Photoinduced Hydrogen Atom Transfer in Salicylic Acid Derivatives Used as Matrix-Assisted Laser Desorption/Ionization (MALDI) Matrices

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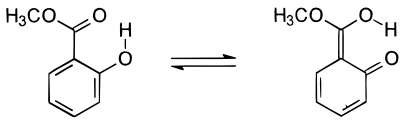
The triplet states of unsubstituted and 5-substituted derivatives of salicylic acid and methyl salicylate, some of which are used as matrices in matrix-assisted laser desorption/ionization (MALDI) mass spectrometry of macromolecules, were studied in acetonitrile and cyclohexane by transient absorption and time-resolved luminescence spectroscopy. The results suggest a tautomeric structure of the triplet states of salicylic acid as well as that of its methyl ester and its 5-hydroxy- and 5-methoxy-substituted derivatives. In this tautomeric structure, the *ortho*-hydroxy hydrogen has been transferred to the carbonyl oxygen. No differences were observed between the triplet−triplet absorption spectra of the acids and the corresponding methyl esters. For the 5-hydroxy- and 5-methoxy- compounds, evidence for long-lived phototautomers was found. The P-type delayed fluorescence of methyl salicylate is consistent with the known tautomer fluorescence at 440 nm, implying a tautomeric structure of the triplet state. Similarly, for the 5-methoxy-substituted compound a unique delayed fluorescence spectrum, red-shifted relative to the prompt fluorescence spectrum, was observed and attributed to excimer or tautomer fluorescence. The results presented here contrast with previous reports on the absence of intramolecular hydrogen atom transfer on the singlet surface of the MALDI matrices 5-methoxy- and 5-hydroxysalicylic acid and their respective methyl esters. The resulting tautomers and their reaction products may be relevant for analyte ionization in MALDI.

Introduction

Since the first description of the anomalously large Stokes shift of the fluorescence of methyl salicylate in 1924 by Marsh[1] and its rationalization by Weller,[2] a good deal of effort has been devoted to the clarification of the photophysics of this and similar *ortho*-hydroxybenzoyl compounds. These compounds undergo a fast[3] intramolecular hydrogen atom transfer reaction in the first excited singlet state to give a tautomer structure **1′** that exhibits a fluorescence with a large Stokes shift.[2−4] This phototautomerization[5] reaction has been the subject of a number of experimental and theoretical investigations.[3,4,6,7]



1 **1'**

Intramolecular hydrogen atom transfer is of fundamental interest as an important primary reaction step in a number of chemically and biologically relevant reactions. It has also been employed for a number of practical purposes: while some compounds undergoing excited-state intramolecular hydrogen atom transfer have thermo-[8] and photochromic[9] properties, others have been used as laser media,[10] as photoprotective agents in polymers, or as charge reduction agents in xerographic toners.[11,12]

Our interest in these compounds originated in their use as matrices in matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS).[13,14] MALDI-MS is a relatively new method for the intact desorption and ionization of large biomolecules (up to several 10^5 Da). It allows for intact volatilization and ionization of these large, polar molecules while avoiding excessive fragmentation. For a typical MALDI experiment, a solution of the analyte (bio)molecule is mixed with a solution of a small organic molecule and then evaporated to give a mixed crystal that contains matrix and analyte in a molar ratio of ca. 10^5:1. The matrix molecule is typically a small aromatic molecule that absorbs strongly at the desorption laser wavelength in the near UV. The interaction between the laser pulse and the matrix results in the desorption of a small volume of material containing neutral and ionic matrix and analyte species. The matrix is suspected to play a crucial role in the ionization of the analyte molecules. Typically, analyte biomolecules, A, are detected as protonated $(A + nH)^{n+}$ or deprotonated $(A - nH)^{n-}$ quasimolecular ions. A number of observations indicate that analyte ionization takes place as a prompt reaction or at least within 10 ns of the desorption laser pulse in the solid to gas-phase transition region above the sample surface, which sets a time frame for matrix excited states that might act as mediators of analyte (de)protonation.

Various mechanisms for the (net) proton transfer from matrix to analyte molecules have been proposed.[15] Photoionization of the matrix M and subsequent hydrogen atom transfer between matrix radical cations and matrix molecules and between

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protonated matrix product ions and analyte molecules A (eqs 1−3)[16] as well as net excited-state proton transfer[17] (eqs 4 and 5) have been suggested.

$$M \xrightarrow{h\nu} M^{+\bullet} + e^- \quad (1)$$

$$M^{+\bullet} \rightarrow (M - H)^{\bullet} + H^+ \quad (2)$$

$$A + H^+ \rightarrow (A + H)^+ \quad (3)$$

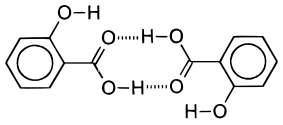
$$M \xrightarrow{h\nu} (M - H)^- + H^+ \quad (4)$$

$$A + H^+ \rightarrow (A + H)^+ \quad (5)$$

Within either model, analogous reactions can be conceived for the formation of negatively charged deprotonated analyte ions. Schlunegger and co-workers[18] pointed out that a variety of good MALDI matrices contain a carbonyl group with a hydroxyl group in the ortho position. Indeed, several *ortho*-hydroxyacetophenones (2,3,4-trihydroxy-,[19] 2,4,6-trihydroxy-,[19] 2,6-dihydroxyacetophenone[20,21]), 3-hydroxy-2-pyridinecarboxylic acid,[22] and various substituted salicylic acid derivatives (SAD)[16,23,24] all contain the *ortho*-hydroxy-carbonyl motif and have successfully been used as matrices for various classes of biomolecules.

We decided to focus our studies on the excited states and reactive intermediates of SAD to elucidate a possible connection between their photochemical and photophysical properties and their exceptional suitability as MALDI matrices. The photophysics of salicylic acid and its methyl ester has been studied, mainly by fluorescence spectroscopy in solution, in the vapor phase and in molecular beam experiments. Time-resolved fluorescence bleaching experiments on methyl salicylate vapor revealed a hydrogen atom transfer along the intramolecular H-bond from the hydroxyl to the carbonyl oxygen in less than 60 fs.[3] The presence of a 5-substituent appears to have a significant influence on the fluorescence spectra of SADs, as described in a number of studies. The compounds 2,5-dihydroxybenzoic acid,[25] 2-hydroxy-5-methoxybenzoic acid[26] and its methyl ester,[27] and 2-hydroxy-5-methylbenzoic acid[26] have been investigated by fluorescence spectroscopy in molecular beam[25,26] and solution[27] experiments. Although the fluorescence spectra of the 5-methyl-substituted compound parallel those of salicylic acid, in particular reproducing the large Stokes shift characteristic of an excited-state tautomerization,[26] the introduction of a more electron-donating 5-methoxy-[26] or -hydroxy-[25] substituent leads to a smaller Stokes shift. For the latter two compounds, no evidence for an excited-state phototautomerization was found in their fluorescence spectra.

