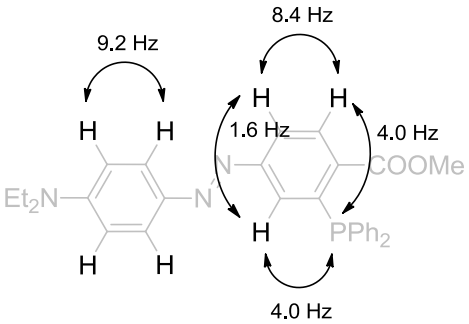


2: Methyl 4-((4-(diethylamino)phenyl)diazenyl)-2-iodobenzoate. To an ice-cold solution of methyl 4-amino-2-iodobenzoate (**1**) (0.32 mmol, 90 mg) in 1N aq. HCl (0.71 mL) and methanol (3.3 mL) was added dropwise a solution of $NaNO_2$ (0.35 mmol, 25 mg) in water (0.13 mL). To the resultant yellow mixture was added slowly a solution of *N*,*N*-diethylaniline (0.30 mmol, 48 μL) and KOH (0.60 mmol, 34 mg) in methanol (0.3 mL) at 0 °C. After stirring for an additional 10 min at this temperature, the reaction mixture was allowed to warm up to rt and stirred for additional 1 h. The mixture was dissolved AcOEt (40 mL) and washed with sat. aq. $NaHCO_3$ (3 x 30 mL) and brine (30 mL). The organic fraction was dried ($MgSO_4$) and the solvent was evaporated. Product **2** was purified by flash chromatography (Silicagel, 40-63 μm, pentane/Et_2O, 1:1, v/v). Yield: 67%. Red oil; R_f = 0.86 (pentane/Et_2O, 1:1, v/v); ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.24 (t, ^3J = 7.2 Hz, 6H, 2 x $CH_2C\mathbf{H_3}$), 3.46 (q, = 7.2 Hz, 4H, 2 x $C\mathbf{H_2}CH_3$), 3.94 (s, 3H, $C\mathbf{H_3}$), 6.71 (d, ^3J = 9.2 Hz, 2H, Ar**H**), 7.81 (dd, ^3J = 8.4 Hz, ^4J = 2.0 Hz, 1H, Ar**H**), 7.86 (d, ^3J = 9.2 Hz, 2H, Ar**H**), 7.92 (d, ^3J = 8.4 Hz, 1H, Ar**H**), 8.41 (d, ^4J = 2.0 Hz, 1H, Ar**H)**; ^{13}C NMR (100 MHz, $CDCl_3$): δ 12.7, 44.8, 52.4, 94.6, 111.0, 121.9, 126.1, 131.6, 133.7, 134.3, 143.1, 151.0, 155.1, 166.6; HRMS (ESI+) calc. for $C_{18}H_{21}IN_3O_2$: 438.0673, found: 438.0675.

3: Methyl 4-((4-(diethylamino)phenyl)diazenyl)-2-(diphenylphosphino)benzoate. A solution of methyl 4-((4-(diethylamino)phenyl)diazenyl)-2-iodobenzoate (**2**) (0.15 mmol, 65 mg), diphenylphosphine (0.24 mmol, 42 μL), palladium(II) diacetate (3.4 mg) and triethylamine (0.24 mmol, 33 μL) in acetonitrile (2.5 mL) was stirred at 85ºC for 3 h. Product **3** was purified by flash chromatography (Silicagel, 40-63 μm, pentane/Et_2O, 4:1, v/v). Yield: 63%; red oil. R_f = 0.68 (pentane/AcOEt, 4:1, v/v);

[3] W. Szymanski, B. Wu, C. Poloni, D. B. Janssen, B. L. Feringa, *Angew. Chem. Int. Ed.* **2013**, *52*, 2068 –2072.
[4] E. Saxon, S. J. Luchansky, H. C. Hang, C. Yu, S. C. Lee, C. R. Bertozzi, *J. Am. Chem. Soc.* **2002**, *124*, 14893 – 14902

