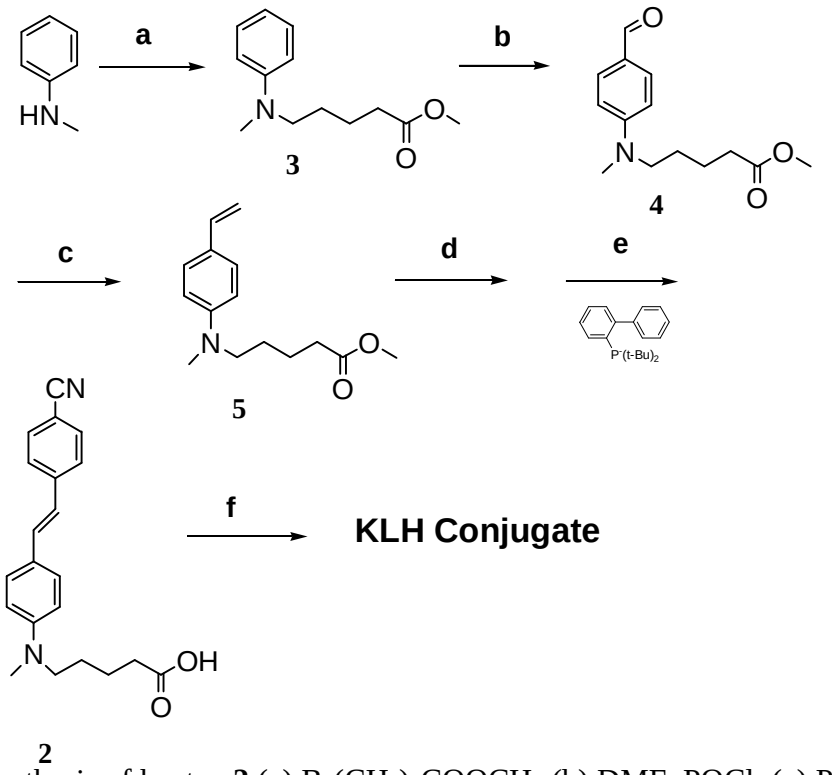
Angewandte Chemie
Eine Zeitschrift der Gesellschaft Deutscher Chemiker

Supporting Information

69451 Weinheim, Germany

The Effects of Antibodies on Stilbene Excited State Energetics

Feng Tian, Erik W. Debler, David P. Millar, Ashok A. Deniz, Ian A. Wilson, Peter G. Schultz



Scheme 1. Synthesis of hapten **2** (a) $Br(CH_2)_4COOCH_3$ (b) DMF, $POCl_3$ (c) Ph_3PCH_3Br, NaH (d) LiOH (e) $Pd_2(dba)_3$, Cs_2CO_3 (f) EDC, NHS, KLH, pH 9.0

Synthesis of methyl 5-(N-methyl-N-phenylamino)pentanoate (3)[1, 2]

N-methylaniline (5.46 g, 50 mmol), methyl 5-bromovalerate (11.1 g, 55 mmol), sodium bicarbonate (8.4 g, 100 mmol) and hexamethyl phosphoramide (HMPA, 50 ml) were added into a 250 ml three neck round bottom flask with a magnetic stirring bar. The reaction mixture was stirred under N_2 at 90 °C for 5 hours. Then the reaction mixture was cooled to room temperature and was mixed with 300 ml of H_2O. The mixture was extracted with diethylether (4x 80 ml). The organic layer was washed by saturated brine and dried over magnesium sulfate. The solvent was evaporated to give 10.5 g (47 mmol) of yellow oil

(yield: 94.8%): ^{1}H NMR (250 MHz): 7.20 (d, 2H, J=8.4 Hz), 6.68 (d, 2H, J=8.4 Hz), 3.64 (s, 3H), 3.32 (t, 2H, J=7.0 Hz), 2.91 (s, 3H), 2.34 (t, 2H, J= 7.0 Hz), 1.62 (m, 4H).