However, 2,5-dihydroxybenzoic acid[24] and its mixtures with 2-hydroxy-5-methoxybenzoic acid[23] were found to be excellent matrices. Unsubstituted salicylic acid also performed well,[28] although its relatively high volatility in the vacuum of the mass spectrometer generally limits its usefulness. One of the best studied matrices for the analysis of proteins is 2,5-dihydroxybenzoic acid.[24] A number of fundamental investigations on the MALDI process have been carried out using this compound.[16,23,25,28−33] Furthermore, its crystal structure[34] is known, as is that of salicylic acid.[35,36] A central element of these crystal structures is the formation of hydrogen-bonded dimers $\mathbf{1_D}$.[37]



$\mathbf{1_D}$

The formation of dimers as well as the dissociation of the carboxyl group[38] can complicate the interpretation of fluorescence spectra of these compounds in solution. In the aprotic organic solvents acetonitrile and cyclohexane studied here, dimers analogous to $\mathbf{1_D}$ are the predominant species in solutions of salicylic acid and various 2- and 5-substituted derivatives. In methyl esters of these acids dimer formation is obviously inhibited. Solutions of methyl esters of SADs have, therefore, been used as reference compounds in these studies to investigate the possible influence of dimer formation on the photophysical properties of the acids.

In polar alcoholic solvents, intermolecular proton transfer to the solvent is observed from the first excited singlet state of a number of SADs, as evidenced by the observation of the carboxylate anion fluorescence upon excitation of the undissociated acid.[11,12,39] In this work, only acetonitrile and cyclohexane were used as solvents, in which hydrogen bonding to the solute and the concomitant effects mentioned above do not play a significant role.

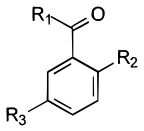
In this study, we have attempted to achieve an improved understanding of the photochemical and photophysical properties of SAD in solution. The ultimate goal of this project is to provide data for a mechanistic understanding of the ionization process in MALDI. Although fluorescence spectroscopy has previously been implemented in the investigation of these compounds, it does not provide a full understanding of the photophysics and photochemistry: it is not inherently suited to investigate the role of phototautomers and triplet excited states in the reactivity and electronic deactivation pathways of these molecules. In this study, we utilized transient absorption spectroscopy to identify the nature of the excited state and transient intermediates of these compounds which may be of relevance to the MALDI process. In particular, we report the observation of triplet phototautomers generated by S_0-S_1 excitation with subsequent intersystem crossing as well as tautomerization on the triplet state surface of the SAD molecule following triplet−triplet energy transfer from a high-energy triplet donor. For SAD with an electron donating 5-hydroxy- or 5-methoxy-substituent, long-lived phototautomers were observed. In summary, all *ortho*-hydroxybenzoyl MALDI matrices studied here exhibit intramolecular hydrogen atom transfer reactions in the excited state. The tautomeric intermediates resulting from these reactions should, therefore, be taken into account when considering candidates for reactive matrix species that mediate analyte ionization.

Experimental Section

Chemicals. The crystalline compounds 2-hydroxybenzoic acid (salicylic acid) (**2**), 2-methoxybenzoic acid (**3**), 2-hydroxy-5-methylbenzoic acid (**5**), 2-hydroxy-5-methoxybenzoic acid (**7**), 2,5-dimethoxybenzoic acid (**9**), 2,5-dihydroxybenzoic acid (**10**), methyl 2,5-dihydroxybenzoate (**11**), 2,5-dihydroxybenzaldehyde (**14**), and 2′,5′-dihydroxyacetophenone (**15**) were purchased from Aldrich (Milwaukee WI) and recrystallized from water before use (Table 1).

The liquid compounds methyl 2-hydroxybenzoate (methyl salicylate) (**1**), methyl 2-methoxybenzoate (**4**), methyl 2-hydroxy-5-methylbenzoate (**6**), methyl 2-hydroxy-5-methoxybenzoate (**8**), and 2′-hydroxyacetophenone (**13**) were purchased from Aldrich in the highest purity available and used as received. Spectroscopy grade solvents were obtained from Fisher Scientific (Pittsburgh, PA). 2,4-Hexadiene was purchased from Acros Organics (Fisher Scientific). All-*trans*-β-carotene was purchased from Aldrich and recrystallized from benzene/methanol before

TABLE 1: Nomenclature for Compounds



R_1	R_2	R_3	
OH	OH	H	**2**
OCH_3	OH	H	**1**
OH	OCH_3	H	**3**
OCH_3	OCH_3	H	**4**
OH	OH	CH_3	**5**
OCH_3	OH	CH_3	**6**
OH	OH	OCH_3	**7**
OCH_3	OH	OCH_3	**8**
OH	OCH_3	OCH_3	**9**
OH	OH	OH	**10**
OCH_3	OH	OH	**11**
H	OH	H	**12**
CH_3	OH	H	**13**
H	OH	OH	**14**
CH_3	OH	OH	**15**

use. The absorption spectrum of the recrystallized material in cyclohexane showed no trace of the common impurity, *cis*-β-carotene.[40] Ground-state absorption spectra were acquired on an HP 8453 diode array UV−Vis spectrophotometer (Hewlett-Packard, Palo Alto, CA). Samples were contained in 10 × 10 mm^2 quartz cuvettes. Absorption coefficients were determined from Beer−Lambert plots in a concentration range from 2×10^{-5} to 2×10^{-3} M.

Transient Absorption Spectroscopy. Experiments were carried out using an apparatus based on an existing design, described elsewhere.[41] The optical bandwidth of the detection setup is <10 nm, the accessible probe wavelength range is 250−825 nm, and the rise time of the system is 45 ns. A digital oscilloscope (LeCroy 9360, LeCroy, Chestnut Ridge, NY) was used to acquire kinetic traces. A shutter and suitably chosen low-wavelength cutoff filters placed between the Xe arc lamp and the sample were used to avoid degradation of the sample by limiting the exposure to the monitoring light, both spectrally and temporally. As pump light sources, the third and fourth harmonics of a Q-switched Nd:YAG laser (266 nm, 355 nm, τ = 6 ns, model GCR 150), a XeCl Excimer laser (308 nm, 10 ns, Lambda Physik, Göttingen, Germany), and an Optical Parametric Oscillator (OPO) with frequency-doubler option (200−345 nm, 365−700 nm, MOPO 710), pumped by the third harmonic of another Q-switched Nd:YAG laser (355 nm, τ = 6 ns, model GCR 230, both YAG lasers and OPO from SpectraPhysics, Mountain View, CA) were used. A known fraction of the laser pulse energy was monitored for every shot with a pyroelectric probe (RjP 735 probe, connected to an Rj7620 energy meter, LaserProbe Inc., Utica, NY). The pulse energy incident on the cuvette was kept below 15 mJ (fluence < 30 mJ cm^{-2}) in all experiments. The cuvette holder served as an aperture of 10 × 5 mm^2 in the excitation beam. The experiment was controlled by laboratory-built timing circuitry as well as digital delay generators (DG 535, Stanford Research Systems, Sunnyvale, CA) and a PowerMacintosh 7200/120 computer with programs written in LabView 4.0 (National Instruments, Austin, TX). An analog/digital I/O board (PCI 1200) and a GPIB board (PCI-GPIB) (both boards from National Instruments) were used to control the experiment and acquire, analyze, and store data. Fitting programs based on a Levenberg−Marquardt algorithm were written in LabView and used to extract kinetic parameters from the data. Samples were contained in 10 × 10 mm^2 cuvettes and all absorbance values stated in the text refer to a path length of 10 mm. Sample absorbances were between 0.1 and 1 at the laser wavelength. The solutions were saturated with air or nitrogen, as stated in the figure captions or the text.

