Excited State Interactions in Flurbiprofen-Tryptophan Dyads

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Abstract

Fluorescence and laser flash photolysis measurements have been performed on two pairs of diastereomeric dyads containing the nonsteroidal anti-inflammatory drug *(S)*- or *(R)*-flurbiprofen (FBP) and *(S)*-tryptophan (Trp), a relevant amino acid present in site I of human serum albumin. The fluorescence spectra were obtained upon excitation at 266 nm, where nearly 60% of the light is absorbed by FBP and 40% by Trp; the most remarkable feature observed in all dyads was a dramatic fluorescence quenching, and the residual emission was assigned to the Trp chromophore. Besides, an exciplex emission was observed as a broad band between 380-500 nm, specially in the case of the *(R,S)*- diastereomers. The fluorescence lifetimes (τ_F) at λ_{em} = 340 nm were clearly shorter in the dyads than in Trp-derived model compounds; by contrast, the values of τ_F at λ_{em} = 440 nm (exciplex) were much longer. On the other hand, the typical FBP triplet-triplet transient absorption spectrum was obtained upon laser flash photolysis, although the

signals were less intense than when FBP was directly excited under the same conditions. The main photophysical events in FBP-Trp dyads can be summarized as follows: 1) most of the energy provided by the incident radiation at 266 nm reaches the excited singlet state of Trp (^{1}Trp*), either *via* direct absorption by this chromophore or by singlet singlet energy transfer from excited FBP (^{1}FBP*); 2) a minor, yet stereoselective deactivation of ^{1}FBP* leads to detectable exciplexes and/or radical ion pairs; 3) the main process observed is intramolecular ^{1}Trp* quenching and 4) the first triplet excited state of FBP can be populated by triplet-triplet energy transfer from excited Trp or by back electron transfer within the charge separated states.

Keywords: Electron transfer, Exciplex, Fluorescence, Laser flash photolysis, Stereodifferentiation.

Introduction

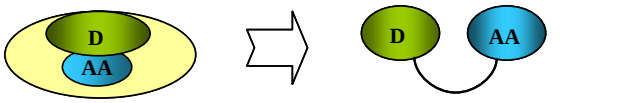
The biological role of proteins frequently involves selective binding to relevant substrates. This is the case of transport proteins that upon binding provide a carrier system for xenobiotics, either in the plasma or across membranes. The most important soluble carrier of drugs in blood is human serum albumin (HSA),[1] a globular protein which accounts for about 60% of the total protein in blood serum. It contains 585 amino acids, in a sequence displaying a total of 17 disulphide bridges, one free thiol and a single tryptophan (Trp) unit.[2-5] The exceptional binding capacity of HSA for a wide range of endogenous and exogenous ligands, together with its abundance, are determining for the pharmacokinetic behavior of drugs. Thus, a detailed knowledge of drug-HSA interactions is essential for the rational design of pharmaceuticals. The pioneering work performed by Sudlow and coworkers three decades ago revealed that there are two major and structurally selective binding sites in HSA, namely site I and site II.[6,7] Although more recent studies have found evidence supporting the existence of several subdomains, consensus still exists that there are basically two high affinity binding sites for small heterocyclic or aromatic compounds in this protein. Site I is also known as the warfarin binding site and contains the only Trp unit, while site II is known as the benzodiazepine binding site and contains Tyr residues.[8,9]

Nonsteroidal anti-inflammatory drugs (NSAIDs) are among the most widely used therapeutic agents. They are prescribed for the treatment of a broad spectrum of pathophysiological conditions including headache, pain caused by degenerative diseases (such as osteo and rheumatoid arthritis) and fever.[10] The main group of NSAIDs are the 2-arylpropionic acid derivatives, such as ibuprofen, naproxen, ketoprofen or flurbiprofen; although they are currently marketed as racemic mixtures (with few exceptions such as naproxen), their anti-inflammatory activity is mainly attributed to the *(S)*-enantiomer.[11] Such drugs bind preferentially (and often stereoselectively) to site II of HSA by means of hydrogen bonding and electrostatic interactions. However, site I is also populated to some extent.[12]

Fluorescence techniques have been employed in the past to gain insight into the interactions between drugs and proteins,[13] with special attention to transport proteins (for example HSA). An interesting feature of intrinsic protein fluorescence is the high sensitivity of tryptophan to its local environment. Thus, changes in Trp emission spectra can be observed in response to protein conformational transitions, ligand binding or subunit association; all these factors can affect the local environment of the indole chromophore. Moreover, Trp is sensitive to collisional quenching, probably due to a tendency of the excited state of indole to act as electron-donor.[14]

Among the NSAIDs, the lowest singlet and triplet excited states of flurbiprofen (FBP) have recently been characterized, and the fluorescence and triplet lifetimes (τ_F and τ_T) have shown to be highly sensitive to the medium.[15] In previous work, we have used FBP triplet excited states to study drug distribution among the solution and the different HSA binding sites.[16,17] On the other hand, the fluorescence behavior of HSA/racemic FBP systems has also been reported.[18]

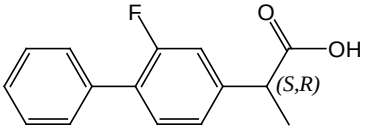
The aim of the present work has been to undertake a systematic fluorescence and laser flash photolysis study on several model dyads containing the chromophores of FBP and Trp, the relevant amino acid present in site I of HSA. This should give information about the operating mechanisms in the excited state interactions using chemically well-defined systems, where the non-covalent supramolecular drug-protein interactions are modeled by the analogous intramolecular processes in covalently linked dyads (Scheme 1and Chart 1). Moreover, a comparison between the behavior of the diastereomeric dyads synthesized from the two FBP enantiomers and the *(S)*-amino acid would provide valuable indications related to the stereodifferentiating interactions with the transport proteins.



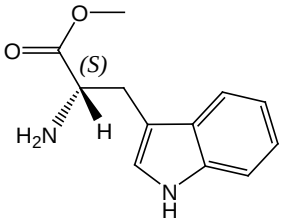
Biological system: non covalent complex drug (D)-amino acid (AA)

Model: covalently bonded dyad

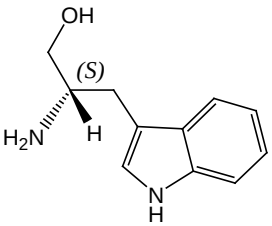
Scheme 1



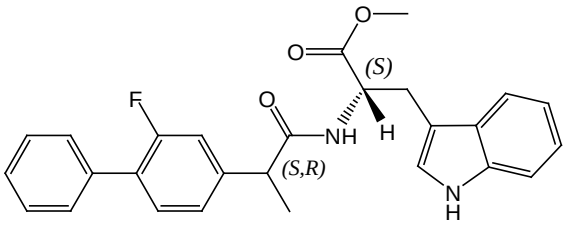
(S)- or *(R)*- Flurbiprofen



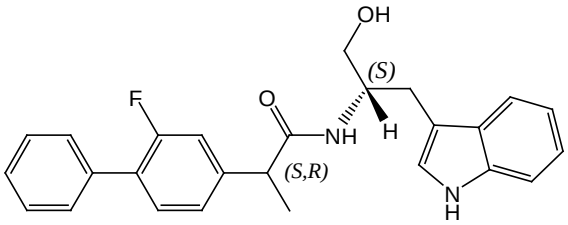
(S)-Tryptophan methyl ester



(S)-Tryptophanol



***(S,S)*-1**
***(R,S)*-1**



***(S,S)*-2**
***(R,S)*-2**

Chart 1

Experimental Section

Materials and solvents. *(S)*- and *(R)*-Flurbiprofen, *(S)*-tryptophan methyl ester hydrochloride, *(S)*-tryptophanol, EDC (1-(3-dimethylaminopropyl)-N-ethylcarbodiimide hydrochloride) and BtOH (1-hydroxybenzotriazole) were commercially available. Their purity was checked by ^{1}H NMR and HPLC analysis. The reagent grade solvents (methanol, dioxane, ethyl acetate, acetonitrile) were used without further purification. Solutions of PBS 0.01 M (pH = 7.4) were prepared by dissolving phosphate buffered saline tablets in deionized water.