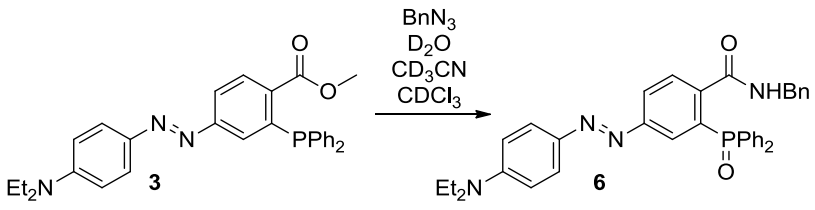




^{1}H NMR (400 MHz, $CDCl_3$): δ 1.21 (t, ^{3}J = 7.2 Hz, 6H, 2 x $CH_2C\mathbf{H_3}$), 3.42 (q, = 7.2 Hz, 4H, 2 x $C\mathbf{H_2}CH_3$), 3.75 (s, 3H, $C\mathbf{H_3}$), 6.66 (d, ^{3}J = 9.2 Hz, 2H, Ar**H**^{a}), 7.33-7.38 (m, 10H, $(C_6\mathbf{H_5})_2P$), 7.40 (dd, J = 4.0 Hz, J = 1.6 Hz, 1H, Ar**H**^{e}); 7.72 (d, ^{3}J = 9.2 Hz, 2H, Ar**H**^{b}), 7.76 (dd, J = 8.4 Hz, J = 1.6 Hz, 1H, Ar**H**^{c}), 8.14 (dd, J = 8.4 Hz, J = 4.0 Hz, 1H, Ar**H**^{d}); ^{1}H{^{31}P} NMR (400 MHz, $CDCl_3$) δ of aromatic protons: 6.66 (d, ^{3}J = 9.2 Hz, 2H, Ar**H**^{a}), 7.33-7.38 (m, 10H, $(C_6\mathbf{H_5})_2P$), 7.40 (d, J = 1.6 Hz, 1H, Ar**H**^{e}); 7.72 (d, ^{3}J = 9.2 Hz, 2H, Ar**H**^{b}), 7.76 (dd, J = 8.4 Hz, J = 1.6 Hz, 1H, Ar**H**^{c}), 8.14 (d, J = 8.4 Hz, 1H, Ar**H**^{d}); ^{31}P{^{1}H} NMR (162 MHz, $CDCl_3$) δ -3.4; ^{13}C NMR (100 MHz, $CDCl_3$): δ 12.6, 44.8, 52.0, 110.9, 119.8, 125.9, 128.5 (d, J = 7.3 Hz), 128.7, 129.7, 131.8 (d, J = 2.6 Hz), 133.6 (d, J = 18.6 Hz), 134.0 (d, J = 20.8 Hz), 137.8 (d, J = 10.7 Hz), 142.0 (d, J = 27.8 Hz), 143.1, 150.7, 155.0, 166.9; HRMS (ESI+) calc. for $C_{30}H_{31}N_3O_2P$: 496.2148, found: 496.2146.

3. 31P NMR study of the Staudinger-Bertozzi ligation of compound 3 with benzyl azide

Compound **3** (18.8 μmol,9.3 mg) was dissolved in CD_3CN (0.4 mL) and $CDCl_3$ (0.4 mL). D_2O (10 μL) was added, followed by benzyl azide (37 μmol, 5 mg). The progress of reaction was followed in time by ^{31}P NMR.