Gated Fluorescence Spectroscopy. Laser-induced delayed fluorescence was probed with a fiber-coupled spectrograph consisting of a grating monochromator (F = 4, 150 grooves/mm grating, λ_{blaze} = 330 nm) with an intensified, gated charge-coupled device (CCD)-array detector (ICCD-MAX, Princeton Instruments, Trenton, NJ). The gate timing was controlled with a digital delay generator (DG 535, Stanford Research Systems, Sunnyvale, CA). The grating spectrograph was calibrated with the Hg lines in the roomlight. No correction was made for the spectral sensitivity of the detector and the monochromator. However, all data reported here were obtained at an identical grating position and are, therefore, directly comparable. The WinSpec software supplied by the manufacturer was used for spectral acquisition and manipulation.

Results

The values for the molar decadic absorption coefficients ϵ_{GS} at the longest wavelength ground-state absorption maxima $\lambda_{max,\,GS}$ of compounds **1**, **2**, **7**, **8**, **9**, **10**, and **11** in acetonitrile solution are given in Table 2. Excitation of 2-hydroxybenzoic acid **2** and its methyl ester **1** at 308 nm in acetonitrile produced identical transient absorption spectra attributable to a single species with an absorption maximum at ca. 440 nm (cf. Figure 1A).[42] In deaerated solution the lifetime of this species was approximately 25 μs.

For both compounds this species was quenched by oxygen (cf. Figure 1B). Quenching was also observed in the presence of 2,4-hexadiene (2,4-HD), a known triplet energy acceptor,[43] with a rate constant k_{ET}(2,4-HD) of (1.2−1.4) × 10^9 M^{-1} s^{-1} (cf. Table 2). From these experiments a tentative assignment of this species as an excited triplet state was made, which was corroborated in a series of experiments described in the following.

Triplet−triplet energy transfer from this species to β-carotene was monitored at 520 nm after excitation of compounds **1** and **2** at 308 nm in deaerated cyclohexane. The rate constants for triplet−triplet energy transfer were determined by fitting the observed kinetic signals to a sum of two exponentials with opposite signs of the pre-exponential factor to account for the natural decay of the β-carotene triplet. The bimolecular rate constants for this energy transfer, k_{ET}(β-carotene) were found to approach the diffusion-controlled limit for both compounds (cf. Table 2).

Using the reverse approach the formation of this species was sensitized by selective excitation[44] of a high triplet energy donor (acetone[45,46] in acetonitrile solution and toluene[45] in acetonitrile or cyclohexane solution) at 266 nm. Under the conditions chosen, the population of the triplet state of **1** or **2** by direct excitation could be neglected.[44] The rate constants k_{ET}(acetone) for triplet−triplet energy transfer from acetone to **1** or **2** in acetonitrile were determined from measurements of the rate of formation of the SAD triplet for several SAD concentrations. The formation of the SAD triplet was monitored at the wavelength of maximum triplet−triplet absorption, $\lambda_{max,TT}$ (cf. Table 2).

The 5-methyl-substituted compounds **5** and **6** were found to exhibit transient absorption spectra similar to those of **1** and **2** upon excitation at 308 nm (data not shown). Compounds **7**, **8**, **10**, and **11** contain a significantly more electron donating 5-methoxy-(**7**, **8**) or 5-hydroxy-(**10**, **11**) substituent. The ob-

TABLE 2: Spectroscopic and Kinetic Data Obtained on Ground and Triplet States of Salicylic Acid Derivatives

	2	**1**	**7**	**8**	**9**	**10**	**11**
$\lambda_{max,\ GS}$/nm[a]	306	305	334	334	319	336	336
$\epsilon_{GS}/10^3\ M^{-1}\ cm^{-1}$[a,b]	3.8	4.0	4.3	4.3	4.0	4.2	4.2
$\lambda_{max,\ TT}$/nm[a,c]	440	440	420	420	390	420	420
$\epsilon_{TT}/10^3\ M^{-1}\ cm^{-1}$[a,d]	4.2	5.4	8.8	9.4	4.9	9.4	8.5
Φ_T[e,f]	0.024	0.046	0.039	0.036	n.d.	n.d.	n.d.
$k_T/10^5\ s^{-1}$[a,g]	0.4	0.7	0.8	2.0	1.0	0.9	1.1
k_{ET} (acetone)$/10^9\ M^{-1}\ s^{-1}$[a]	0.50	0.51	3.9	3.8	1.9	3.1	3.6
k_{ET} (2,4-HD)$/10^9\ M^{-1}\ s^{-1}$[a]	1.4	1.2	0.3	1.4	2.8	1.0	1.5
k_{ET} (β-carotene)$/10^9\ M^{-1}\ s^{-1}$[e]	7.1	7.4	6.6	6.4	n.d.	n.d.	n.d.

[a] In acetonitrile. [b] Estimated error ± 5%. [c] Estimated error ± 5 nm. [d] Estimated error < ± 15%. [e] In cyclohexane. λ_{exc} = 308 nm. [f] Estimated error ± 0.01. [g] Determined after direct excitation.

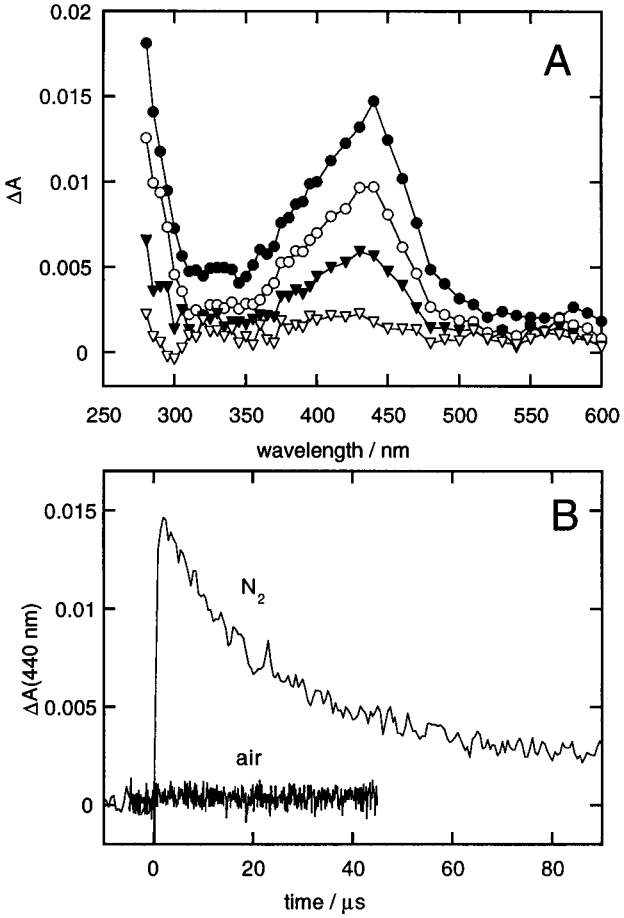


Figure 1. Transient absorption of **2** in acetonitrile, excited at 308 nm. A: Spectra at 2−3 μs (solid circles), 10−11 μs (open circles), 25−26 μs (solid triangles), and 80−81 μs (open triangles) after the laser pulse in degassed acetonitrile. B: Comparison of kinetic traces at 440 nm in N_2- and air-equilibrated acetonitrile. The absorbance of the sample at 308 nm was ca. 0.45 (ca. 120 μM **2**) and the laser fluence per pulse was ca. 16 mJ cm^{-2}.