General. IR Spectra were obtained with a FTIR instrument; ν_{max} (cm^{-1}) is given for the main absorption bands. ^{1}H NMR Spectra were measured in $CDCl_3$ (or, when indicated, in CD_3OD) using a 300 MHz apparatus; chemical shifts are reported in δ (ppm) values, using TMS as internal standard. Combustion analyses were performed at the Instituto de Química Bio-Orgánica of the CSIC in Barcelona. Isolation and purification were done by preparative layer chromatography on silica gel Merck, using hexane/ethyl acetate as eluent.

Synthesis of the substrates. To a solution of 0.8 mmol of (*S)*- or (*R)*- flurbiprofen, in acetonitrile (20 mL), 0.8 mmol of EDC and 0.8 mmol of BtOH were added as solids. The mixture was maintained under stirring, and then 0.8 mmol of *(S)*-TrpMe or *(S)*-TrpOH in 2 mL

of acetonitrile were added. After three hours the solvent was removed under vacuum; the crude solid was dissolved in methylene chloride, washed consecutively with diluted $NaHCO_3$, 1 M HCl and brine. Final purification was done by preparative layer chromatography followed by recrystallization.

Fluorescence measurements. Emission spectra were recorded on a spectrofluorometer system, provided with a monochromator in the wavelength range 200-900 nm. The solutions were placed into 10 x 10 mm^2 quartz cells with a septum cap and were purged with nitrogen for at least 15 min before the measurements. The absorbance of the samples at the excitation wavelength was kept lower than 0.1. For time resolved fluorescence decay measurements, the conventional single photon counting technique was used. All the experiments were carried out at room temperature (22 ºC).

Laser flash photolysis experiments. Laser flash photolysis experiments were performed by using a Q-switched Nd:YAG laser (266 nm, 4 mJ per pulse, 5 ns fwhm) coupled to a mLFP-miniaturized equipment. All transient spectra were recorded employing 10 x 10 mm^2 quartz cells with 4 mL capacity and were bubbled during 30 min with N_2 before acquisition. The absorbance of the samples was 0.2 at the laser wavelength. All the experiments were carried out at room temperature.

N-[2-(S)-(2-Fluoro[1,1'-biphenyl]-4-yl)-propanoyl]-(S)-tryptophan methyl ester, (S,S)-1; 73 %; white crystals; UV λ_{max} (log ε): 222 (4.5), 248 (4.2), 280 sh (3.9), 290 sh (3.7); FTIR ν: 3408, 3059, 2976, 2951, 2922, 1736, 1653, 1543, 1282, 1219, 744, 698. ^{1}H NMR δ: 8.02 (s, 1H), 7.54-6.99 (m, 12H), 6.83 (d, 1H, *J* = 2.4 Hz), 5.95 (d, 1H, *J* = 7.8 Hz), 4.93-4.87 (m, 1H), 3.67 (s, 3H), 3.51 (q, 1H, *J* = 7.2 Hz), 3.38-3.24 (m, 2H), 1.51 (d, 3H, *J* = 7.2 Hz); ^{13}C NMR δ: 173.4, 172.4, 161.6, 158.3, 142.5, 142.4, 136.3, 135.7, 131.2, 129.2, 128.8, 128.0, 127.9, 123.9, 122.8, 122.6, 120.0, 118.8, 115.8, 115.4, 111.6, 110.2, 53.3, 52.6, 46.8, 27.6, 18.5; Anal. Calcd. for $C_{27}H_{25}FN_2O_3$: C 72.96; H 5.67; N 6.30. Found: C 72.62; H 5.73; N 6.10.

N-[2-(R)-(2-Fluoro[1,1'-biphenyl]-4-yl)-1-propanoyl]-(S)-tryptophan methyl ester, (R,S)-1 : 75 %; white crystals; UV λ_{max} (log ε): 222 (4.5), 248 (4.2), 280 sh (3.9), 290 sh (3.7); FTIR ν: 3406, 3055, 3002, 2976, 2952, 2935, 1722, 1651, 1523, 1211, 764, 737, 700. ^{1}H NMR δ: 7.95 (s, 1H), 7.58-7.01 (m, 12H), 6.62 (d, 1H, *J* = 2.1 Hz), 5.96 (d, 1H, *J* = 7.8 Hz), 4.98-4.92 (m, 1H), 3.70 (s, 3H), 3.51 (q, 1H, *J* = 7.2 Hz), 3.31-3.18 (m, 2H), 1.48 (d, 3H, *J* = 7.2 Hz). ^{13}C NMR δ: 172.8, 172.3, 161.3, 158.0, 142.6, 142.5, 136.0, 135.4, 130.9, 129.0, 128.9, 128.5, 127.7, 123.6, 122.6, 122.3, 119.7, 118.4, 115.5, 115.2, 111.2, 109.7, 52.7, 52.4, 46.5, 27.4, 18.4; Anal. Calcd. for $C_{27}H_{25}FN_2O_3$: C 72.96; H 5.67; N 6.30. Found: C 72.60; H 5.70; N 6.13.

N-[2-(S)-(2-Fluoro[1,1'-biphenyl]-4-yl)-propanoyl]-(S)-tryptophanol, (S,S)-2: 75 %; white crystals; UV λ_{max} (log ε): 223 (4.4), 248 (4.1), 280 sh (3.8), 290 sh (3.6); FTIR ν: 3411, 1645, 1556, 1457, 1414, 742, 698. ^{1}H NMR (CD_3OD) δ: 7.64-6.97 (m, 13H), 4.25-4.16 (m, 1H), 3.62 (q, 1H, *J* = 7.2 Hz), 3.53 (d, 2H, *J* = 5.4 Hz), 3.07 (dd, 1H, J_1 = 14.5 Hz, J_2 = 6.0 Hz), 2.92 (dd, 1H, J_1 = 14.5 Hz, J_2 = 6.9 Hz), 1.34 (d, 3H, *J* = 7.2 Hz). ^{13}C NMR δ: 175.2, 161.4, 158.1, 143.7, 143.6, 137.0, 135.9, 130.6, 130.4, 130.5, 128.8, 128.3, 127.5, 123.6, 122.9, 121.2, 118.5, 118.4, 115.1, 111.2, 111.1, 63.2, 52.5, 45.9, 26.5, 17.7; Anal. Calcd. for $C_{27}H_{25}FN_2O_3$: C 72.96; H 5.67; N 6.30. Found: C 72.62; H 5.73; N 6.10.

N-[2-(R)-(2-Fluoro[1,1'-biphenyl]-4-yl)-propanoyl]-(S)-tryptophanol, (R,S)-2: 73 %; white crystals; UV λ_{max} (log ε): 223 (4.4), 248 (4.1), 280 sh (3.8), 290 sh (3.6); FTIR ν: 3367, 3051, 1633, 1529, 1485, 1419, 766, 741, 702. ^{1}H NMR (CD_3OD) δ: 7.53-6.89 (m, 13H), 4.29-4.21 (m, 1H), 3.67-3.53 (m, 3H), 2.97 (dd, 1H, J_1 = 15.0 Hz, J_2 = 6.3 Hz), 2.85 (dd, 1H, J_1 = 15.0 Hz, J_2 = 7.8 Hz), 1.42 (d, 3H, *J* = 7.2 Hz); ^{13}C NMR δ: 176.6, 162.9, 159.6, 145.1, 145.0, 138.4, 137.4, 132.1, 132.0, 130.4, 129.8, 129.4, 129.0, 125.0, 124.5, 122.6, 119.9, 119.8, 116.4, 116.1, 112.5, 64.9, 53.8, 47.3, 27.9, 19.1; Anal. Calcd. for $C_{27}H_{25}FN_2O_3$: C 72.96; H 5.67; N 6.30. Found: C 72.62; H 5.73; N 6.10.

