

Picosecond Fluorescence Lifetime Standards for Frequency- and Time-Domain Fluorescence

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We characterized a series of dimethylamino-stilbene derivatives as standards for time-domain and frequency-domain lifetime measurements. The substances have reasonable quantum yields, are soluble in solvents available with a high purity, and do not show significant sensitivity to oxygen quenching. All the fluorophores displayed single exponential intensity decays, as characterized by frequency-domain measurements to 10 GHz. The decay times vary from 880 to 57 ps, depending on structure, solvent, and temperature, which is a useful range for modern picosecond time-domain or gigahertz frequency-domain instruments. These fluorophores may be used either to test an instrument or as reference compounds to eliminate color effects. We also characterized two-fluorophore mixtures, with the decay times spaced twofold (150 and 300 ps), with varying proportions. These mixtures are useful for testing the resolution of other time- and frequency-domain instrumentation. The excitation wavelength ranges from 260 to 430 nm, and the emission from 350 to 550 nm. The decay times are independent of the excitation and emission wavelengths.

KEY WORDS: Fluorescence spectroscopy; lifetime; 4,4'-substituted stilbenes; time-resolved fluorescence; time-domain fluorescence; frequency-domain fluorescence.

INTRODUCTION

Measurements of time-resolved fluorescence are used widely in chemical, biochemical, and biophysical research [1–3]. Such measurements are performed using time-domain [4, 5] or frequency-domain [6–10] methods. In either event it is often desirable and/or necessary to measure the decays of known fluorophores with known

lifetimes, in order to validate the data from the complex apparatus [11,12]. Furthermore, time-domain (TD)⁴ and frequency-domain (FD) methods are often performed against reference compounds [13–16], instead of scattered light, in order to avoid the wavelength-dependent time response of photomultiplier tubes (PMTs) [17–20]. While these wavelength-dependent effects appear to be less pronounced with the newer microchannel plate (MCP) PMTs, it is still necessary to verify that such effects are not present or to avoid such effects by the use of lifetime standards with emission at the desired wavelength.

Unfortunately, there are few available lifetime standards [11,12,15,16,21], and some of those which have been proposed, like quinine, have been found to be unsuitable [20, 21]. Additionally, many of the proposed substances have lifetimes which are too long to allow measurements at high modulation frequencies or are difficult to use due to the effects of oxygen quenching [22] or the lifetimes are too long to provide a reasonable test

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⁴ Abbreviations used: FD, frequency domain; TD, time domain; MCP PMT, microchannel plate photomultiplier tube; DXS, 4-dimethylamino-4'-X-stilbene (where X can be M, F, B, or C for methoxy, fluoro, bromo, or cyano, respectively); HPLC, high-performance liquid chromatography.

of the apparatus. In particular, there appear to be no accepted standards with picosecond or subnanosecond lifetimes.

In the present paper we describe a series of dimethylamino-stilbene derivatives which display lifetimes ranging from 57 to 880 ps. Importantly, all the intensity decays were found to be single exponentials, so that the observed value should be independent of the measurement method. Also, in spite of the short decay times, these standards display reasonable fluorescence intensity, so that they can be easily observed free of background fluorescence and/or scatter from the solvent. The solvents are readily available in pure form. The excitation wavelengths cover the most widely used wavelengths from 260 to 430 nm, and due to the short lifetimes the values are not sensitive to quenching by dissolved oxygen. And finally, we have characterized two-component mixtures of these standards, which we propose as a resolution standard for double-exponential decays.

MATERIALS AND METHODS

Although the proposed lifetime standards are not commercially available, the syntheses and purifications are easy and can be done in any chemical/biochemical laboratory. The details about synthesis and purification of *trans*-dimethylamino stilbenes are presented in the Appendix. These compounds were all found pure on reverse phase HPLC. The solvents were spectral grade and used without further purification. Optical densities at the excitation wavelengths were near 0.1, resulting in probe concentrations near $10^{-4}M$.