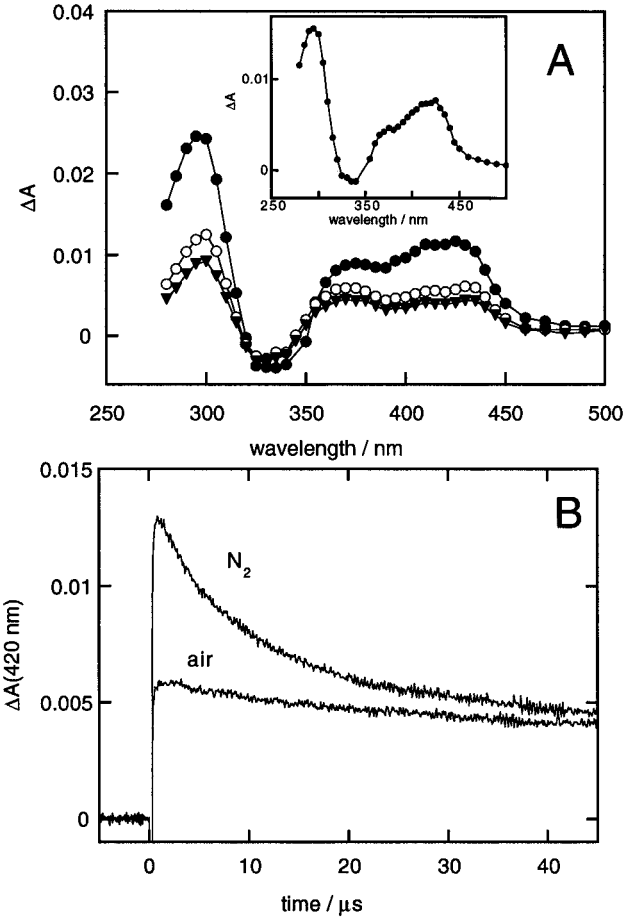


Figure 2. Transient absorption of **7** in acetonitrile, excited at 355 nm. A: Spectra at 0.8−1.2 μs (solid circles), 19−20 μs (open circles), 79−80 μs (solid triangles) after the laser pulse in degassed acetonitrile. The inset shows the difference between the spectra at the first and last time points. B: Comparison of kinetic traces at 420 nm in N_2- and air-equilibrated acetonitrile. The absorbance of the sample at 355 nm was ca. 0.7 (ca. 270 μM **7**) and the laser fluence per pulse was ca. 20 mJ cm^{-2}.

served transient absorption spectra of the latter compounds are similar to each other but different from those of **1**, **2**, **4**, and **5** and more complex. As a representative example, the transient absorption spectrum of **7**, upon excitation at 355 nm in acetonitrile, is given in Figure 2A.

In addition to a longer-lived species that is not quenched by oxygen and will be discussed in a later section, a shorter-lived species with absorption maxima at 300 and 420 nm, with a shoulder at 370 nm (see difference spectrum in the inset in Figure 2A), was found to be quenched by oxygen and by 2,4-hexadiene (cf. Table 2). This latter species was therefore identified as the triplet state. This assignment was confirmed in experiments analogous to those described above for **1** and **2**. The determined rate constants for these reactions (k_{ET}(2,4-HD), k_{ET}(β-carotene), and k_{ET}(acetone)) are listed in Table 2.

Triplet−triplet absorption spectra of **1**, **2**, **7**, **8**, **10**, and **11** were also acquired after sensitization with acetone. Their spectral profiles are identical to that of the oxygen-sensitive species observed after direct excitation of the corresponding SAD at 308 (**1**, **2**) or 355 nm (**7**, **8**, **10**, **11**). The triplet−triplet absorption spectra of **1** and **2** are indistinguishable[42] and exhibit a maximum at ca. 440 nm (cf. Figure 3A). The triplet−triplet absorption spectra of the 5-substituted **7**, **8** (5-methoxy-), **10**, and **11** (5-

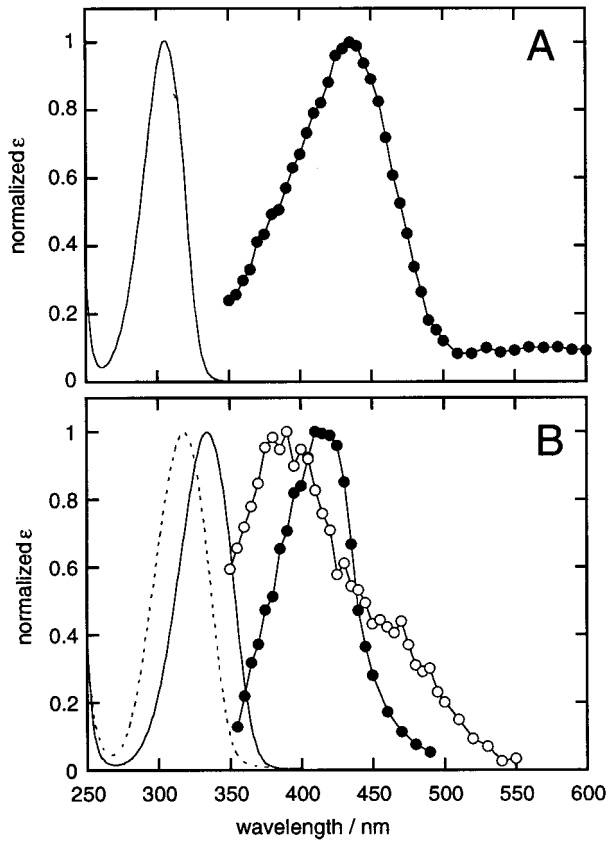


Figure 3. Normalized ground state- and triplet−triplet absorption spectra of **1**, **7**, and **9**. A: Ground-state absorption spectrum of **1** (solid line) and triplet−triplet absorption spectrum of **1** (solid circles) after photosensitization with acetone in N_2-equilibrated acetonitrile (λ_{exc} = 266 nm). The absorbance of the sample at 266 nm was ca. 1, the concentration of **1** was ca. 1 mM, and the laser fluence per pulse was ca. 6 mJ cm^{-2}. B: Ground-state absorption spectra of **7** (dashed line) and **9** (solid line) and triplet−triplet absorption spectra of **7** (solid circles) and **9** (open circles) after photosensitization with acetone in N_2-equilibrated acetonitrile. For the sample of **7**, the absorbance at 266 nm was ca. 0.5, and the concentration of **7** was ca. 350 μM. For the sample of **9**, the absorbance at 266 nm was ca. 1, and the concentration of **9** was ca. 60 μM. The laser fluence was ca. 2 mJ cm^{-2} pulse^{-1} for both samples.

hydroxy-) all resemble each other closely,[42] but differ slightly from those of **1** and **2**, which do not carry a 5-substituent (cf. Figure 3). The triplet−triplet absorption maxima for **7**, **8**, **10**, and **11** lie at ca. 420 nm. In the triplet−triplet spectra of all compounds **1**, **2**, **7**, **8**, **10**, and **11** a weak (ϵ_{TT} < 0.05−0.10 $\epsilon_{TT,max}$) red tail, extending beyond 825 nm, is observed (data not shown).[47] For 7-hydroxy-1-indanone, a similar long-wavelength transient absorption has previously been ascribed to the triplet−triplet absorption of a tautomer analogous to **1′**, as inferred from two-step laser-induced fluorescence measurements.[48] For the SAD investigated here, the shape of the transient spectra was found to be independent of both the solvent polarity (acetonitrile, cyclohexane) and the triplet photosensitizer used (acetone in acetonitrile, toluene in acetonitrile or cyclohexane), further supporting the assignment as triplet states and ruling out a contribution of parallel reactions, e.g., excited-state proton transfer to the hydrogen bonding acetone molecule[46] or hydrogen abstraction by the acetone carbonyl triplet.