Synthesis of methyl 5-(N-(4-formylphenyl)-N-methylamino)pentanoate (4)[2]

Under N_2, anhydrous DMF (11.7 g, 160 mmol) was added into a 100 ml 3-neck round bottom flask with a magnetic stirring bar and cooled with an ice bath. Then phosphorous oxychloride (6.02 g, 40 mmol) was added dropwise with stirring. The reaction mixture turned light pink. After 20 min, compound **3** (8.5 g, 40 mmol) was added dropwise. The color of the reaction mixture turned yellow-green. The reaction mixture was stirred at 110 °C for 2 hours and then the reaction mixture was cooled and poured onto ice (100 g). The mixture was neutralized (pH 7.0) with saturated sodium acetate and store at 4 °C overnight. The precipitate was filtered, washed with water and dried under vacuum to give 6.4 g (25.7 mmol) of brown solid (yield: 64%): ^{1}H NMR (250 MHz), 9.74 (s, 1H), 7.74 (d, 2 H, J=9.2 Hz), 6.70 (d, 2H, J=9.2 Hz), 3.69 (s, 3 H), 3.45 (t, 2H, 6.6 Hz), 3.07 (s, 3H), 2.38 (t, 2 H, 6.6 Hz), 1.70 (m, 4H).

Synthesis of methyl 5-(N-methyl-N-(4-vinylphenyl)amino)pentanoate (5)

Sodium hydride (60 mg, 2.5 mmol) and dry DMSO (1 ml) was added into a 25 ml 3-neck round bottom flask with a magnetic stirring bar. The mixture was heated at 80 °C for 30 min until the generation of gas bubble was ceased. Then the mixture was cooled on an ice bath. Methyl triphenylphosphonium bromide (0.893 g, 2.5 mmol) dissolved in 2 ml of dry DMSO was added dropwise. After 20 min, the reaction mixture was warmed to room temperature. Then, compound **4** (0.62 g, 2.5 mmol) dissolved in 2 ml dry DMSO was added dropwise. The reaction mixture was stirred for 16 hr at room temperature, poured into H_2O (150 ml) and extracted with diethylether (4x 50 ml). The organic layer was washed with water, saturated brine and dried over sodium sulfate. The diethylether was evaporated and the residue was purified by silica gel flash chromatography (80% n-hexane/20% EtOAc) to afford 0.2 g (0.81 mmol, yield: 32%) of colorless oil (R_f=0.44,). ^{1}H NMR (250 MHz): 7.26 (d, 2H, J=8.5 Hz), 6.64 (m, 1H), 5.5

(m, 1H), 4.89 (m, 1H), 3.64 (s, 3H), 3.33 (t, 2H, J= 6.8 Hz), 2.94 (s, 3H), 2.36 (t, 2H, J=6.8 Hz), 1.66 (m, 4H); HRMS (MLADI-FTMS), expected (M+1) for $C_{15}H_{21}O_2N$: 248.1645, observed: 248.1648.

Synthesis of 5-(N-(4-(4-cyanostyryl)phenyl)-N-methylamino)pentanoic acid (2)

Lithium hydroxide monohydrate (84 mg, 2.0 mmol) was dissolved in the mixture of methanol and water (volume ratio 2:1, 1 ml). This solution and compound **5** (124 mg, 0.5 mmol) were added into a 10 ml round bottom flask with a magnetic stirring bar. The reaction was stirred at room temperature for 2 hr. TLC analysis of the reaction mixture indicated that the hydrolysis reaction was complete. The reaction mixture was neutralized with 0.1 M acetic acid and the solvent was evaporated. The residue was extensively dried under high vacuum. The resulting white solid, 4-bromobenzonitrile (182 mg, 1.0 mmol), $pd_2(dba)_3$ (6.87 mg, 0.0075 mmol), P ligand (8.94 mg, 0.06 mg) and Cs_2CO_3 (244 mg, 0.75 mmol) were added to a 10 ml 2-neck round bottom flask with a magnetic stirring bar. The mixture was dried under high vacuum. Under N_2, dry dioxane (2 ml) was added. Then, the mixture was stirred at 75 °C for 22 hours. The solvent was removed and the residue was purified by silica gel flash chromatography ($CHCl_3$ with 7% HOAc) to afford 150 mg (0.45 mml) bright yellow solid (yield: 90%). ^{1}H NMR (400 MHz): 7.58 (d, 2H, J=8.5Hz), 7.52 (d, 2H, J=8.5Hz), 7.41 (d, 2H, J=8.9Hz), 7.14 (d, 1H, J=16.2Hz), 6.86 (d, 1H, J=16.2Hz), 6.67 (d, 2H, J=8.9Hz), 3.38 (t, 2H, J=6.8 Hz), 2.98 (s, 3H), 2.41 (t, 2H, J=7.0Hz), 1.67 (m, 4H). HRMS (MALDI-FTMS) (M+H): expected for $C_{21}H_{22}O_2N_2$: 335.1754, observed: 335.1764.