Frequency-domain measurements were performed on the 10 GHz instrument described previously [10], which is a modification of previous instruments [8,9] by the addition of an intrinsically modulated picosecond laser source and a high-speed MCP PMT. Excitation wavelengths near 300 nm were obtained from a frequency-doubled cavity-dumped rhodamine 6G (R6G) dye laser, and wavelengths near 350 nm from a pyridine 2 dye laser. The geometrical factors are not trivial when picosecond lifetimes are measured [23]. Therefore special attention was given to eliminate reflections from cuvette surfaces by absorbing glass plates located inside the cuvette. The plates were angled so the reflected laser beam, as well as fluorescence, was directed to the top of the cuvette and were not visible by the detector. Magic-angle polarization conditions were used to eliminate the effects of rotational diffusion on the intensity decays [24]. The emission was isolated using Corning cutoff

filters 3-79 or 3-75, and thin (1-mm) neutral density filters were used to compensate for the differences in optical delays between emission and scatterer. Measurements with interference filters are possible using compensation of optical delays. It should be noted that in viscous polar solutions, the lifetimes of stilbenes can strongly depend on the observation wavelength [25]. We measured almost the same lifetimes of DCS in toluene through 440- and 480-nm interference filters (113 and 116 ps), which suggests that spectral relaxation does not have a significant effect on the decay times under our experimental conditions.

Fluorescence spectra of the reference compounds were measured before and after the lifetime measurements, and we have found no changes in shapes or intensities. Also, the steady-state intensities of the samples were stable during the lifetime measurements. However, we observed some decrease in the fluorescence intensity for highly concentrated solutions and unattenuated laser light, so we recommend using diluted solutions and attenuated (or expanded) laser beams. The solid samples of the reference compounds are stable and can be stored at room temperature.

The frequency responses were fit to the single- and double-exponential models for the intensity decay

$$I(t) = \sum_{i=1}^2 \alpha_i \exp(-t/\tau_i) \quad (1)$$

where α_i are the preexponential factors and τ_i the associated decay times, using the procedures of nonlinear least-squares [26] as applied to the frequency-domain data [27,28]. More specifically, the experimental phase (ϕ_ω) and modulation (m_ω) values at each modulation frequency ($\omega = 2\pi$ times frequency in hertz) were compared with the calculated values ($\phi_{\omega c}$ and $m_{\omega c}$), so as to minimize the value of reduced χ_R^2 ,

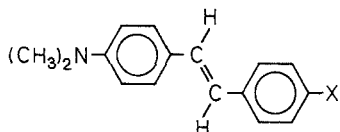
$$\chi_R^2 = \frac{1}{\nu} \left[\frac{\phi_\omega - \phi_{\omega c}}{\delta\phi} \right]^2 + \frac{1}{\nu} \left[\frac{m_\omega - m_{\omega c}}{\delta m} \right]^2 \quad (2)$$

In this expression ν is the number of degrees of freedom and $\delta\phi = 0.2$ and $\delta m = 0.005$ are the uncertainties in the phase and modulation measurements. These values are estimated from several years of experience with our instrumentation. In the case of a multiexponential analysis, the fractional intensity of each component to the total stationary intensity is given by

$$f_i = \frac{\alpha_i \tau_i}{\sum_j \alpha_j \tau_j} \quad (3)$$

RESULTS

The reference compounds consist of derivatives of 4-dimethylamino-stilbene, in which the 4' position is occupied by groups of varying electronegativity (Scheme I). The 4' group is varied from the electronegative fluorine to the electron-donating cyano group. The absorption and emission spectra of these compounds in various solvents are shown in Figs. 1–3. These spectra allow selection of a suitable standard, given the desired excitation and emission wavelengths. One notices sensitivity of the emission spectra to solvent polarity, as in seen for DMS in Fig. 1 and for DCS in Fig. 3. This behavior is probable due to charge separation in the excited state [29–32], and one may expect these compounds to display time-dependent solvent relaxation and/or wavelength-dependent lifetimes [25]. However, such effects are not significant in these solvents, presumably because the process is very rapid at these temperatures. Hence, one can use these compounds for all emission wavelengths, with the same value for the reference lifetimes.



	X
DMS	OCH ₃
DFS	F
DBS	Br
DCS	CN

Scheme I. Structures of the dimethylamino *trans*-stilbene picosecond lifetime standards.

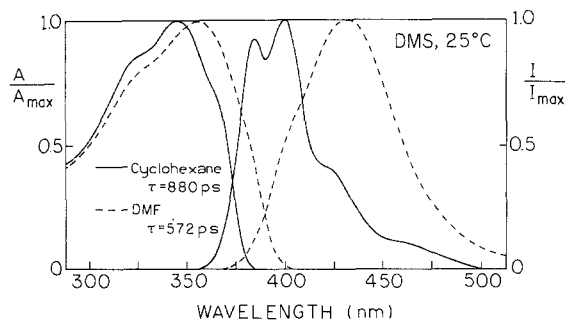


Fig. 1. Absorption (A) and fluorescence (I) spectra of DMS in cyclohexane (—) and N,N -dimethylformamide (---) at 25°C.