Results and Discussion

Fluorescence measurements on FBP/HSA supramolecular systems

In the absence of protein, the fluorescence spectra of *(S)*- or *(R)*- FBP in phosphate buffer (PBS)/air at λ_{exc} = 266 nm were expectedly identical (λ_{max} = 310 nm);[15] they are shown in Figure 1A. The emission of HSA alone was also recorded; it displayed the typical maximum due to the Trp residue (λ_{max} = 340 nm).[14] As shown in Figure 1A, the spectra recorded for equimolar mixtures of *(S)*- or *(R)*- FBP and HSA were less intense and contained the contributions of both drug and protein (Trp) emissions, although the latter was clearly predominating. The dual fluorescence from two distinct excited species is in good agreement with the fact that the decays measured at 310 nm (maximum of FBP emission) and 340 nm (due to the Trp chromophore of HSA) are clearly different, as it can be seen in Figure 1B.

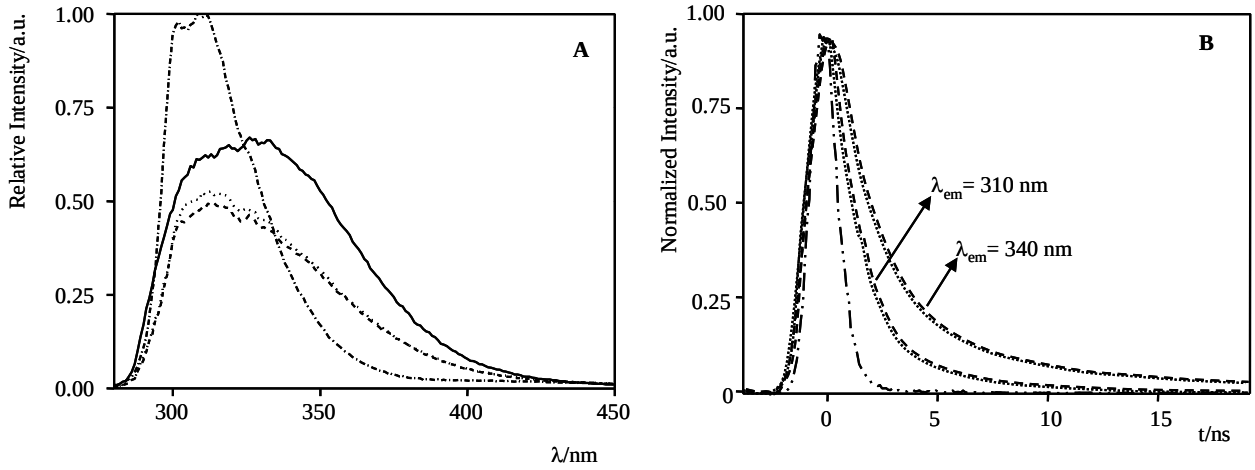


Figure 1 A. Fluorescence emission (λ_{exc}= 266 nm) of (*S*)- or *(R)*- FBP (-·-·), HSA (——), (*S*)-FBP/HSA 1:1 (- - -) and (*R*)-FBP/HSA 1:1 (·····) in 0.01 M PBS. **B.** Decay of (*S*)-FBP/HSA 1:1 (- - -) and (*R*)-FBP/HSA 1:1 (·····) in 0.01 M PBS at λ_{em} = 310 and 340 nm, respectively. Lamp emission (—··—). FBP and HSA concentration were $1.0 \cdot 10^{-5}$ M.

Photophysical studies on FBP-Trp dyads in acetonitrile

In order to obtain further information on the excited state interactions between FBP and Trp, several model dyads containing *(S)*- or *(R)*-FBP and the main fluorophore of HSA (Trp) were synthesized by conventional methods and studied by fluorescence (steady-state and time resolved) and laser flash photolysis. In these well defined systems, the two chromophores are covalently linked through an amide bridge. The chemical structures of the parent compounds, together with those of the dyads are presented in Chart 1. Due to their poor solubility in aqueous media, the experiments were carried out in acetonitrile.

The first selected dyads were *(S,S)*-**1** and *(R,S)*-**1**, two diastereomeric amides obtained by condensation of *(S)*- or *(R)*- flurbiprofen and *(S)*-tryptophan methyl ester (TrpMe). Their UV-absorption spectra are shown in Figure 2; they were identical to the added spectra of the corresponding isolated FBP and Trp units, at the same concentrations. This reveals the absence of any significant ground-state intramolecular interactions between the two moieties.

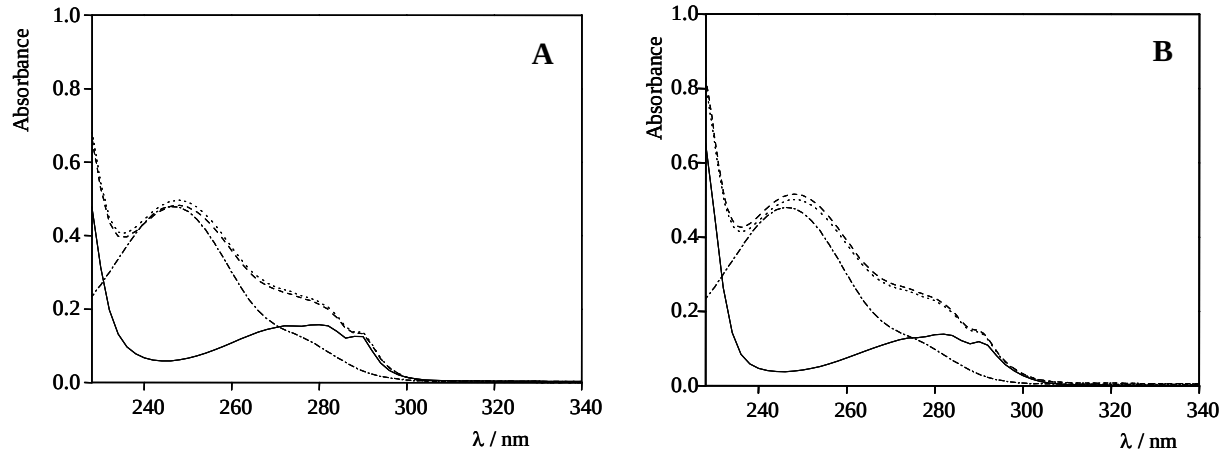


Figure 2 A. UV-absorption spectra of *(S)*-FBP (-·--·), *(S)*-TrpMe (——), *(S,S)*-**1** (- - -) and *(R,S)*-**1** (·····) in acetonitrile at 2.5 10^{-5} M concentration. **B** UV-absorption spectra of *(S)*-FBP (-·--·), *(S)*-TrpOH (——), *(S,S)*-**2** (- - -) and *(R,S)*-**2** (·····) in acetonitrile at 2.5 10^{-5} M concentration.

The fluorescence spectra of *(S,S)*-**1** and *(R,S)*-**1** were obtained upon excitation at 266 nm, where nearly 60 % of the light is absorbed by the biphenyl and 40 % by the indole chromophore,

using isoabsorptive solutions (A_{266} = 0.08) of *(S)*-FBP and *(S)*-TrpMe as models for comparison. The most remarkable feature observed in both dyads was a dramatic quenching (see Figure 3A); the residual emission was assigned to the Trp chromophore (λ_{em}= 340 nm)[14] rather than to FBP. Moreover, a possible exciplex emission was observed as a broad band between 380-500 nm (Figure 3B), specially in the case of *(R,S)*-**1**. The fluorescence lifetimes at λ_{em} = 340 nm were clearly shorter in the dyads (*ca.* 0.9 ns) than in *(S)*-TrpMe (6.4 ns), indicating a dynamic quenching. By contrast, the values of τ_F at λ_{em} = 440 nm were much longer (7.6 and 5.1 ns for *(S,S)*-**1** and *(R,S)*-**1**, respectively).