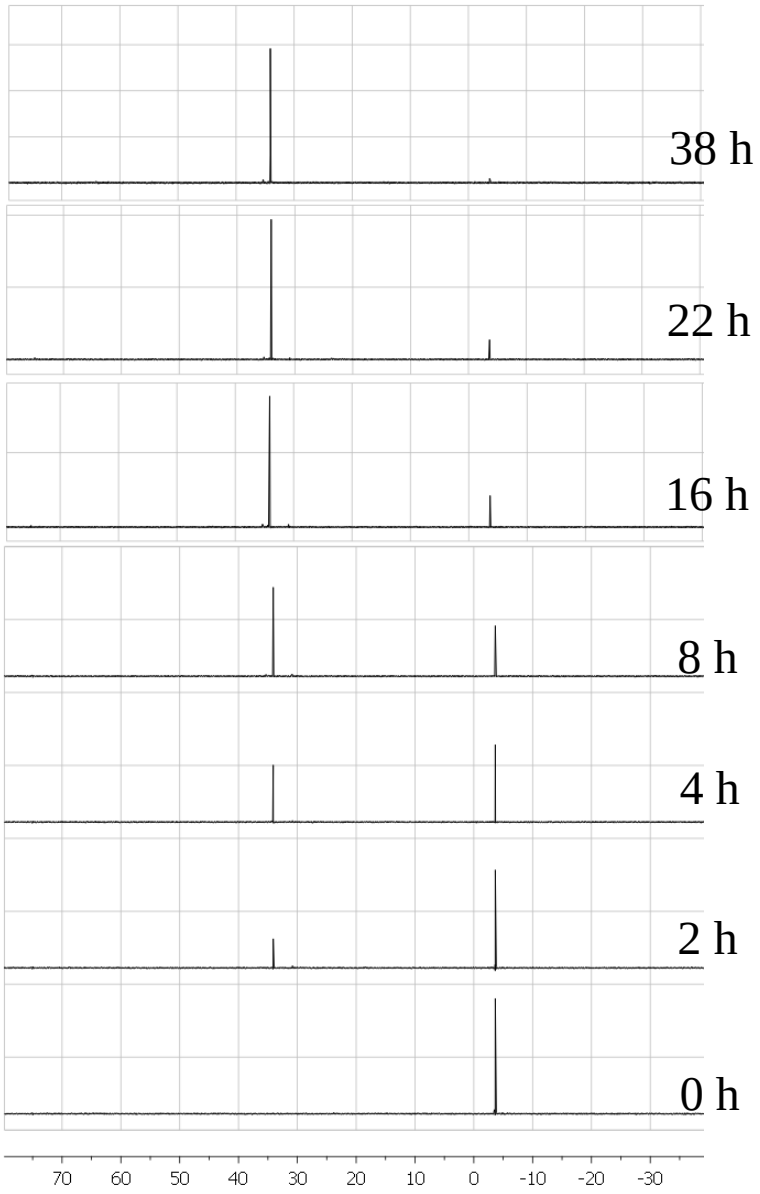
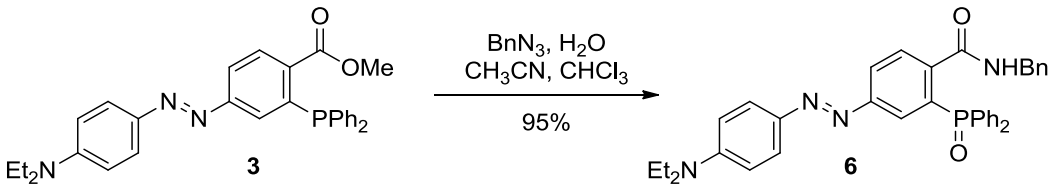


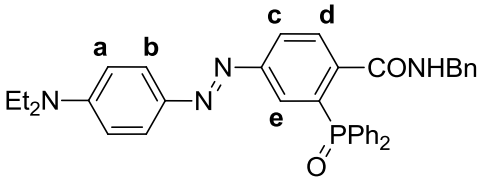
Figure 1. ^{31}P NMR spectra obtained from monitoring the reactions of compounds **3-5** with 2 equiv. of benzyl azide.

4. Synthesis of compound 6 *via* Staudinger-Bertozzi ligation



6: (E)-N-benzyl-4-((4-(diethylamino)phenyl)diazenyl)-2-(diphenylphosphoryl)-benzamide. A solution of methyl 4-((4-(diethylamino)phenyl)diazenyl)-2-(diphenylphosphino)benzoate (**3**) (81 μmol, 40 mg) and benzyl azide (0.24 mmol, 32 mg) in acetonitrile (1.5 mL), chloroform (1.5 mL) and water

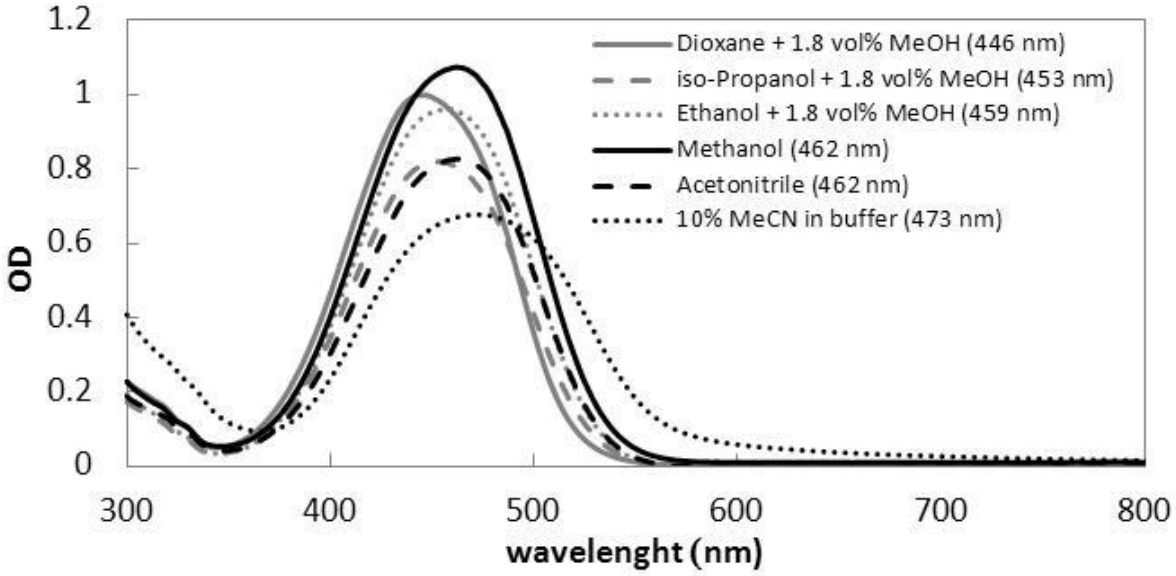
(100 μL) was stirred at rt overnight. The reaction mixture was diluted in AcOEt and dried ($MgSO_4$). The solvent was evaporated and product **4** was purified by flash chromatography (Silicagel, 40-63 μm, pentane:AcOEt, 1:1 to 0:1, v/v). Yield: 95%; red solid. R_f = 0.05 (pentane/AcOEt, 4:1, v/v);