The intersystem crossing quantum yields Φ_T of **1**, **2**, **7**, and **8** (**10** and **11** are insoluble in cyclohexane) were determined by sensitization of the β-carotene triplet in N_2-equilibrated cyclohexane after excitation at 308 nm and are listed in Table 2. The β-carotene triplet signal obtained through photosensitization with naphthalene (Φ_T = 0.75)[49] was used as a reference for these determinations. The relative values for the SAD were $\Phi_{T,1}/\Phi_{T,2}$ = 1.9, $\Phi_{T,7}/\Phi_{T,2}$ = 1.6, and $\Phi_{T,8}/\Phi_{T,2}$ = 1.5.

To corroborate the nature of triplet quenching by oxygen we attempted to detect the near-infrared phosphorescence from the singlet molecular oxygen ($O_2(^1\Delta_g)$) that is normally formed in such reactions.[50] The $O_2(^1\Delta_g)$ phosphorescence from a reference solution (phenalenone, quantum yield Φ_Δ = 0.95 [51]) was easily detected in acetonitrile when a germanium diode detector was used, whereas no $O_2(^1\Delta_g)$ phosphorescence was detected from SAD solutions of equal absorbance at the pump laser wavelength. The detection sensitivity of the setup used allows a detection of quantum yields of $O_2(^1\Delta_g)$ formation of Φ_Δ > 0.05. Therefore, the absence of $O_2(^1\Delta_g)$ phosphorescence from SAD solutions is consistent with the values for the triplet quantum yields, Φ_T < 0.05, determined by sensitization of the β-carotene triplet (cf. Table 2).

The molar decadic triplet−triplet absorption coefficients, ϵ_{TT}, of **1**, **2**, **7**, **8**, **9**, **10**, and **11** at the blue maximum $\lambda_{max,TT}$ of the triplet−triplet absorption spectrum were determined by sensitization of the SAD triplet with acetone in degassed acetonitrile at various laser pulse energies *E* and by comparison with signals obtained from the triplet−triplet absorption of an optically matched benzophenone solution, employed as actinometer. For the evaluation of these data, corrections for the competition between triplet−triplet energy transfer to the SAD acceptor and natural decay of the acetone donor triplet as well as the decay of the acceptor triplet were taken into account. The values for $\epsilon_{TT}(\lambda_{max,\,TT})$ are stated in Table 2.

Structure of the Triplet State. To investigate the influence of the *ortho*-hydroxy group on the photochemistry of SAD, studies of compounds **3**, **4**, and **9** were carried out. In these *ortho*-methoxy-compounds the possibility for intramolecular hydrogen atom transfer to the carbonyl oxygen is absent. Indeed, appreciable differences were observed between the transient absorption spectra of **3** or **4** and those of the corresponding *ortho*-hydroxy compounds **2** or **1**.

From the kinetics observed at various probe wavelengths it was concluded that the transient absorption spectra of **3** and **4** in N_2-equilibrated acetonitrile consist of several primary species (data not shown). These transient species were not observed in air-equilibrated solutions due to quenching by oxygen.[52] Unfortunately, it did not prove possible to sensitize the triplet state of **3** or **4**.[53] However, the transient absorption spectra of **2** and **3** show significant differences. Whereas the latter has a maximum at 380 nm, the former has a maximum transient absorption at 440 nm that could clearly be assigned to the triplet state (cf. Figure 3A). In both cases, the transient absorption spectrum of the corresponding methyl ester is identical to that of the acid.

The triplet−triplet absorption spectra of **7** and **9** (cf. Figure 3B) were compared to investigate the effect of an electron-donating 5-methoxy-substituent, which was reported to have a considerable effect on the fluorescence emission spectra. The triplet state of both compounds was sensitized by acetone triplet in deaerated acetonitrile solution. Replacement of the *ortho*-hydroxy substituent with a methoxy group changes both the absorption maximum and the profile of the triplet−triplet absorption spectrum. The maximum of the triplet−triplet absorption lies at 420 nm for the *ortho*-hydroxy-compound **7** and at 390 nm for the *ortho*-methoxy-substituted compound **9**. We interpret these differences between a hydroxy and a methoxy substituent in the *ortho*-position to the carbonyl group as

evidence for an intramolecular rearrangement that is only possible in the case of the hydroxy-compound.

These findings support the assignment of the species giving rise to the observed triplet−triplet absorption spectra of the *ortho*-hydroxy compounds as intramolecularly hydrogen-transferred triplet tautomers, in agreement with other studies that have suggested the tautomeric character of the triplet states of other 2-hydroxybenzoyl-compounds.[54−56]

Delayed Fluorescence. To further confirm the structure of the observed triplet species, the spectra of the P-type delayed fluorescence resulting from triplet−triplet annihilation in degassed cyclohexane were obtained. The SAD triplet state was sensitized by triplet−triplet energy transfer from toluene to achieve much higher SAD triplet concentrations at moderate laser fluences than could be achieved by direct excitation, due to the low quantum yields for intersystem crossing[57] (reactions 6−12):

$$^1\text{Toluene} \xrightarrow{h\nu\ (266\ \text{nm})} {}^1\text{Toluene}^* \xrightarrow{\text{ISC}} {}^3\text{Toluene}^* \quad (6)$$

$$^3\text{Toluene}^* + {}^1\text{SAD} \rightarrow {}^1\text{Toluene} + {}^3\text{SAD}^* \quad (7)$$

$$^3\text{SAD}^* \xrightarrow{\text{ISC}} {}^1\text{SAD} \quad (8)$$

$$^3\text{SAD}^* + {}^3\text{SAD}^* \rightarrow {}^{1,3,5}(\text{SAD}\cdot\text{SAD})^*\ [58,59] \quad (9)$$

$$(\text{SAD}\cdot\text{SAD})^* \rightarrow {}^1\text{SAD}^* + {}^1\text{SAD} \rightarrow 2({}^1\text{SAD}) + h\nu \quad (10)$$

$$^1(\text{SAD}\cdot\text{SAD})^* \rightarrow {}^1\text{SAD} + {}^1\text{SAD} + h\nu_{\text{excimer}} \quad (11)$$

$$^1\text{SAD} \xrightarrow{h\nu\ (266\ \text{nm})} {}^1\text{SAD}^* \rightarrow {}^1\text{SAD} + h\nu \quad (12)$$

The delayed fluorescence spectrum of **1** comprises a single band with a maximum at 440 nm (Figure 4A), which has previously been assigned to the S_1'-S_0' emission of the hydrogen-transferred tautomer form **1′**.[3] The triplet−triplet absorption of the same sample containing **1** and toluene in degassed cyclohexane was monitored at 440 nm and is displayed in Figure 4B. From ca. 1.5 μs after the laser pulse on, reaction 7 is essentially complete and reactions 8 and 9 determine the SAD triplet concentration: the kinetic behavior of the delayed fluorescence emitted from the sample at 440 nm (cf. Figure 4B) indicates decay by first- and second-order processes.The delayed fluorescence spectrum displayed in Figure 4A was, therefore, obtained by integration over a time window from 1.7 to 91.7 μs [60] after the laser pulse using the gated spectrograph. The horizontal lines in Figure 4B and the inset mark the time interval in which the delayed fluorescence spectrum in Figure 4A was acquired. The spectrally and temporally integrated signal of the delayed fluorescence was found to be proportional to the square of the absorbed pump light energy, as expected for reaction 9.[61] The negative signal in the inset in Figure 4B and the spike in the delayed fluorescence trace immediately after the laser pulse are due to the response of the photomultiplier tube (PMT) to the prompt fluorescence of **1**. In control experiments, no fluorescence was observed in this time interval after the laser pulse from air-equilibrated solutions containing **1** and toluene. Here, quenching of the toluene and acceptor triplets by oxygen is the dominant effect, precluding the observation of other triplet reactions. Deaerated or air-equilibrated solutions of **1** alone or toluene alone also did not exhibit delayed fluorescence, as expected (data not shown). Direct excitation of solutions of **1** alone at 266 nm gives rise to prompt fluorescence spectra (reaction 12, cf. Figure 4A). These