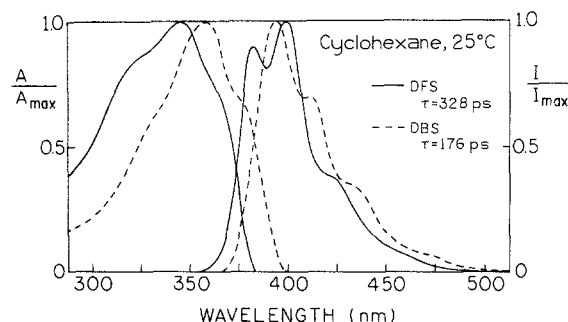


Fig. 2. Absorption and fluorescence spectra of DFS (—) and DBS (---) in cyclohexane at 25°C.

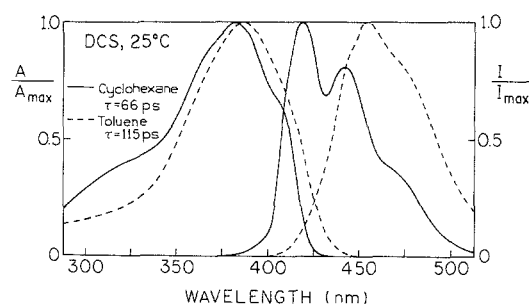


Fig. 3. Absorption (A) and fluorescence (I) spectra of DCS in cyclohexane (—) and toluene (---) at 25°C.

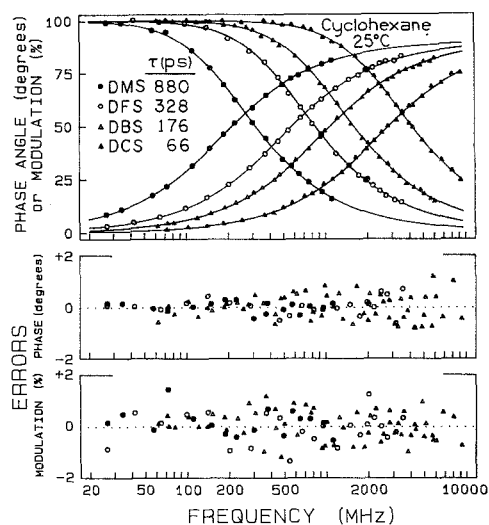


Fig. 4. Phase and modulation data for DMS (●), DFS (○), DBS (△), and DCS (▲) in cyclohexane at 25°C. The solid lines show the best single-exponential fits to the data. The lower panels show the deviations in phase and modulation from the single-exponential fits.

Another advantage of these compounds is the Stokes' shift between absorption and emission (Figs. 1–3). Because of these shifts there is little opportunity for reabsorption of the emitted light (i.e., radiative transport), which can alter the decay times for strongly overlapping absorption and emission such as for fluorescein [33].

In order to provide a range of decay times, the four stilbene derivatives were each characterized in different solvents. The four frequency responses, all in cyclohexane at 25°C, are shown in Fig. 4. In each case the entire frequency response was measured, with the frequencies extending to 10 GHz in the case of DCS. It is important to notice that all the decays could be fit to a single exponential. The values of χ_R^2 for the single-exponential fits are reasonable (Table I) and did not decrease for the double-exponential analysis. Also, the residuals are randomly distributed (Fig. 4, lower panels), again supporting the single-exponential model. As may be expected, the χ_R^2 values increase as the decay times decrease, which probably reflects increased uncertainties

and/or errors in these data as the quantum yields decrease. However, the double-exponential fits do not result in decreased values of χ_R^2 , which suggests that the errors are random.

At present many picosecond laser systems use a R6G dye laser, which frequency-doubled yield excitation wavelengths in the 285- to 310-nm range. Hence, it is of interest to determine the lifetimes of these standards for these excitation wavelengths. These decay times, as observed for specific modulation frequencies, are summarized in Table II. In all cases the decays appear to be single exponentials, as evidenced by the agreement between the phase and the modulation lifetimes. Importantly, the decay times are identical for excitation at 365 nm (Table I) and 305 nm (Table II).

For convenience, the single-exponential decay times are summarized in Fig. 5. This presentation shows the range of decay times available for the series of standards and for each standard. The uncertainty in the lifetime is comparable to the width of the lines.