.

Steady-state spectral measurements

Absorption spectra were measure on a HP 8453 spectrometer in PBS (10 mM sodium phosphate, 150 mM NaCl, pH 7.0), 5% DMF cosolvent, at 21 °C. The dissolved oxygen was not purged. For all the absorption measurements, the concentrations of hapten **1** and **2** were kept at 10 μM in the presence of excess antibody binding sites. The absorption spectra of antibody solutions were used as background for correcting the spectra of the corresponding antibody-hapten complex solutions.

Fluorescence spectra were measured on FluoroMax-2 fluorimeter. In a typical experiment, the slits were at 5/5, the spectrum was recorded from a wavelength that is 10 nm longer than the excitation wavelength to 700 nm with increment of 2 nm. The integration time is 1 s. The concentration of **2** was kept at 10 nM, the concentration of **1** was kept at 100 nM, and antibody concentrations were kept around 10 μM. Fluorescence spectra of antibody solutions were used as background to correct the fluorescence spectra of corresponding antibody-hapten complexes.

Quantum yields were measured by following the instruction at: http://www.jobinyvon.com/usadivisions/Fluorescence/applications/quantumyieldstrad.pdb. The standard for the fluorescence quantum yield determinations of the different antibody-**2** complexes was quinine bisulfate in 0.5 N H_2SO_4 (Φ_f =0.55). With the excitation wavelength at 380 nm, the fluorescence spectra of different antibody-**2** complexes were recorded at concentrations: 10 nM, 20 nM, 30 nM, 40 nM and 50 nM with the integration time 1 s and increment of 2 nm. The slits were set at 2/3. The fluorescence spectra of quinine bisulfate were recorded at concentrations: 10 nM, 20 nM, 30 nM, 40 nM and 50 nM. The standard for the fluorescence quantum yield determination of 11G10-**1** was sulforhodamine in ethanol (Φ_f =1.00). With the excitation wavelength at 532 nm, the fluorescence spectrum of the 11G10-**1** complex was recorded at antibody concentrations: 2 μM, 4 μM, 6 μM, 8 μM, and 10 μM with the integration time 1 s and increment of 2 nm. The slits were set at 2/3. The fluorescence spectrum of sulforhodamine 101 was recorded at concentrations: 0.95 nM, 1.85 nM, 2.80 nM, 3.75 nM and 4.65 nM.