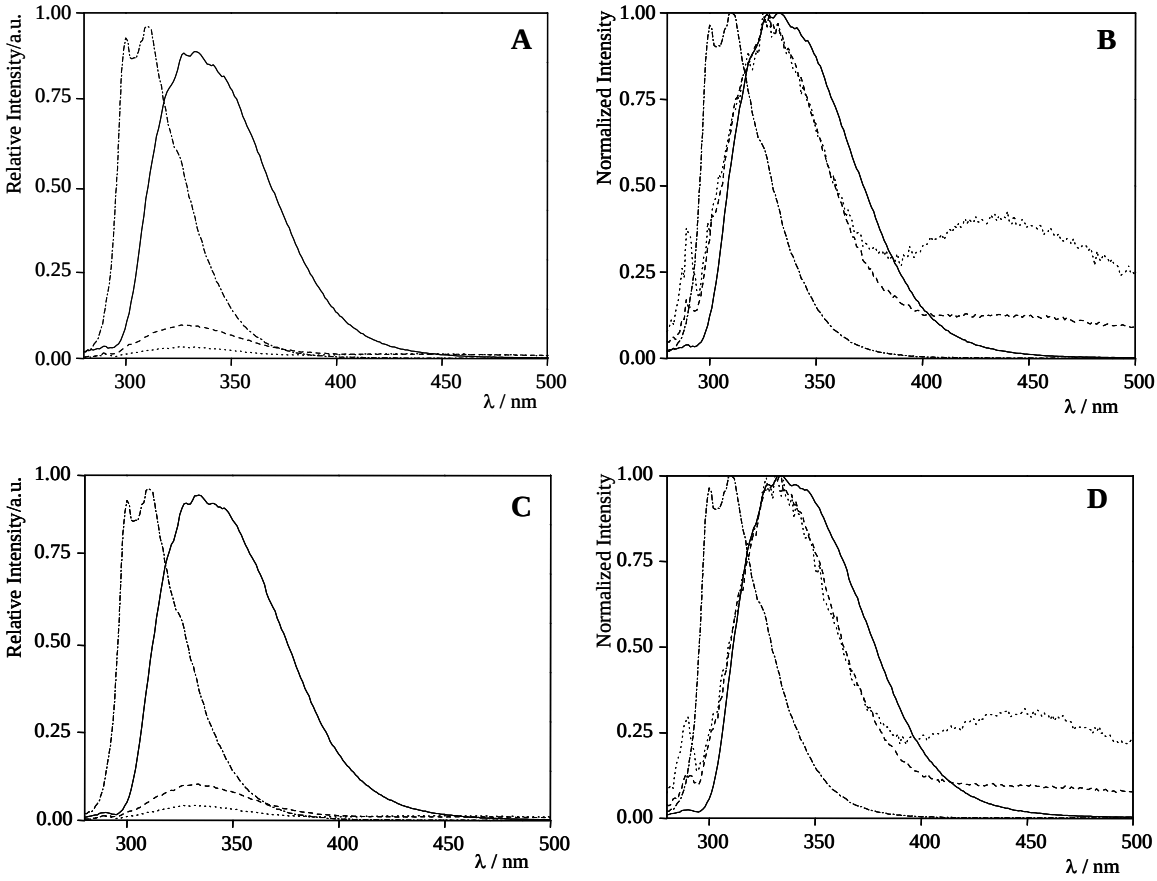


Figure 3. A Fluorescence spectra of *(S)*-FBP (- · -), *(S)*-TrpMe (——), *(S,S)*-**1** (- - -) and *(R,S)*-**1** (·····). **B** Normalized fluorescence spectra of *(S)*-FBP (- · - ·), *(S)*-TrpMe (——), (*S,S*)-**1** (- - -) and (*R,S*)-**1** (·····). **C** Fluorescence spectra of *(S)*-FBP (- · -), *(S)*-TrpOH (——), *(S,S)*-**2** (- - -) and *(R,S)*-**2** (·····). **D** Normalized fluorescence spectra of *(S)*-FBP (- · - ·), *(S)*-TrpOH (——), (*S,S*)-**2** (- - -) and (*R,S*)-**2** (·····). All the experiments were carried out in deaerated acetonitrile, at 1.0· 10^{-5} M concentration, using 266 nm as the excitation wavelength.

On the other hand, the typical FBP T-T transient absorption spectrum was obtained upon LFP of *(S,S)*- and *(R,S)*-**1** at 266 nm, although the signals were less intense than when FBP was directly excited under the same conditions (Figure 4A). The triplet lifetimes (decays at 360 nm shown in Figure 4B) were the same in the dyads as in FBP (*ca.* 24 μs for the three compounds), indicating the lack of intramolecular interaction in the excited triplet state.

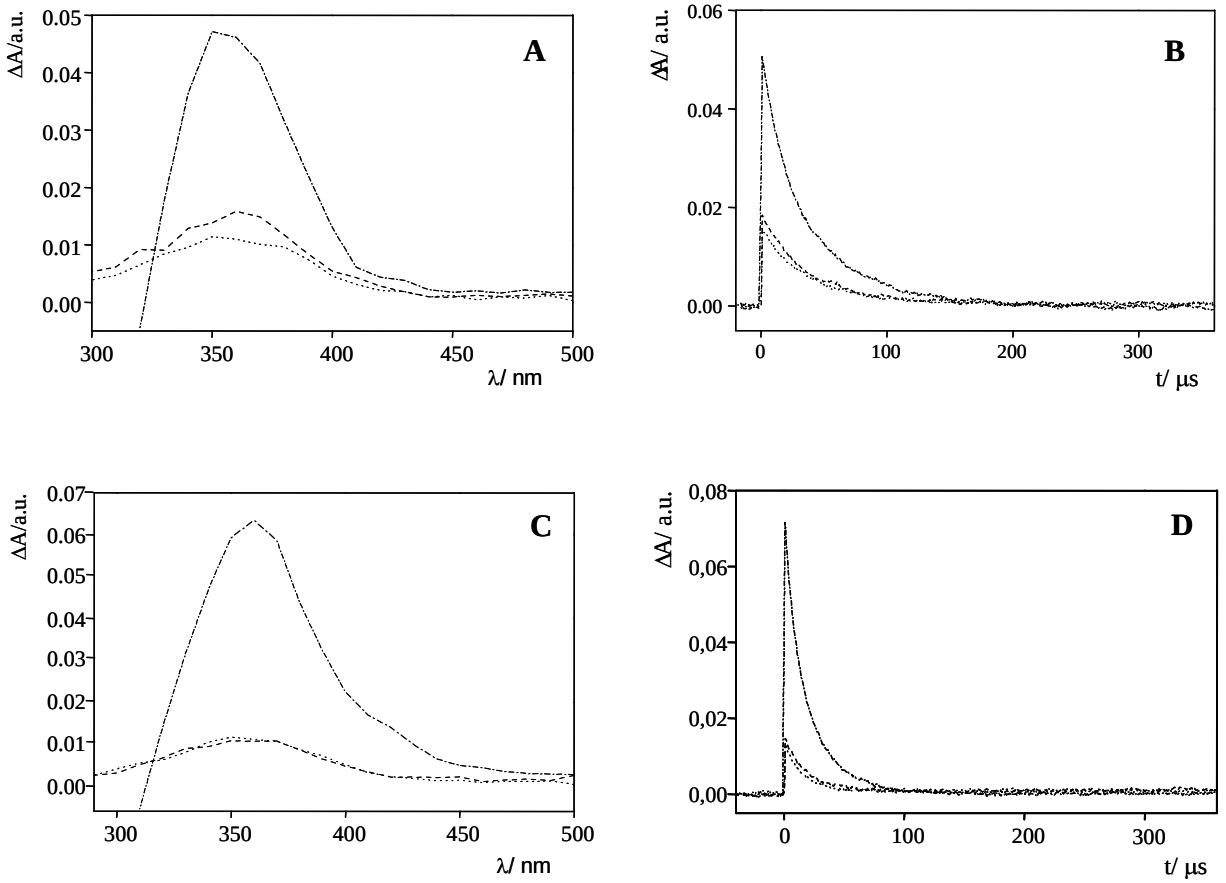


Figure 4: **A** Laser flash photolysis of *(S)*-FBP (- · - · -), *(S,S)*-**1** (- - -) and *(R,S)*-**1** (·····) in MeCN/N_2.: Transient spectra obtained 6μs after the laser pulse. **B**: Decays monitored at 360 nm. **C**: Laser flash photolysis of *(S)*-FBP (- · - · -), *(S,S)*-**2** (- - -) and *(R,S)*-**2** (·····) in MeCN/N_2. Transient spectra obtained 6 μs after the laser pulse. **D**: Decays monitored at 360 nm.