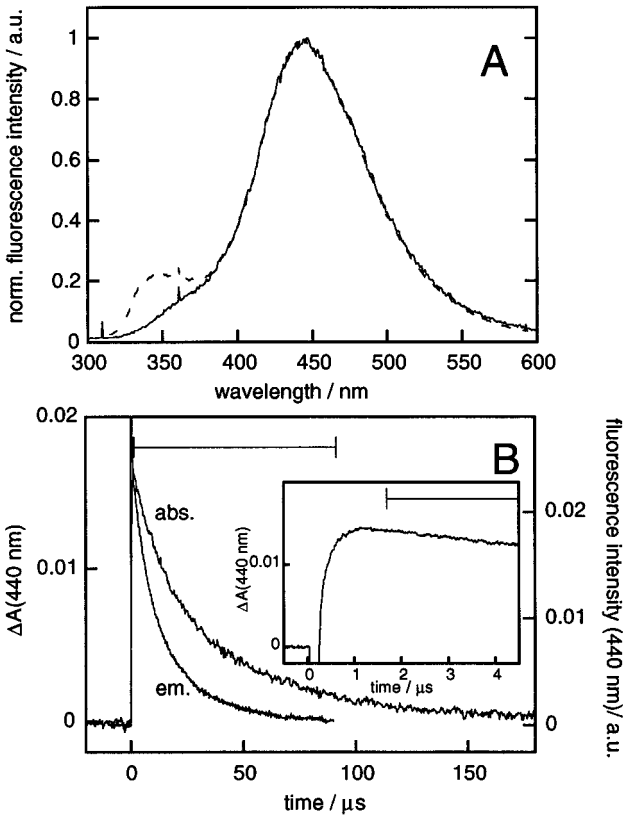
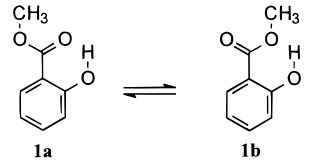


Figure 4. Delayed fluorescence from triplet states of **1**. A: Time-integrated delayed fluorescence spectrum (solid line) of a solution containing toluene and ca. 420 μM **1** in deaerated cyclohexane integrated over the time interval (1.7−91.7 μs) after the laser pulse. The absorbance at 266 nm was ca. 1. The dashed line depicts a prompt fluorescence spectrum from an air-equilibrated solution containing ca. 420 μM **1** alone, excited at 266 nm with a laser fluence of ca. 4 mJ cm^{-2} pulse^{-1}. Both spectra are normalized. Note that the normalized spectra coincide for λ > ca. 380 nm. B: Transient absorption at 440 nm (absorbance) due to the triplet of **1** and time-resolved trace of the delayed fluorescence emitted from the sample at 440 nm (emission) in N_2-equilibrated solution. The fluorescence data were acquired with the monochromator-PMT detector of the flash photolysis setup and inverted for easier comparison. Same sample and conditions as for the delayed fluorescence spectrum in A. The inset shows the transient absorption at 440 nm on a shorter time scale under otherwise identical conditions.

spectra contain two bands: in addition to the band at λ_{em} = 440 nm, an emission at λ_{em} = 330 nm is observed.[2,3,62−65]



1a **1b**

In the literature, [2,3,62−65] this latter emission has been ascribed to the fluorescence of rotamer **1b**[66] of **1** that cannot tautomerize upon electronic excitation to S_1. The delayed fluorescence spectrum coincides with the long-wavelength band of the prompt (tautomer) fluorescence spectrum of **1**. This indicates that the delayed fluorescence is emitted from the tautomer structure **1′** (according to eqs 9 and 10).

In the case of **8**, the interpretation is less straightforward. For samples containing toluene and **8** in degassed cyclohexane, a unique delayed fluorescence spectrum is observed in a time window from 1.7 to 91.7 μs after the laser pulse (cf. Figure

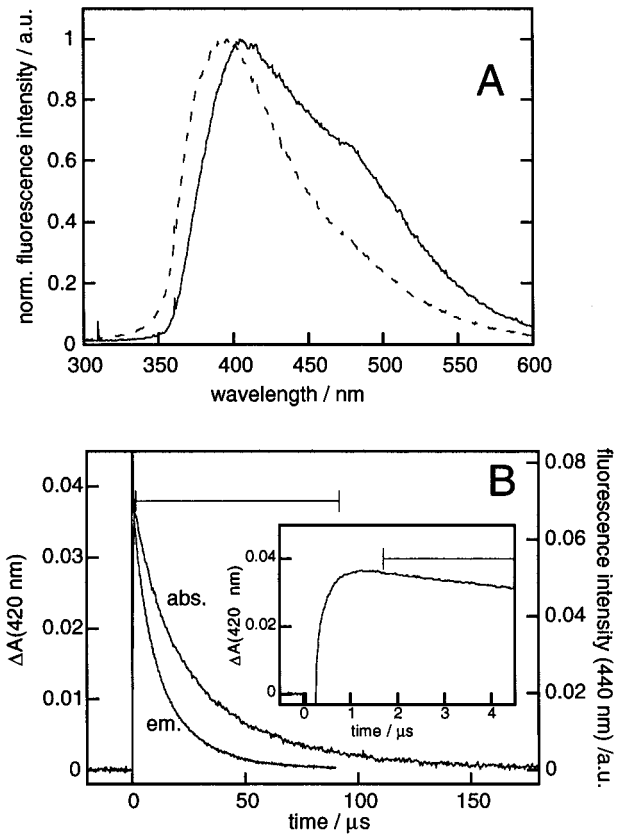
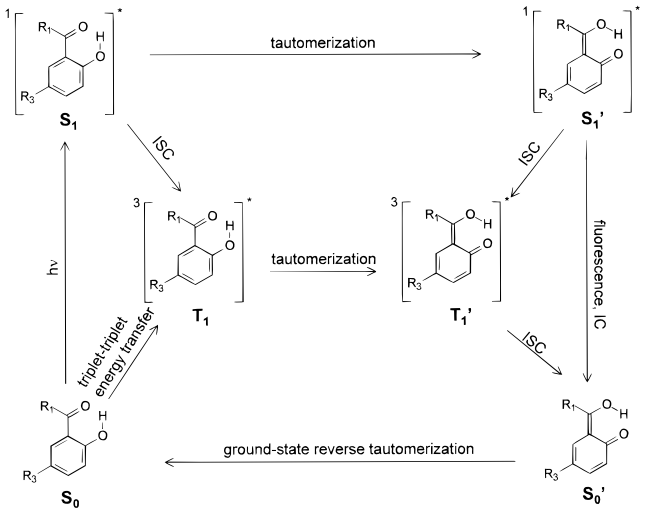


Figure 5. Delayed fluorescence from triplet states of **8**. A: Time-integrated delayed fluorescence spectrum (solid line) of a solution containing toluene and ca. 440 μM **8** in deaerated cyclohexane integrated over the time interval (1.7−91.7 μs) after the laser pulse. The absorbance at 266 nm was ca. 0.9. The dashed line depicts a prompt fluorescence spectrum from an air-equilibrated solution containing ca. 440 μM **8** alone, excited at 266 nm with a laser fluence of ca. 4 mJ cm^{-2} pulse^{-1}. Both spectra are normalized. B: Transient absorption at 420 nm (absorbance) due to the triplet of **8** and time-resolved trace of the delayed fluorescence emitted from the sample at 440 nm (emission) in N_2-equilibrated solution. Same sample and conditions as for the delayed fluorescence spectrum in A. The inset shows the transient absorption at 420 nm on a shorter time scale under otherwise identical conditions. For details, see text and Figure 4.