^{1}H NMR (400 MHz, $CDCl_3$): δ 1.22 (t, ^3J = 7.2 Hz, 6H, 2 x $CH_2C\mathbf{H_3}$), 3.44 (q, = 7.2 Hz, 4H, 2 x $C\mathbf{H_2}CH_3$), 4.14 (s, ^3J = 5.2 Hz, 2H, $C\mathbf{H_2}NH$), 6.66 (d, ^3J = 9.2 Hz, 2H, Ar$\mathbf{H}^{\mathbf{a}}$), 7.20-7.30 (m, 5H, Ar**H**), 7.44-7.53 (m, 4H, Ar**H** + Ar$\mathbf{H}^{\mathbf{e}}$), 7.55-7.62 (m, 2H, Ar**H**), 7.65-7.73 (m, 6H, Ar**H** + Ar$\mathbf{H}^{\mathbf{b}}$), 8.01 (ddd, *J* = 8.0 Hz, *J* = 2.0 Hz, *J* = 2.0 Hz, 1H, Ar$\mathbf{H}^{\mathbf{c}}$), 8.14 (dd, *J* = 8.0 Hz, *J* = 4.0 Hz, 1H, Ar$\mathbf{H}^{\mathbf{d}}$), 9.23 (t, ^3J = 5.1 Hz, 1H, N**H**); ^{1}H{^{31}P} NMR (400 MHz, $CDCl_3$) δ of aromatic protons: 6.66 (d, ^3J = 9.2 Hz, 2H, Ar$\mathbf{H}^{\mathbf{a}}$), 7.20-7.30 (m, 5H, Ar**H**), 7.44-7.53 (m, 4H, Ar**H** + Ar$\mathbf{H}^{\mathbf{e}}$), 7.55-7.62 (m, 2H, Ar**H**), 7.65-7.73 (m, 6H, Ar**H** + Ar$\mathbf{H}^{\mathbf{b}}$), 8.01 (dd, *J* = 8.0 Hz, *J* = 2.0 Hz, 1H, Ar$\mathbf{H}^{\mathbf{c}}$), 8.14 (d, *J* = 8.0 Hz, 1H, Ar$\mathbf{H}^{\mathbf{d}}$), 9.23 (t, ^3J = 5.1 Hz, 1H, N**H**); ^{31}P{^{1}H} NMR (162 MHz, $CDCl_3$) δ 35.5; ^{13}C NMR (100 MHz, $CDCl_3$): δ 12.6, 44.1, 44.8, 110.9, 124.4, 124.5, 125.9, 127.1, 128.0, 128.4, 128.5, 128.6, 128.7 (d, *J* = 12 Hz), 129.7, 130.4, 130.7, 131.5, 131.8 (d, *J* = 8 Hz), 132.3 (d, *J* = 3 Hz), 133.0 (d, *J* = 10 Hz), 137.7, 140.3 (d, *J* = 8 Hz), 142.9, 150.8, 153.3 (d, *J* = 13 Hz), 167.0 (d, *J* = 3 Hz). HRMS (ESI+) calc. for $C_{36}H_{36}N_4O_2P$: 587.2570, found: 587.2570; HRMS (ESI+) calc. for $C_{36}H_{35}N_4O_2PNa$: 609.2390, found: 609.2385; UV-Vis (30 x 10^{-6} M in methanol): λ_{max} = 462 nm, ε_{462} = 28.0 x 10^3 M^{-1} cm^{-1}.