In view of the short fluorescence lifetime of the Trp chromophore in *(S,S)*-**1** and *(R,S)*-**1** (close to the detection limit of our instrument) and in order to minimize possible errors, two new

diastereomeric dyads were synthesized starting from *(S)*-tryptophanol (*(S)*-TrpOH), a Trp analog with a longer lived singlet excited state (8.1 ns).

The fluorescence spectra of solutions of *(S)*-FBP, *(S)*-TrpOH, *(S,S)*-**2** and *(R,S)*-**2** in acetonitrile are shown in Figure 3C. Again, a strong quenching was observed for both dyads, and the remaining emission was attributed to the Trp chromophore. The fluorescence lifetimes were shorter in the dyads (*ca.* 1.1 ns in both cases) than in the model *(S)*-TrpOH, indicating intramolecular dynamic quenching. After normalization of the three emissions, the exciplex was observed as a broad band of low intensity in the interval between 380 and 500 nm (Figure 3D); a significant stereodifferentiation in the exciplex lifetimes (measured at 440 nm) was observed, with values of 6.7 ns for *the (S,S)*- and 5.0 ns for the *(R,S-)* isomer, respectively.

As regards the laser flash photolysis studies on (*S,S*)-**2** and (*R,S)*-**2** in acetonitrile, the only signal observed was the typical FBP T-T absorption spectrum (Figure 4C). The triplet lifetimes were the same in the dyads as in FBP (*ca.* 24 μs for the five compounds); thus, no intramolecular interaction appeared to take place in the excited triplet state.

The main deactivation processes that can take place upon excitation of a dyad containing covalently linked FBP and Trp subunits are indicated in Scheme 2 and can be summarized as follows. Initial excitation at 266 nm (i) could lead to the first singlet excited state of either FBP or Trp, as determined from the UV absorption spectra (Figure 2). Singlet-singlet energy transfer (SSET) from ^{1}FBP* (E_s = 99 kcal mol^{-1})[15] to the Trp moiety (E_s = 96 kcal mol^{-1})[19] would be thermodynamically allowed (ii). Radiative deactivation from ^{1}Trp* or ^{1}FBP* is represented in (iii) and (iv), respectively. Routes (v) and (vi) correspond to intersystem crossing (ISC) processes. Intramolecular quenching (Q) of the first excited singlet state of FBP or Trp, to give exciplexes or radical ion pairs, is illustrated by processes (vii) and (viii). Back electron transfer (BET) from the radical ion pairs could afford the FBP and/or Trp first triplet excited state (ix). Finally, triplet-triplet energy transfer (TTET) from Trp (E_T = 71 kcal mol^{-1})[20] to FBP (E_T = 65 kcal mol^{-1})[15] would also be possible (x).

(i) $FBP\text{-}Trp \xrightarrow{h\nu} {}^{1}FBP^{*}\text{-}Trp + FBP\text{-}{}^{1}Trp^{*}$

(ii) $^{1}FBP^{*}\text{-}Trp \xrightarrow{SSET} FBP\text{-}{}^{1}Trp^{*}$

(iii) $FBP\text{-}{}^{1}Trp^{*} \xrightarrow{-h\nu'} FBP\text{-}Trp$

(iv) $^{1}FBP^{*}\text{-}Trp \xrightarrow{-h\nu''} FBP\text{-}Trp$

(v) $FBP\text{-}{}^{1}Trp^{*} \xrightarrow{ISC_{Trp}} FBP\text{-}{}^{3}Trp^{*}$

(vi) $^{1}FBP^{*}\text{-}Trp \xrightarrow{ISC_{FBP}} {}^{3}FBP^{*}\text{-}Trp$

(vii) $^{1}FBP^{*}\text{-}Trp \xrightarrow{Q_{FBP}} {}^{1}(FBP\text{-}Trp)^{*}$ and/or $FBP^{+\cdot}\text{-}Trp^{-\cdot}$

(viii) $FBP\text{-}{}^{1}Trp^{*} \xrightarrow{Q_{Trp}} {}^{1}(FBP\text{-}Trp)^{*}$ and/or $FBP^{+\cdot}\text{-}Trp^{-\cdot}$

(ix) $FBP^{+\cdot}\text{-}Trp^{-\cdot} \xrightarrow{BET} {}^{3}FBP^{*}\text{-}Trp$ and/or $FBP\text{-}{}^{3}Trp^{*}$

(x) $FBP\text{-}{}^{3}Trp^{*} \xrightarrow{TTET} {}^{3}FBP^{*}\text{-}Trp$

Scheme 2

The relative energies of the different excited states of FBP-Trp dyads are shown in Figure 5, together with the most relevant excited state processes. The fluorescence quantum yields obtained using the free amino acid Trp as standard (ϕ_F = 0.13 in water)[19] together with the corresponding rate constants for the models (*(S)*-FBP, *(S)*-TrpMe, *(S)*-TrpOH) and for compounds *(S,S)*-**1**, *(R,S)*-**1**, *(S,S)*-**2** and *(R,S)*-**2** are given in Table 1.

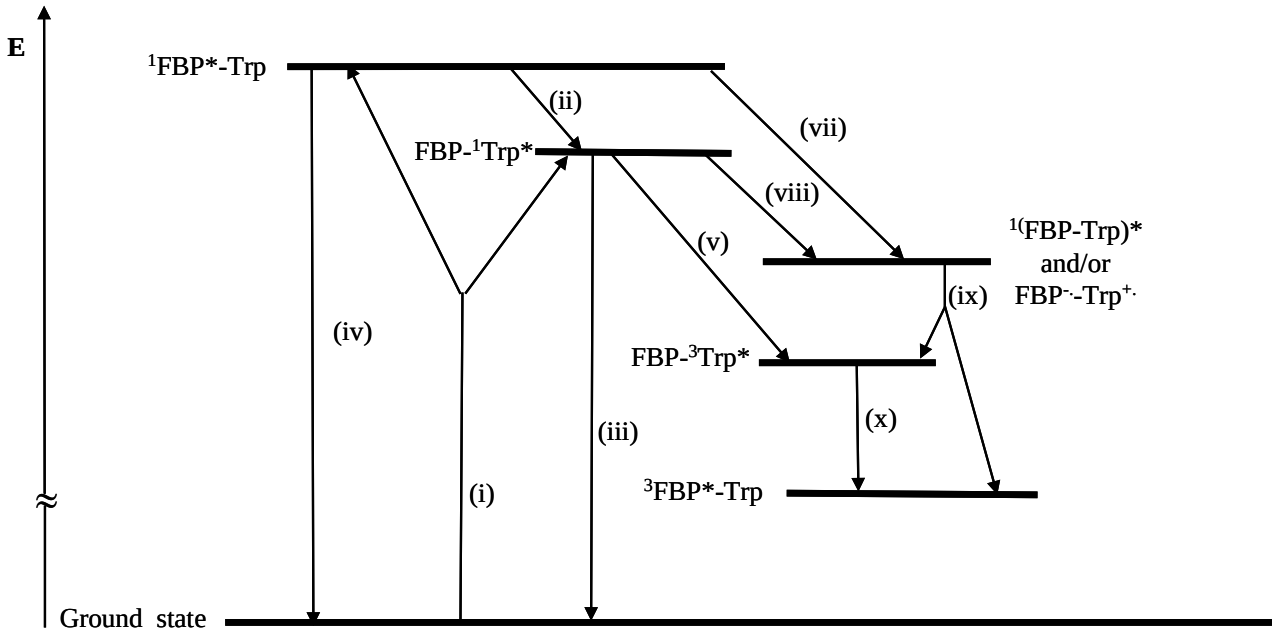


Figure 5. Qualitative energetic diagram for the different excited states and reactive intermediates generated upon excitation of FBP-Trp dyads.