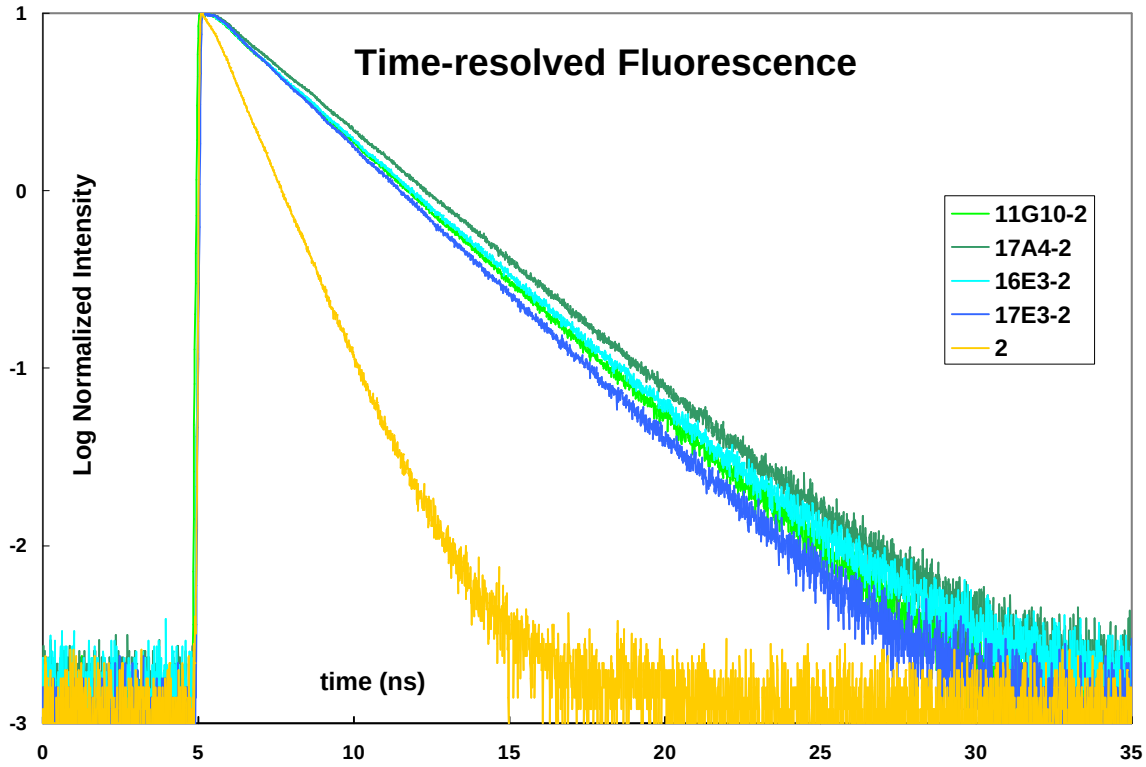
The K_d values were measured by fluorescence titration. The parameters used for different antibody complexes are listed below:

complex	11G10-**2**	16E3-**2**	17A4-**2**	17E3-**2**	11G10-**1**
λ_{ex} (nm)	440	420	420	400	532
λ_{em} (nm)	485	465	480	450	684

Time-resolved spectroscopy

All time-resolved measurements were made using the time-correlated single-photon counting method.[3] Antibody complexes were prepared using 10 μM of mAb and 5 μM of **2** in PBS (10 mM sodium phosphate, 150 mM NaCl, pH 7.4), with 5% DMF cosolvent. Measurements were obtained by using repetitive excitation by a pulsed laser (frequency doubled output of a Coherent Mira Titanium-Sapphire laser, ca. 2 ps pulsewidth, repetition rate of 3.8 MHz at 440 nm. Fluorescence emission was measured at right angles to the excitation beam and by using a polarizer set at the magic angle (54.7 relative to vertical). Emission was detected at 575 nm (free **2**), 525 nm (11G10-**2** and 17A4-**2**), and 500 nm (16E3-**2** and 17E3-**2**) by using a microchannel plate photomultiplier (Hamamatsu R3809U-01) and standard time-correlated single-photon counting electronics. Emission decays were recorded in 4096 channels with a time increment of 10.2 ps/channel and were normalized relative to the number of counts recorded in the peak channel (approximately 50,000). The instrument response function was recorded using scattered light (440 nm) from a dilute suspension of nondairy coffee creamer.

Emission decays were fit using non-linear least squares regression following convolution of the fitting function with the instrument response function. A sum of one or more exponentials was used for the fitting function and the goodness of fit was determined by examination of χ^2 and of the weighted residuals. The intensity decays measured at each wavelength could be satisfactorily fit by mono-exponential decays: $I(t) = I_0 \exp(-t/\tau_f)$.