Table I. Intensity Decays Analysis of Picosecond Fluorescence Lifetime Standards

Compound	No.	Solvent	ϕ^a	T (°C)	n	τ_i (ps)	f_i	χ_R^2	$\frac{\chi_R^2 (1 \text{ exp})}{\chi_R^2 (2 \text{ exp})}$
DMS	1	C ^b	0.59	25	1 ^c	880 (2.0)	1	1.0	0.98
	2	C	—	37	1	711 (1.5)	1	1.4	0.98
	3	T	0.32	25	1	740 (1.9)	1	0.9	1.04
	4	T	—	5	1	921 (2.0)	1	1.4	1.00
	5	DMF	0.27	25	1	572 (1.9)	1	2.3	1.03
	6	EA	0.15	25	1	429 (1.7)	1	1.8	1.02
DFS	7	C	—	25	1	328 (1.0)	1	2.1	0.97
	8	C	—	37	1	252 (0.7)	1	2.0	1.04
	9	T	0.16	25	1	305 (0.8)	1	2.2	0.99
DBS	10	T	—	5	1	433 (0.9)	1	1.2	0.99
	11	C	0.11	25	1	176 (0.6)	1	3.2	0.99
	12	C	—	37	1	133 (0.4)	1	3.8	0.98
	13	T	0.12	25	1	168 (0.5)	1	3.9	1.03
DCS	14	T	—	5	1	248 (0.6)	1	1.7	0.99
	15	C	0.06	25	1	66 (0.2)	1	4.2	0.98
	16	C	—	37	1	57 (0.3)	1	7.2	0.98
	17	T	0.06	25	1	115 (0.4)	1	4.2	0.99
	18	T	—	5	1	186 (0.6)	1	3.8	1.03
DBS + DFS (50:50)		T	—	25	2	164 (6.8)	0.49 ^d		
						303 (16)	0.51	2.1	9.7
DBS + DFS (90:10)		T	—	25	2	168 (4.8)	0.88		
						294 (78.0)	0.12	1.7	1.90
DBS + DFS (10:90)		T	—	25	2	146 (29)	0.07		
						301 (5.8)	0.93	1.9	2.4

^aQuantum yields, from Refs. 30–32.

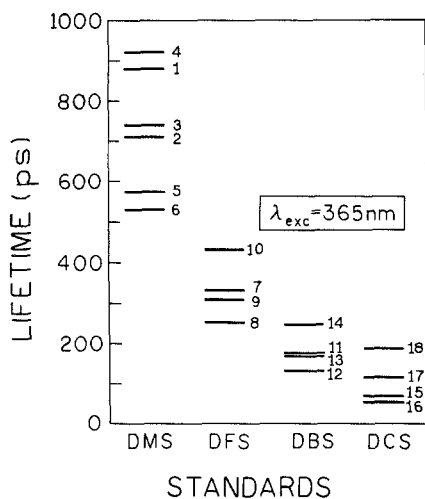
^bC, cyclohexane; T, toluene; DMF, dimethylformamide; EA, ethyl acetate. The excitation wavelength was 365 nm.

^cNumber of exponential components.

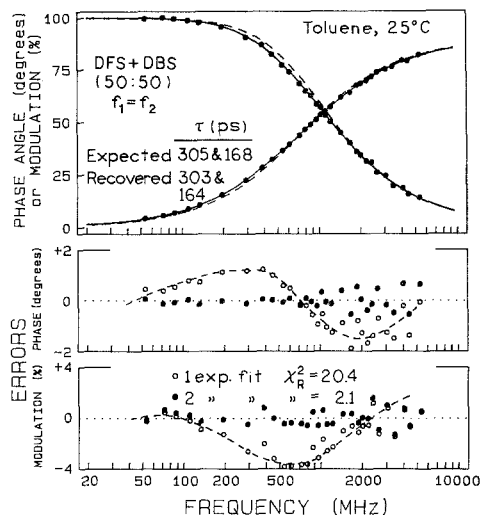
^dCalculated from Eq. (3).

Table II. Phase and Modulation Lifetimes of Picosecond Fluorescence Standards Obtained for Selected Frequencies with Excitation at 305 nm

Compound	Solvent	Frequency (MHz)	Phase (degree)	τ_{ϕ} (ps)	Modulation	τ_m (ps)
DMS	C	338	61.4	864	0.474	874
		490	66.2	737	0.406	732
	DMF	565	63.7	570	0.427	597
		EA	687	66.4	532	0.400
DFS	C	2007	75.2	300	0.258	296
	T	1172	66.0	305	0.414	298
DBS	C	2114	66.8	175	0.401	173
	T	2030	64.3	165	0.424	167
DCS	C	4292	59.4	63	0.492	66
	T	2850	63.7	113	0.438	115


Fig. 5. Lifetimes of picosecond fluorescence standards. The conditions are given by numbers in Table I.