The fluorescence spectra of *(S,S)*-**1**, *(R,S)*-**1,** *(S,S)*-**2** and *(R,S)*-**2** (Figure 3) show that emission occurs exclusively from the Trp chromophore (λ_{max} = 340 nm).[14] Then, k_Q for the Trp unit (step (viii) in Scheme 2) was determined by means of equation 1:

$$k_Q = \frac{1}{\tau_F} - \frac{1}{\tau_{F(model)}} \qquad \text{equation 1}$$

where τ_F is the fluorescence lifetime in the dyad and $\tau_{F\ (model)}$ is that of the model *(S)*-TrpMe or *(S)*-TrpOH. Thus, k_Q was found to be 9.54×10^8 s^{-1} for both diastereomers of **1** and 7.86×10^8 s^{-1} in the case of dyads **2.** From the experimental ϕ_F and τ_F values, the k_F corresponding to process (iii) was obtained using equation 2:

$$k_F = \phi_F/\tau_F \qquad \text{equation 2}$$

Taking into account the above data, the quantum yield for intramolecular dynamic quenching ($\phi_{Q(dyn)}$) was estimated through equation 3:

$$\phi_{Q\,(dyn)} = \frac{k_Q}{k_F} \times \phi_F \qquad \text{equation 3}$$

These ϕ_Q values were identical for all dyads (0.87). As the Trp chromophore is absorbing only *ca.* 40% of the incident photons at 266 nm (see Figures 2A and 2B), the obtained ϕ_Q clearly confirm an efficient singlet-singlet energy transfer from FBP to Trp (process (ii) in Scheme 2), which should be thermodynamically favored in view of the relative singlet energies of the isolated chromophores (see above). In spite of the lack of stereodifferentiation in the quenching of ^{1}Trp*, the spectra shown in Figures 3A and 3C were clearly different for each pair of diastereomers. From the area under the emission curves, the values obtained for overall quenching ($\phi_{Q\,(ov)}$) were 0.88 and 0.94 (for *(S,S)*-**1** and *(R,S)*-**1**) and 0.89 and 0.95 (for *(S,S)*-**2** and *(R,S)*-**2**). Thus, the "dynamic" and the "overall" fluorescence quenching have *ca.* the same values for the *(S,S)*-dyads, but they were somewhat different for the *(R,S)*-diastereomers. This can be due to a minor, yet stereoselective deactivation of ^{1}FBP* leading to exciplexes and/or radical ion pairs (route (vii) in Scheme 2), which is more important for the *(R,S)*-diastereomers than for the corresponding *(S,S)*-dyads. The exciplexes were actually observed after magnification of the emission spectra between 380-500 nm as a broad band of low intensity (Figures 3B and 3D); they were expectedly much more intense in the case of the *(R,S)*-diastereomers.

As regards the Trp fluorescence quenching mechanism, it could involve in principle either electron transfer or exciplex formation. Application of the Weller equation[21] taking into account the Trp singlet energy[20] and the relevant redox potentials,[22] allowed us to estimate the ΔG values corresponding to the two possible pathways; both were found to be exergonic (ΔG_{ET} = -15 kcal mol^{-1} and ΔG_{EXC} = –10 kcal mol^{-1}). All values would be *ca.* 3 kcal mol^{-1} more negative for FBP quenching, due to the higher singlet energy of this chromophore.

Another point of interest was the origin of the FBP triplet. Its quantum yield (ϕ_T) was determined from the LFP experiments and found to be in the range 0.1-0.3. Triplet generation

could in principle occur through several pathways: a) intersystem crossing from ^{1}FBP (Scheme 2, process (vi)), which can be safely ruled out, since no fluorescence from this chromophore was detected; b) triplet-triplet energy transfer from ^{3}TrpMe (process (x) in Scheme 2). Although this process is exergonic (ΔG *ca.* -4 kcal mol^{-1}), its contribution must be minor, since the intersystem crossing quantum yield for conversion of ^{1}Trp* to ^{3}Trp* in the dyads (process (v) in Scheme 2) has to be in the same order as ϕ_F; c) back electron transfer in the radical ion pairs (Scheme 2, process (ix)) resulting from quenching of the excited singlets (ΔG *ca.* -12 kcal mol^{-1}). This is indeed the most feasible pathway, as the added $\phi_{Q\,(ov)}$ and ϕ_T clearly exceed the unity.

Table 1. Photophysical properties of *(S)*-FBP, *(S)*-TrpMe, *(S)*-TrpOH and the dyads in acetonitrile or 1,4-dioxane, in deaerated media.[a]

Compound	Conditions[b]	ϕ_F	τ_F [c] (ns)	$k_F \cdot 10^{-8}$ (s^{-1})	$k_Q \cdot 10^{-8}$ (s^{-1})	$\phi_{Q(dyn)}$	$\phi_{Q(ov)}$	ϕ_T[d]	τ_T (μs)
(S)-FBP	1	0.21	1.7	1.24	-	-	-	0.71	24
(S)-TrpMe	1	0.34	6.4	0.53	-	-	-	n.d.	-
(S,S)-**1**	1	0.04	0.9	0.44	9.54	0.87	0.88	0.26	24
(R,S)-**1**	1	0.02	0.9	0.22	9.54	0.87	0.94	0.22	24
(S)-TrpOH	1	0.38	8.1	0.47	-	-	-	n.d.	-
(S,S)-**2**	1	0.04	1.1	0.36	7.86	0.87	0.89	0.19	24
(R,S)-**2**	1	0.02	1.1	0.18	7.86	0.87	0.95	0.10	24
(S)-FBP	2	0.25	1.8	1.38	-	-	-	0.70	26
(S)-TrpMe	2	0.31	4.2	0.74	-	-	-	n.d.	-
(S,S)-**1**	2	0.08	1.2	0.67	5.95	0.71	0.74	0.15	14
(R,S)-**1**	2	0.03	1.2	0.25	5.95	0.71	0.88	0.13	14
(S)-TrpOH	2	0.35	5.4	0.64	-	-	-	n.d.	-
(S,S)-**2**	2	0.08	1.5	0.53	4.82	0.73	0.77	0.19	17
(R,S)-**2**	2	0.05	1.5	0.33	4.82	0.73	0.86	0.13	17

[a] In general, errors were lower than 5% of the stated values; [b] Conditions 1: Acetonitrile, λ_{exc} = 266 nm; conditions 2: 1,4-dioxane, λ_{exc} = 266 nm; [c] Measured at 310 nm for *(S)*-FBP and at 340 nm for the other compounds; [d] n.d.: non detected.

Photophysical studies on FBP-Trp dyads in 1,4-dioxane

In order to disfavor the occurrence of intramolecular electron transfer, fluorescence measurements were also performed in 1,4-dioxane, whose dielectric constant is lower than that of acetonitrile (2.2 *vs* 37.5).[23] The UV-absorption spectra of *(S,S)*-**1** and *(R,S)*-**1**, (Figure 6A) were identical to the added spectra of the isolated FBP and Trp units, at the same concentrations, in accordance with the lack of any significant ground-state intramolecular interactions between the two moieties.