5A). In control experiments analogous to those described for **1**, the delayed fluorescence is absent (data not shown). The kinetic traces for the triplet−triplet-absorption and the time-resolved delayed fluorescence are shown in Figure 5B, respectively. The spectrally and temporally integrated signal of the delayed fluorescence was found to be proportional to the square of the absorbed pump light energy[61] and both the fluorescence and the triplet−triplet-absorption were found to decay by a mixture of first- and second-order processes. The delayed and prompt fluorescence spectra of **8** exhibit striking differences (cf. Figure 5A): the emission spectrum of the delayed fluorescence (λ_{max} = 405 nm) is red-shifted relative to the prompt fluorescence (λ_{max} = 396 nm)[67] of the nontautomerized singlet and has a different profile. The delayed fluorescence is, therefore, not emitted from the same species as the prompt fluorescence. The discussion section of this paper considers several candidates for the species emitting the delayed fluorescence.

Note that delayed fluorescence with similar kinetic behavior was also observed from cyclohexane solutions containing toluene and the carboxylic acids **2** or **7**, respectively.

SCHEME 1



Long-Lived Species. The transient absorption spectra of the *ortho*-hydroxy-SAD with an electron-donating 5-methoxy (**7**, **8**) or 5-hydroxy-substituent (**10**, **11**) further exhibit a similar feature with absorption maxima at 300, 370, and 430 nm. The corresponding species is observed in air- and nitrogen-equilibrated samples and decays with a lifetime of several hundred microseconds. In the case of **7**, its spectrum corresponds to the last time point in the transient absorption spectrum in Figure 2A. The amplitude of this species is proportional to the laser pulse energy and its lifetime does not depend on the ground-state concentration.

This species was not observed for compounds **1** and **2**. However, in the transient absorption spectra of **12**,[54] **13**,[54] **14**, and **15** (this work, data not shown) long-lived transients with similar characteristics are observed. This species is assigned to the phototautomer S_0' formed after relaxation of the singlet or triplet tautomer. It is longer lived in water at pH = 1 [68] and in acetonitrile than in benzene. Its lifetime presumably corresponds to a thermal reverse hydrogen atom transfer to yield the more stable ground-state tautomer S_0. A similar solvent-dependent stabilization of phototautomer structures has been reported for *ortho*-methylacetophenone.[69]

Discussion

In this study, we investigated the reactive intermediates of salicylic acid and its derivatives by transient absorption and fluorescence spectroscopy in solution. In particular, we found evidence for the formation of triplet phototautomers, in which the *ortho*-hydroxy hydrogen has been transferred to the carbonyl oxygen. Triplet−triplet absorption spectra of the *ortho*-hydroxy compounds **1**, **2**, and **7** differ appreciably from those of the corresponding *ortho*-methoxy compounds **3**, **4**, and **9**, respectively. The difference between these latter spectra and those of **1**, **2**, and **7** is, therefore, evidence for the tautomeric structure T_1' of the triplet states of the *ortho*-hydroxy derivatives **1**, **2**, **7**, **8**, **10**, and **11**. For the *ortho*-methoxy compounds **3**, **4**, and **9**, the intramolecular hydrogen atom transfer to give tautomers S_1' or T_1' (cf. Scheme 1) is not possible and their triplet−triplet absorption spectra are assigned as those of the nontautomerized triplet state T_1.

In the fluorescence spectra of compounds **1** and **2** evidence for an equilibrium between two different rotamers (**1a**, **1b**) of the ground-state had been found.[2,3,62−65] The kinetics of the triplet decay can be fitted with the same monoexponential decay

for all probe wavelengths. Therefore, the existence of two (kinetically) distinct long-lived triplet states can be ruled out. A fast rotational equilibrium between **1a** and **1b**, however, may not be observable with the time resolution of our experiment. Tautomerization **1** → **1′** would only occur from rotamer **1a**, replacing the single bond in **1a** with a double bond in **1a′**, whereas the triplet state of rotamer **1b** would remain free to rotate, rapidly depopulating a presumed triplet state of rotamer **1b** by sequential rotation and tautomerization (**1b** → **1a** → **1a′**). However, the time resolution of our experiment is insufficient to elucidate this further.

In a gated fluorescence spectroscopy experiment on solutions of methyl salicylate, **1**, the spectrum of the delayed fluorescence emitted after triplet−triplet annihilation was found to coincide with that of the known tautomer fluorescence $S_1' \rightarrow S_0'$ (emitted only from the S_1' state of rotamer **1a**). Fluorescence from the first excited singlet state of **1b**, readily observed in the prompt fluorescence spectrum, is absent in the delayed fluorescence spectrum (cf. Figure 4A). The excimer formed in the collision of two triplet states of **1** (cf. eq 9), therefore, dissociates into an excited singlet state of **1** and a ground-state molecule (cf. eq 10). From the exact coincidence of the long-wavelength band of the prompt and delayed fluorescence emission spectra, it can be concluded that the excited singlet state populated in this reaction is in the tautomer form S_1' (the excited state of rotamer **1a**). It appears likely that the triplet state giving rise to the delayed fluorescence is in the same rotameric and tautomeric configuration as the singlet state formed from it in reactions 9 and 10.

The delayed fluorescence spectrum of methyl 2-hydroxy-5-methoxybenzoate, **8**, was found to differ more dramatically from the prompt fluorescence (of the nontautomerized singlet) and could not be unequivocally assigned. The red-shifted fluorescence emission band of **8** had not been observed in several fluorescence studies of this and similar SADs with an electron-donating 5-substituent.[25−27] Within the framework of reactions 6−12 suggested above, alternative explanations can be found for this behavior. Two molecules of **8** in the first excited triplet state form an excited collision complex[58] and, upon its dissociation, the fluorescence of one of the partners of the collision complex is observed. If the triplet is in the tautomeric form T_1', as supported by other data (vide supra), this might render an excited tautomer singlet state S_1' accessible, thus explaining the red-shifted fluorescence emission spectrum (similar to the case of compound **1**, where the delayed fluorescence clearly only consists of tautomer fluorescence). Alternatively, the delayed fluorescence may be dissociative excimer fluorescence, according to eq 11. Further work will be needed to discern these possibilities. It is, however, clear that in the collision of two excited triplet states of **8**, a new singlet surface becomes accessible. The significant red-shift of the emission indicates that this latter singlet state has lower energy than that populated by direct excitation.