Crystallography

Fab 11G10 was produced by papain digest as previously described.[4] Crystallization experiments were performed by the sitting drop vapor diffusion method at 22.5°C. The Fab, concentrated to 15mg/ml, was crystallized in presence of 2-fold molar excess of stilbene derivative **1** from 17% PEG 4,000 and 0.2M ammonium sulfate. For data collection, the crystal was flash-cooled to 100K using 25% glycerol as a cryoprotectant. Data were collected in-house using a rotating anode as x-ray source and processed and scaled with HKL2000 (Table 1). [5] The structure of 11G10 was determined by molecular replacement using Phaser.[6] The model was refined by alternating cycles of model building with the program O[7] and refinement with Refmac5. [8] The final statistics are shown in Table 1. The quality of the structure was analyzed using the programs MolProbity[9], WHAT IF,[10] and PROCHECK.[11] Figure **2** was prepared with PyMol.[12] The coordinates and the structure factors are deposited at the PDB under accession code 2G2R.

Table 1. Data collection and refinement statistics

	11G10-**1**
Space group	C2
Unit cell dimensions (Å)	a=164.8, b=53.8, c=110.4 ß=94.8º
Resolution range (Å)	50.0-2.75 (2.81-2.75)*
Unique reflections	25,496
Completeness (%)	99.2 (98.5)
R_{sym}†	0.14 (0.55)
<I/s >	9.1 (1.9)
R_{cryst}‡/ R_{free}‡	0.20/0.25
No. of refined amino acid residues/ water molecules/ stilbene molecules/ sulfate ions	876/120/2/3
Rmsd¶ bond length (Å)/ angle (º)	0.011/ 1.3
Average B-values protein/ water/ stilbene/ sulfate ($Å^2$)	32.7/ 23.8/ 40.9/ 64.2
Ramachandran plot most favored/ additionally allowed/ generously allowed/ disallowed‖ (%)	90.5/8.9/0.3/0.3

* Highest resolution shell.

† $R_{sym} = S_{hkl}S_i \mid I_i(hkl) - \langle I_i(hkl)\rangle \mid / S_{hkl}S_i \mid I_i(hkl) \mid$

‡ $R_{cryst} = S_{hkl} \mid \mid F_c(hkl)\mid - \mid F_o(hkl)\mid\mid / S_{hkl} \mid F_o(hkl) \mid$

§ R_{free} is calculated as for R_{cryst}, but from 5% of the data that was not used for refinement.

¶ Root-mean-square deviation.

‖ Val^{L51} and Val^{A51} are the only residues in a disallowed region, but both have well-defined electron density. They are in a ? turn, as commonly observed in other antibody structures.

Reference

[1] E. Juaristi, J. D. Reyna *Tetrahedron Lett.* **1984**, *25*, 3521.
[2] Q. Zheng, G. S. He, T. Lin, P. N. Prasad, *J. Mater. Chem.* **2003**, *13*, 2449.
[3] G. A. C. J. N. Demas, *J. Phys. Chem.* **1971**, *75*, 991.
[4] E. Harlow, D. Lane, *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, N. Y., **1988**.

[5] Z. Otwinowski, W. Minor, *Methods Enzymol.* **1997**, *276*, 307.
[6] L. C. Storoni, A. J. McCoy, R. J. Read, *Acta Crystallogr. D* **2004**, *60*, 432.
[7] T. A. Jones, J. Y. Zou,S. W. Cowan, M. Kjeldgaard, *Acta Crystallogr. A* **1991**, *47*, 110.
[8] G. N. Murshudov, A. A.Vagin, E. J. Dodson, *Acta Crystallogr. D* **1997**, *53*, 240.
[9] S. C. Lovell, I. W. Davis, W. B. Arendall 3rd, P. I. de Bakker, J. M. Word, M. G. Prisant, J. S. Richardson, D. C. Richardson, *Proteins* **2003**, *50*, 437.
[10] G. Vriend, *J. Mol. Graph.* **1990**, *8*, 52.
[11] R. A. Laskowski, M. W. MacArthur, D. S. Moss, J. M. Thornton, *J. Appl. Crystallogr.* **1993**, *26*, 283.
[12] W. L. DeLano, DeLano Scientific, San Carlos, CA, USA, **2002**.