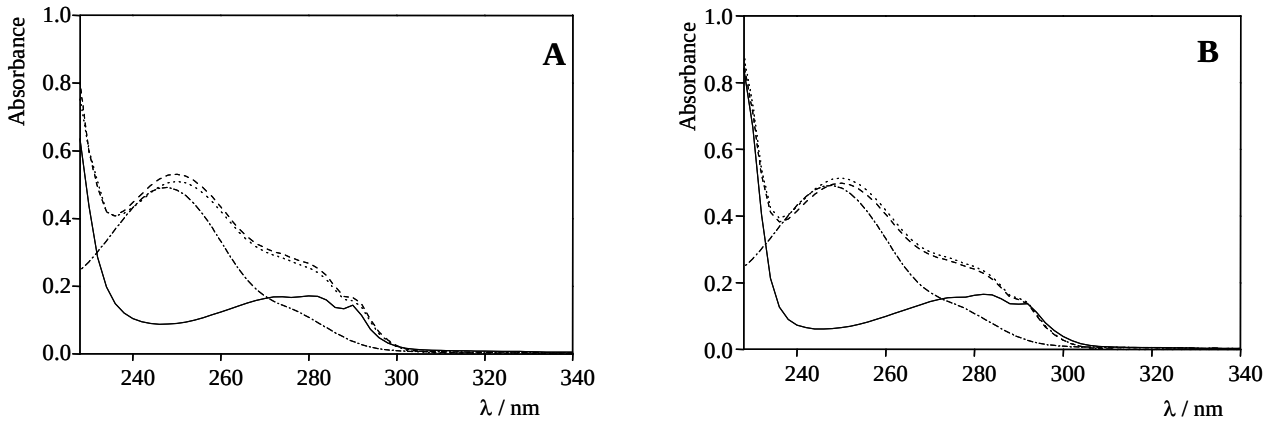


Figure 6 A. UV-absorption spectra of *(S)*-FBP (– · – ·), *(S)*-TrpMe (——), *(S,S)*-**1** (– – –) and *(R,S)*-**1** (·····) in 1,4-dioxane at 2.5 10^{-5} M concentration. **B** UV-absorption spectra of *(S)*-FBP (– · – ·), *(S)*-TrpOH (——), *(S,S)*-**2** (– – –) and *(R,S)*-**2** (·····) in 1,4-dioxane at 2.5 10^{-5} M concentration.

The most remarkable feature in the fluorescence spectra of *(S,S)*-**1**, *(R,S)*-**1,** *(S,S)*-**2** and *(R,S)*-**2** upon excitation at 266 nm was a dramatic quenching (see Figures 7A,C). The remaining emission was assigned to the Trp chromophore. By contrast, the possible exciplex emission (band between 380-500 nm, Figures 7B,D) was less intense in 1,4-dioxane. The fluorescence lifetimes at λ_{em} = 340 nm were clearly shorter in the dyads (between 1.2 and 1.5 ns) than in *(S)*-TrpMe (4.2 ns) or in *(S)*-TrpOH (5.4 ns), indicative of a dynamic quenching; at λ_{em} = 440 nm, τ_F was the same for both diastereomers of each pair (1.9 ns for **1** and 3.0 ns for **2**).

Concerning the LFP experiments, the typical FBP T-T transient absorption spectrum was obtained upon excitation at 266 nm;[15] the signals were less intense than when FBP was directly excited under the same conditions (Figure 8A,C). The triplet lifetimes (decays at 360 nm shown in Figure 8B,D) were shorter in the dyads (14 μs) than in FBP, indicating some degree of intramolecular interaction (may be exciplex formation) in the excited triplet state.

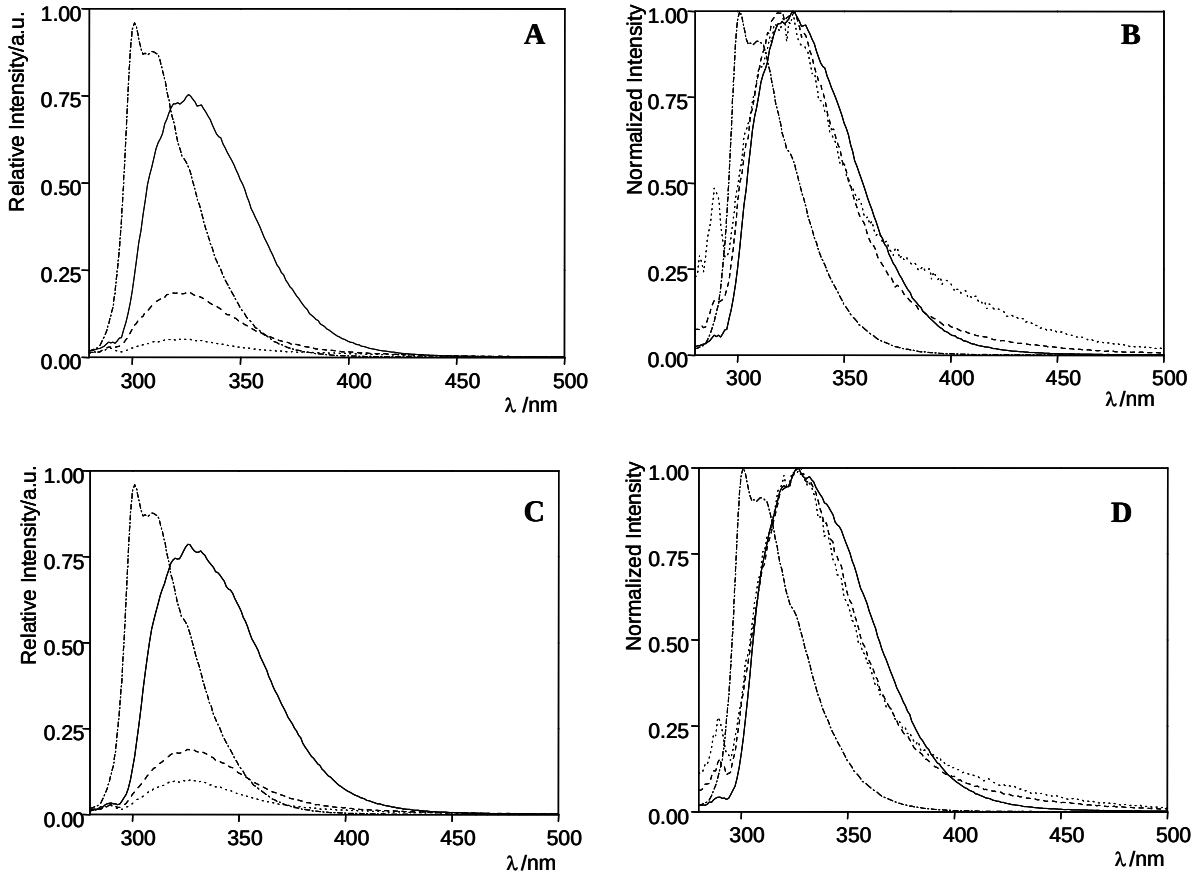


Figure 7. A Fluorescence spectra in deaerated 1,4-dioxane of *(S)*-FBP (-·--·-), *(S)*-TrpMe (——), *(S,S)*-**1** (- - -) and *(R,S)*-**1** (·····); **B** Normalized fluorescence spectra in deaerated 1,4-dioxane of *(S)*-FBP (-·--·-), *(S)*-TrpMe (——), *(S,S)*-**1** (- - -) and *(R,S)*-**1** (·····); **C** Fluorescence spectra in deaerated 1,4-dioxane of *(S)*-FBP (-·--·-), *(S)*-TrpOH (——), *(S,S)*-**2** (- - -) and *(R,S)*-**2** (·····); **D** Normalized fluorescence spectra in deaerated 1,4-dioxane of *(S)*-FBP (-·--·-) , *(S)*-TrpOH (——), (*S,S*)-**2** (- - -) and (*R,S*)-**2** (·····).