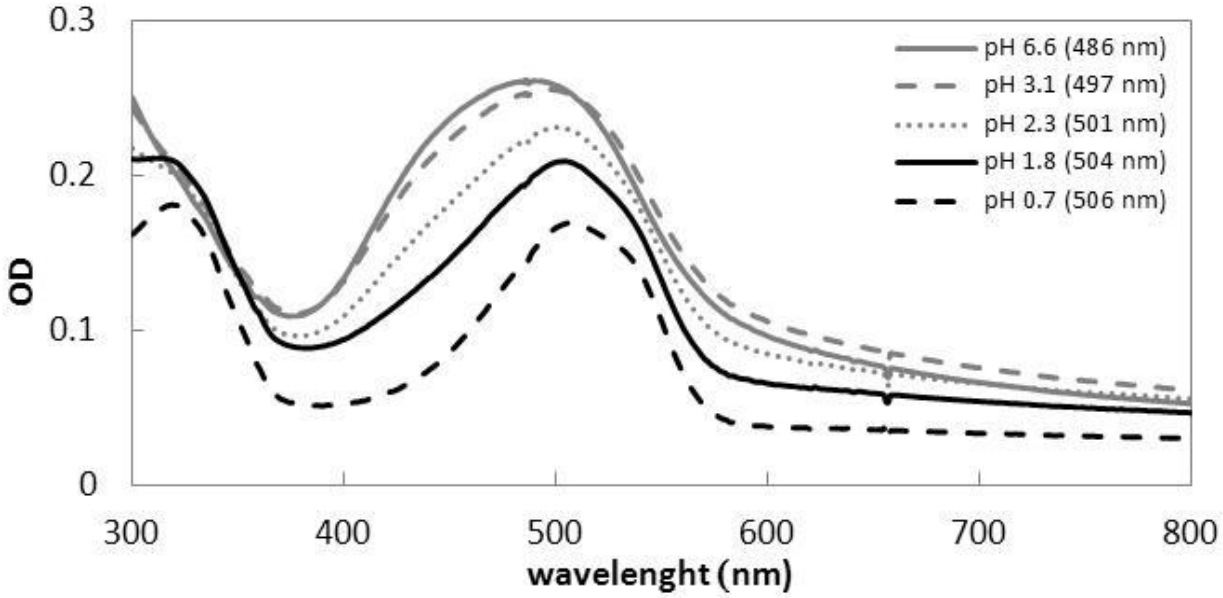
5. Solvatochromism of compound 6

UV-Vis spectra of compound 6 (30 x 10^{-6} M) were measured in different solvents at rt; buffer = 5 mM TRIS•SO4, pH 7.2. In case of dioxane, *iso*-propanol and ethanol, a stock solution of compound **6** in methanol was used and diluted with respective solvent. Collected spectra are presented below.



6. Acidochromism of compound 6

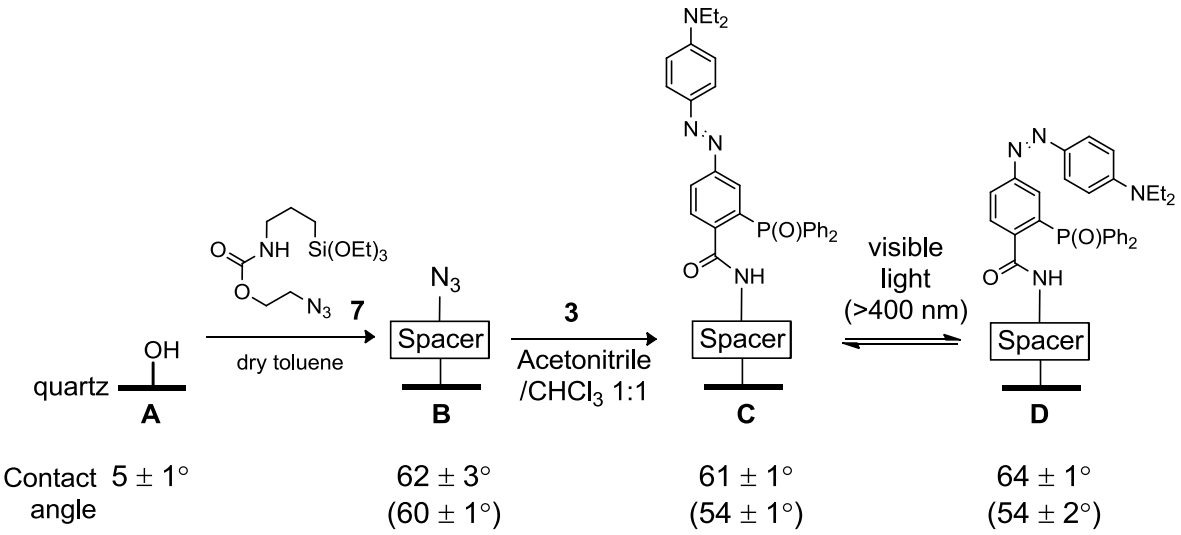
The solution of compound **6** (10 x 10^{-6} M in 10% MeCN / water, 3 mL) at rt was gradually acidified by drop-wise addition of small volume of 1N aq. HCl. Collected spectra are presented below.