The successful use of photosensitization to generate the triplet phototautomers reveals a previously unknown alternative tautomerization pathway entirely on the triplet surface ($T_1 \rightarrow T_1'$) for the *ortho*-hydroxy SAD studied here. This triplet tautomerization is independent of the occurrence of intramolecular hydrogen atom transfer in the singlet state, which has been reported for **1** and **2**,[2−4,6] but not for **7**,[26] **8**,[27] and **10**.[25] Similar triplet intramolecular hydrogen atom transfer reactions are well-known to occur for various *ortho*-alkyl ketones.[69−73] A comparison of the relative reactivity of excited singlet and triplet states of acetone toward intermolecular hydrogen atom abstraction has been published by Nau et al.[74]

The transient absorption spectra reveal the formation of a phototautomer of the 5-hydroxy- or 5-methoxy-substituted compounds **7**, **8**, **10**, and **11**, corroborating the tautomerization in the excited state. For the unsubstituted compounds **2** and **1**, no phototautomer was observed and it is suspected that the phototautomers of the latter compounds are much less stable and shorter-lived than those of the 5-substituted compounds and, therefore, not observed on the time scale of our experiment. This can be rationalized in terms of the electron density at the enolic hydroxy-group that is formed by the hydrogen atom transfer to the carbonyl oxygen being higher for the compounds **7**, **8**, **10**, and **11** with an electron-donating 5-substituent (which remains conjugated to the carbonyl oxygen even in the tautomer) than for **1** and **2**. Therefore, the reverse hydrogen atom transfer $S_0' \rightarrow S_0$ is less favored and hence slower for these compounds than for the unsubstituted compounds **1** and **2**.

No difference between the triplet−triplet absorption spectrum of the acid vs that of the corresponding methyl ester was observed for any of the compounds studied, showing that carboxyl dimer formation in aprotic solvents does not influence the formation of the observed triplet state, which we assigned as tautomer triplet T_1'. This is of importance for an extrapolation of these results to the reactions in the microcrystalline MALDI sample, where the solvent is absent, but carboxyl dimers form a central element of the crystal structure.[34−36]

The results presented in this current communication together with the published literature lead us to propose the following scheme for the role of excited states in the phototautomerization of SAD (cf. Scheme 1).

In the case of **2**, excitation of the $S_0 \rightarrow S_1$ transition is followed by a rapid[3] hydrogen atom transfer $S_1 \rightarrow S_1'$ in the first excited singlet state. This S_1' tautomer of the singlet state gives rise to the well-known tautomer fluorescence $S_1' \rightarrow S_0'$. Intersystem crossing $S_1' \rightarrow T_1'$ occurs from the excited singlet tautomer S_1'. In the case of **1**, this sequence of tautomerization and intersystem crossing has previously been proposed based on time-resolved electron paramagnetic resonance (EPR) data.[56] A comparison of the relative time scales of tautomerization $S_1 \rightarrow S_1'$(<60 fs)[3] and decay of the singlet tautomer S_1' (<1 ns)[4] also supports this sequence.

Transient absorption spectra of triplet tautomers T_1' have been reported for several *ortho*-hydroxybenzoyl compounds, such as **12**, **13**,[54] and 7-hydroxy-1-indanone.[48] The triplet−triplet absorption spectra of these tautomers[48,54] are similar to the spectra of the *ortho*-hydroxy SAD triplets observed here. A weak long-wavelength tail in the triplet−triplet absorption spectrum of 7-hydroxy-1-indanone[75] is also in agreement with the observations in this report, as is the observation of delayed tautomer fluorescence of 7-hydroxy-1-indanone. Chou et al. observed the delayed fluorescence as background in a two-step laser-induced fluorescence experiment while investigating the fluorescence emitted after reverse intersystem crossing.[48]

Phototautomers with absorption spectra similar to those of **7**, **8**, **10**, and **11** have previously been observed for other *ortho*-hydroxybenzoyl compounds, such as **12** and **13**,[54] and for *ortho*-methyl acetophenone.[76]

Conclusions

This study focused on the solution photophysics of SADs, an important class of molecules among the various aromatic *ortho*-hydroxycarbonyl compounds used as MALDI matrices. Evidence for a reversible intramolecular rearrangement in the

excited state in polar and apolar aprotic solvents is presented. Although the phototautomerization of unsubstituted methyl salicylate or salicylic acid in the first excited singlet state is well established, we present new results indicating the formation of triplet tautomers of these compounds as well as their respective 5-hydroxy- and 5-methoxy-substituted derivatives. These tautomeric triplet states were found to be populated by either intersystem crossing from the first excited singlet state or by triplet−triplet energy transfer. Using the latter approach, we demonstrated that tautomerization can also occur on the triplet state surface, without the involvement of excited singlet states of the SAD.

The phototautomers discussed here may act as intermediates in the reactions leading to analyte ionization and should be taken into account in a description of photochemical and photophysical processes relevant for MALDI. Work currently in progress explores the role of excited-state tautomers in MALDI, in particular their reactivity toward intermolecular electron, proton, and hydrogen atom transfer as well as an extension of these studies to crystalline samples.

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(45) E_T(acetone) = 332 kJ mol^{-1}, E_T(toluene) = 347 kJ mol^{-1} (see ref 43).
(46) In a number of reports, complex formation between **2** (Joshi, H. C.; Tripathi, H. B.; Pant, T. C.; Pant, D. D. *Chem. Phys. Lett.* **1990**, *173*, 83−86), **5** (see ref 26), and **7** (see ref 26) and diethyl ether has been observed and was found to significantly change the photophysical behavior due to hydrogen bonding between diethyl ether and the SAD. To rule out a similar effect in the presence of acetone, the comparison with toluene was carried out, where this hydrogen bonding cannot occur. The similarity of the spectra showed that complex formation with acetone is not relevant here (see also ref 57).
(47) This shoulder is not observed upon direct excitation, where the transient absorbance change due to triplet−triplet absorption is much lower (low quantum yield of intersystem crossing, cf. Table 1). The signal-to-noise ratio in the setup used does not permit the observation of ΔA values this low.
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(52) Although not unequivocally identified, it is assumed that the primary species formed is the triplet state while other species observed correspond to its reaction products. This is supported by the absence of these signals in aerated solution, where the triplet state is quenched by oxygen.
(53) No signal was observed after sensitization with acetone or toluene (see ref 45). The high absorption of **3** and **4** at 266 nm (ϵ_{GS} = ca. 8000

M^{-1} cm^{-1}) precluded the use of concentrations high enough to intercept a significant fraction of the short-lived toluene triplets.

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(57) Acetone could not be used for these experiments, because acetone-delayed fluorescence was observed between 350 and 450 nm even in solutions containing only acetone (for a fluorescence spectrum of acetone, see e.g. ref 74). ^{3}(Acetone)* also efficiently abstracts hydrogen from cyclohexane and could only be used in acetonitrile.

(58) ^{1,3,5}(SAD·SAD)* symbolizes the general excited dimeric collision complex of two excited triplet SAD molecules. The theoretically possible spin multiplicities of the complex are 1, 3, and 5 (see ref 59). Only the dissociation of the singlet complex ^{1}(SAD·SAD)* can give rise to a fluorescent excited singlet ^{1}SAD*.

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(61) In these experiments, the laser fluence and the concentration of **1** or **8** were kept constant, but the concentration of toluene and thus the absorbance A at 266 nm was varied ($0.05 < A(266\ \text{nm}) < 1$). Then, $(1-10^{-A(266\ \text{nm})})$ is proportional to the absorbed energy. Log−log-plots of $(1-10^{-A(266\ \text{nm})})$ vs the spectrally and temporally integrated intensity of the delayed fluorescence had a slope of 1.9 for both compounds, corroborating the postulated second-order process.

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(67) This compares with $\lambda_{max} = 395$ nm in the spectrum in ref 27. Note that the fluorescence emission data in the present work are not corrected for the spectral response of the instrumentation.

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