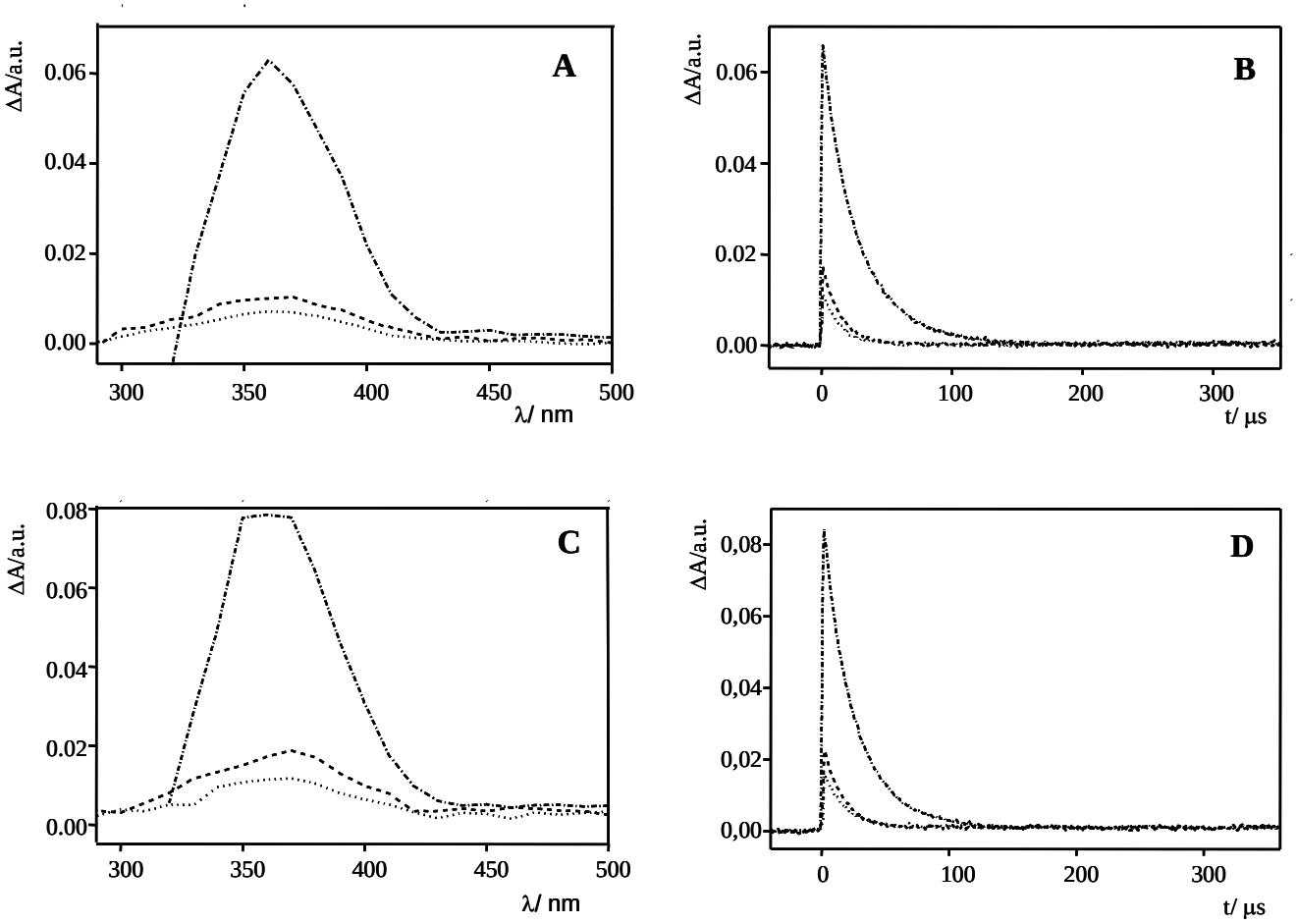


Figure 8. Laser flash photolysis of *(S)*-FBP (——), *(S,S)*-**1** (- - - - -) and *(R,S)*-**1** (·····) in 1,4-dioxane/N_2. **A**: Transient spectra obtained 3 μs after the laser pulse. **B**: Decays monitored at 360 nm. Laser flash photolysis of *(S)*-FBP (——), *(S,S)*-**2** (- - - - -) and *(R,S)*-**2** (·····) in 1,4-dioxane/N_2.**C**: Transient spectra obtained 3 μs after the laser pulse. **D**: Decays monitored at 360 nm.

The relevant quantum yields and rate constants (obtained as detailed above for the corresponding data in acetonitrile) are given in Table 1.

Also in this solvent, emission occurs exclusively from the Trp chromophore (λ_{max} = 340 nm). The k_Q for the Trp unit (step (viii) in Scheme 2), using equation 1, was found to be 5.95 × 10^8 s^{-1} for dyads **1** and 4.82 × 10^8 s^{-1} in the case of **2.** From ϕ_F and τ, and using equation 2, the k_F corresponding to process (iii) was obtained. The quantum yield values for intramolecular

dynamic quenching ($\phi_{Q(dyn)}$), estimated through equation 3, were identical for each couple of dyads (0.87 for **1** and 0.73 for **2**). Again, as the Trp chromophore is not absorbing all the incident light (only *ca.* 40% of the photons at 266 nm, see Figures 6A and 6B), the obtained ϕ_Q corroborate an efficient singlet-singlet energy transfer from FBP to Trp (process (ii) in Scheme 2). The spectra shown in Figures 7A and 7C were clearly different for each pair of diastereomers, in spite of the lack of stereodifferentiation in dynamic quenching of ^{1}Trp*. The overall quenching data ($\phi_{Q\ (ov)}$) were 0.74 and 0.88 (for *(S,S)*-**1** and *(R,S)*-**1**) and 0.77 and 0.86 (for *(S,S)*-**2** and *(R,S)*-**2**). Thus, the "dynamic" and the "overall" fluorescence quenching are substantially different for the *(R,S)*-diastereomers. Such stereodifferentiation process is attributed to deactivation of ^{1}FBP* leading to exciplexes and/or ion pairs (route (vii) in Scheme 2).

Application of the Weller equation[21] taking into account the Trp singlet energy[19] and the relevant redox potentials,[22] allowed us to estimate the ΔG values corresponding to the two possible pathways (ΔG_{ET} = +10 kcal mol^{-1} and ΔG_{EXC} = -6 kcal mol^{-1}). Thus, stereodifferentiation can be made higher in dioxane by slowing down the electron transfer process and increasing the exciplex involvement.

Finally, it must be noted that the triplet quantum yields (ϕ_T) determined from the LFP experiments are between 0.1 and 0.2, and that the overall quenching quantum yields ($\phi_{Q\ (ov)}$) fall in the range 0.7-0.9. As the added $\phi_T + \phi_{Q\ (ov)}$ values do not exceed the unity, there is no need to invoke in this case back electron transfer in the radical ion pair as the origin of triplet formation. This agrees well with the fact that electron transfer quenching would be endergonic in 1,4-dioxane, as stated in the above paragraph.

Conclusions

In summary, for the FBP-Trp dyads studied, the main photophysical events can be summarized as follows: 1) most of the energy provided by the incident radiation at 266 nm reaches the ^{1}Trp*, either *via* direct absorption by this chromophore or by SSET from ^{1}FBP*; 2) a minor, yet stereoselective deactivation of ^{1}FBP* leads to detectable exciplexes and/or radical ion pairs; 3) the main process observed is intramolecular ^{1}Trp* quenching of and 4) the first triplet excited state of FBP can be populated by TTET from ^{3}Trp* or by BET from the charge separated states. The singlet excited state processes observed in the dyads explain the fluorescence quenching and the predominating Trp-like emission of HSA/FBP supramolecular systems. By contrast, the lack of relevant interactions in the excited triplet states of the dyads can not be correlated with the laser flash photolysis studies on HSA/FBPMe conjugates,[16] where the dramatic lengthening of the triplet lifetimes within the protein binding sites has to be attributed to the particular properties of the microenvironment provided by the tertiary structure of these biomolecules (*i. e.* conformational restrictions, inhibition of self-quenching or triplet-triplet annihilation, protection from attack by oxygen or other reagents, etc).

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Suggestion for the table-of-contents entry

Excited State Interactions in Flurbiprofen-Tryptophan Dyads

Ignacio Vayá, M. Consuelo Jiménez and Miguel A. Miranda**