7. Lewis acid titration of compound 6

To a solution of compound **6** (10 x 10^{-6} M in 10% MeCN / water, 3 mL) at rt were gradually added small volumes of 1 M aq. $CaCl_2$ or 1 M aq. NaCl. The results obtained for the absorption maximum shift are presented in the manuscript in Figure 3.

8. Modification of azide-decorated quartz surface with compound 3

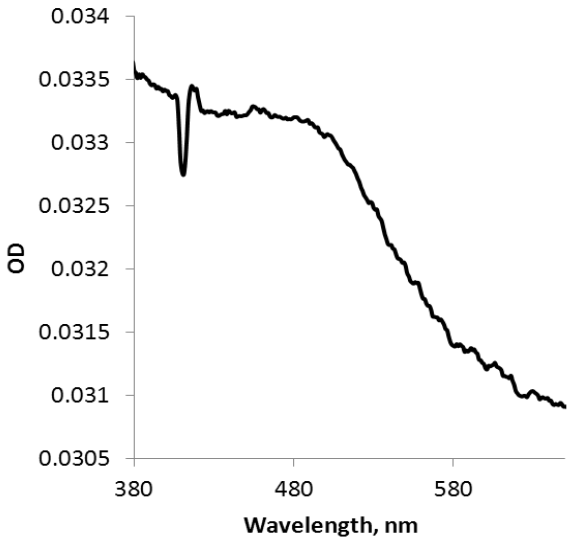


CAUTION: Piranha solution, used in this procedure, is extremely energetic and potentially explosive. It must be handled with extreme caution. (For handling and disposal of piranha solution, see e. g. http://www.ehs.utoronto.ca/Assets/ehs+Digital+Assets/ehs3/Chemical+Safety/Piranha+2010.pdf)

Quartz microscopy coverslips were cleaned using piranha solution (conc. H_2SO_4 : 30% H_2O_2, 3:1, v/v), rinsed with water (2x), dried under N_2 stream and cleaned by a plasma cleaner (1 min, 100% O_2, 1.5 mbar).

Cleaned coverslips were immersed in a 2 mM solution of compound **6** in dry toluene during 1 d at rt. Afterwards, the coverslips were sonicated in toluene and methanol (1 min each) and then dried under N_2 stream. Azide-functionalized quartz coverslips were immersed in a 0.5 mM solution of compound **3** in acetonitrile/chloroform (1:1) for 3 d at rt, sonicated in acetonitrile (1x) and Et_2O (3x), and then dried under N_2 stream.

UV-Vis spectrum obtained for modified quartz slide C is presented below.



9. Kinetics of thermal *cis-trans* isomerisation of compound 6

The solutions for laser flash photolysis measurements were placed in 1×1 cm quartz cuvette, and degassing was performed by bubbling argon through the solution. The concentrations were chosen so that the absorbance of the solution at the excitation wavelength (532 nm) were around 0.5. UV-Vis spectra have been measured both before and after irradiated by 532 nm pulse laser and no change has been observed. The results are presented in the manuscript, Figure 4b and 4c.

The changes in the UV-vis spectrum after irradiation in methanol at 200 K are presented below.