In most biochemical samples the intensity decays are not single exponentials. In fact, considerable fluorescence experimentation is directed toward resolution of the intensity decay of multiexponential or nonexponential processes. Hence, it seems valuable to have a multiexponential reference sample with known decay times. For this purpose we propose mixtures of DBS and DFS in toluene, which show decay times of 303 and 164 ps. Frequency-domain data for the 50:50 mixture ($f_1 = f_2$) are presented in Fig. 6. Evidently, the data (\bullet) cannot be fit to the single-exponential model (---; $\chi_R^2 = 20.4$), analysis in terms of two decay times results in a reduction of χ_R^2 to 2.1 and recovery at the expected decay times and amplitudes (Table I). In this example the decay times are twofold different, and both are subnanosecond. We suggest this mixture as a modestly difficult


Fig. 6. Phase and modulation data for mixture DBS:DFS (50:50) in toluene at 25°C. The solid line shows the best two component fit to the data and the dashed line shows the best single-exponential fit to the data. The lower panels show the deviation in phase and modulation for double (\bullet)- and single (\circ)-exponential fits.

sample, which may be used to test the resolution of other instruments.

Also included in Table I are the results for two more difficult mixtures. In these cases, we varied the amplitudes of the two lifetimes (164 and 303 ps) to be at the ratio 90:10 or 10:90. This mimics the often-encountered situation where one questions whether a decay time is present at some low amplitude. As seen in Table I we were able to recover both decay times. As may be expected, the uncertainties are somewhat larger for the trace component in each mixture.

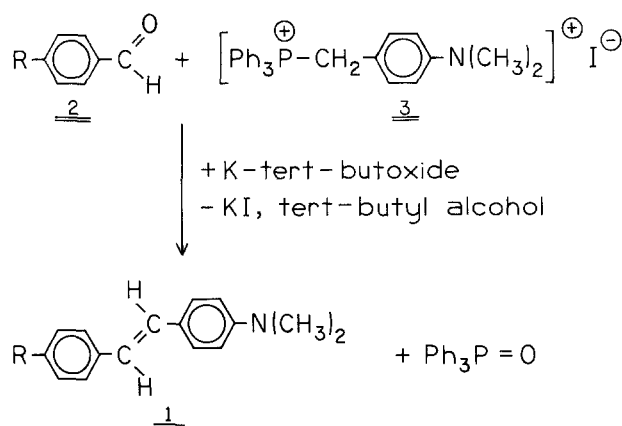


Fig. 7. Synthesis of *trans*-stilbene picosecond lifetime standards.

Table III. Donor-acceptor Substituted *trans*-Stilbenes by Wittig Olefination Reaction

X	Yield ^a (n % of theory)	Melting point (°C)	Recryst. from
CN	32	257–259	Ethanol
Br	38	236	Benzene
Cl	38	221–222	Benzene
F	43	188–189	Hydrocarbon mixture with boiling-point range 70–95°C
OCH ₃	68	183–184	Hydrocarbon mixture with boiling-point range 70–95°C

^aPure product.

DISCUSSION

We hope that these standards are valuable for the fluorescence community. These substances are not commercially available, but synthesis and purification are straightforward. Small amounts of these compounds can be provided by CFS. These same compounds may also serve as standards for decays of fluorescence anisotropy. Such work is in progress.

APPENDIX

The stilbene derivatives can be obtained in different ways: by Knoevenagel condensation [34], Grignard reaction [35], or Meerwein arylation. We used the Wittig reaction [36] between **2** and **3** as shown in Fig. 7. One-

tenth mole (4-dimethylaminobenzyl)-triphenylphosphonium iodide (**3**) heated in 500 ml dried benzene with 0.1 mol potassium *t*-butoxide in an argon atmosphere and refluxed for 10 min, followed by the addition of 0.1 mol freshly purified aldehyde (**2**). The mixture is heated for another 15 min with stirring. After cooling the reaction mixture is shaken with 300 ml water. The benzene layer is separated, and dried with sodium sulfate and then evaporated under vacuum. The raw product (**1**) was recrystallized from ethanol and then the solvent listed in Table III.

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