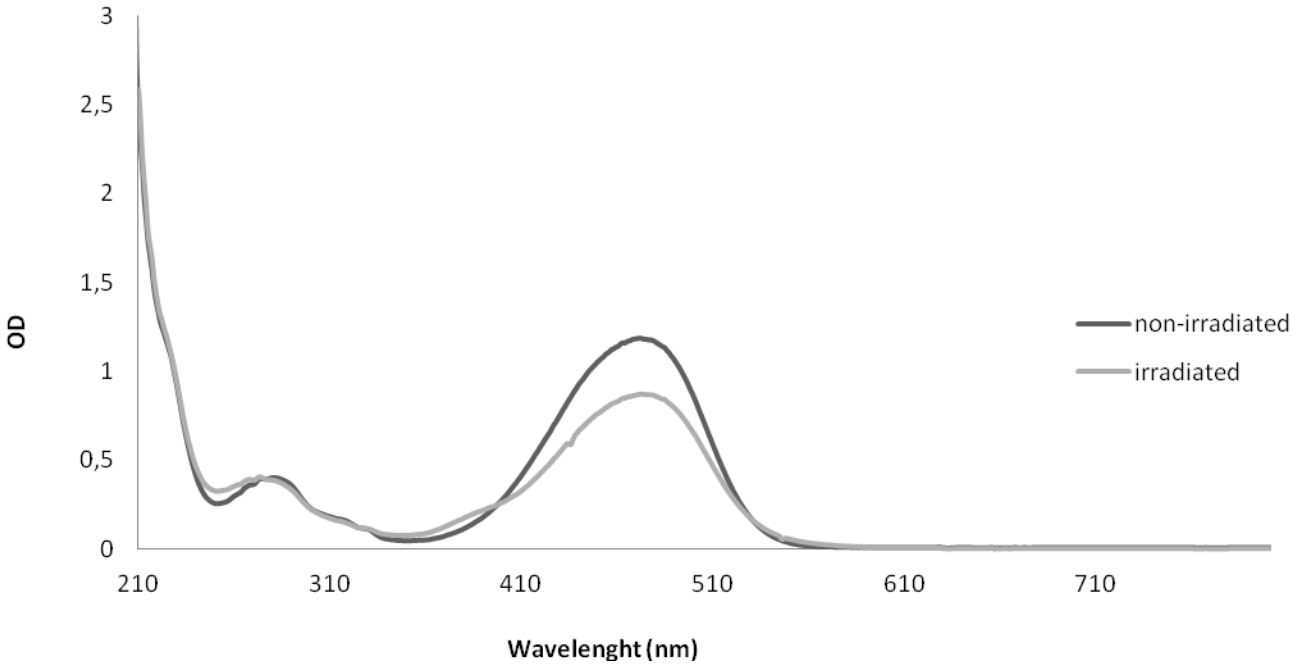


Figure 2. Compound **6** (3.0 x 10^{-5} M) in methanol at 200 K. The *cis* form was generated by irradiation with a Hg Lamp with 400 nm long pass filter;

10. Preparation, analysis and Staudinger ligation to a model peptide

10.1. HPLC analysis: RP-HPLC was carried out with Shimadzu equipment.

Eluent A: 0.1 % TFA in acetonitrile.

Eluent B: 0.1 % TFA in water.

Gradient: 0 - 3 min, 10 % A;

3 - 58 min, 1.54% A min^{-1}. Final: 95% A.

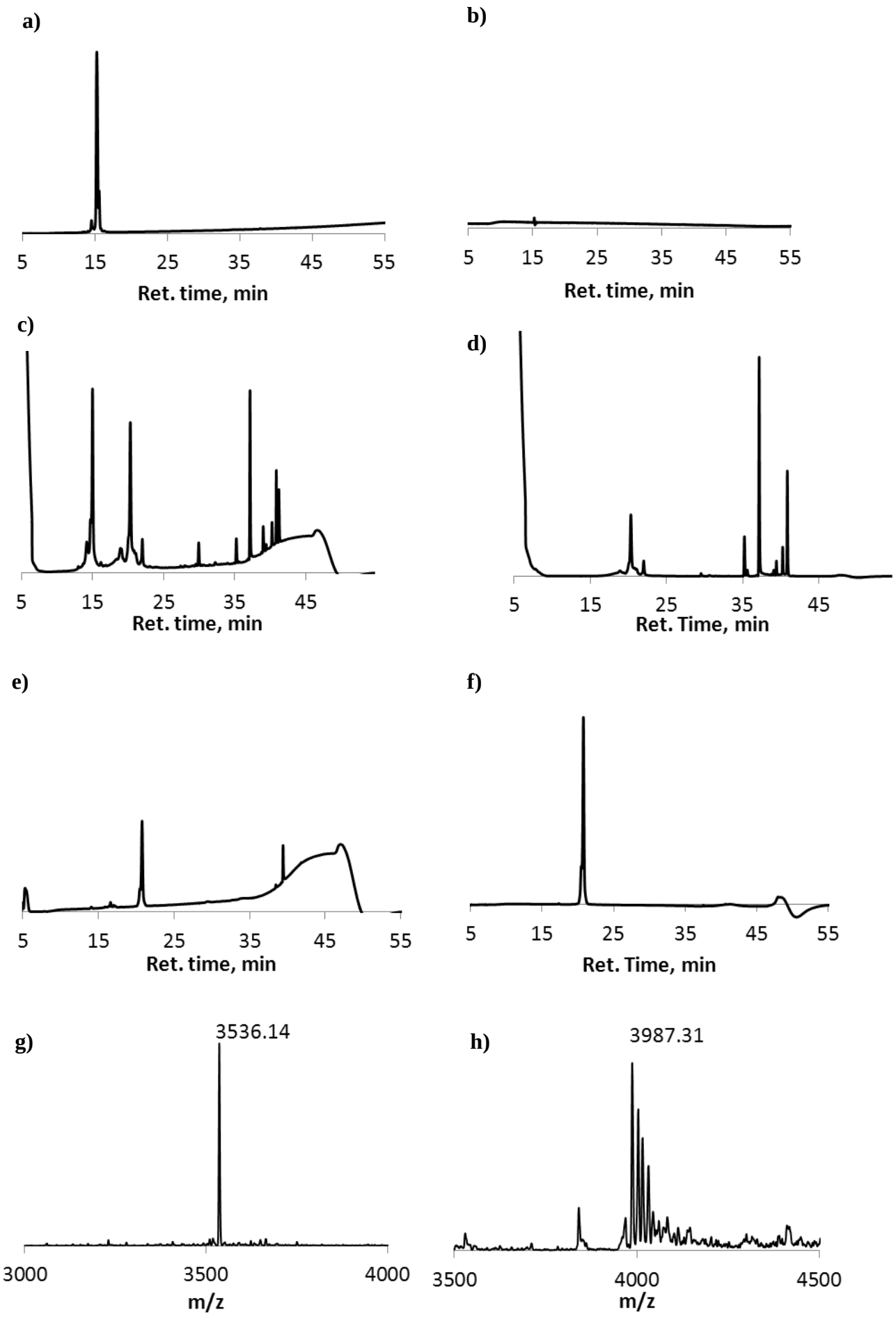
For analytical RP-HPLC, a XTerra C18 3.0x150mm column (Waters) was used with a flow rate of 0.5 mL min^{-1} and for semi-preparative RP-HPLC, a XTerra Prep C18 7.8x150mm column (Waters) was used with 1 mL min^{-1}.

10.2. Preparation of [Aha^{27}]-Sp1-f3 (H_2N-KKFACPECPKRFMRSDHLSKHIKTHQXKK-NH_2), (X = L-azidohomoalanine)

The peptide was synthesized on a 0.1 mmol scale by standard protocol of Fmoc chemistry with the peptide synthesizer CEM Liberty, with CEM Discover microwave. Sieber resin was used (0.69 mmol/g). The coupling step was performed with Fmoc-protected amino acid (5 eq), HBTU (5 eq) and DIPEA (10 eq) in DMF. The Fmoc-deprotection step was performed with 5% piperazine in DMP. Cleavage from the resin was performed with TFA:EDT:H_2O:TIS 94/1/2.5/2.5 for 2h. The product was precipitated with diethyl ether and isolated by centrifugation. The crude peptide was purified by semi-preparative RP-HPLC. Ret. time: 15.24 min. ESI-MS: 3536.14 (M+1).

10.3. Peptide 1: Staudinger ligation on [Aha^{27}]-Sp1-f3

0.2 mg (0.06 μmol) **[Aha^{27}]-Sp1-f3** was dissolved in 0.1mL H_2O and a solution of compound **3** (0.34 mg, 0.6 μmol, in 0.4 mL DMF) was added. The reaction mixture was stirred for 2 d at rt under N_2 atmosphere. 0.2 mL H_2O was added and the reaction mixture was washed with DCM (0.5 mL) 3 times. The aqueous layer was freeze-dried and the product purified by semi-preparative RP-HPLC. Ret. time: 20.73 min. ESI-MS: 3987.31 (M+1).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Ret. time, min
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3000
3500
4000
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h)
3987.31
3500
4000
4500
m/z

Figure 3. a) HPLC trace of purified **[Aha^{27}]-Sp1-f3** at 220 nm. b) HPLC trace of purified **[Aha^{27}]-Sp1-f3** at 500 nm. c) HPLC trace of crude reaction mixture at 220 nm. d) HPLC trace of crude reaction mixture at 500 nm. e) HPLC trace of purified **Peptide 1** at 220 nm. f) HPLC trace of purified **Peptide 1** at 500 nm. g) Deconvoluted ESI-MS spectra of purified **[Aha^{27}]-Sp1-f3**. h) Deconvoluted ESI-MS spectra of purified **Peptide 1**.