Effect of the Binding Sites of Human Serum Albumin on the Efficiency and Photostationary State Isomer Ratios of the Photoisomerization of Bilirubin

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The quantum yields of the isomerization and photostationary state isomer ratios of the photoisomerization of (4*Z*,15*Z*)-bilirubin IXα (*ZZ*-BR) were determined in an aqueous buffered solution in the presence of human serum albumin (HSA) at a molar ratio of [*ZZ*-BR]/[HSA] from 0.5 to 2. The BR isomer compositions in the photostationary state were constant at [BR]/[HSA]=0.5—0.7. With increasing [*ZZ*-BR]/[HSA] from 0.8 to 2, the *ZZ*-BR composition in the photostationary state increased from 58 to 75%, but the *ZE*-BR composition decreased from 39 to 22%. The quantum yields in the isomerization of *ZZ*-BR to *ZE*-BR ($\Phi_{ZZ\rightarrow ZE}$) and a cyclized product, lumirubin (LR), ($\Phi_{ZZ\rightarrow LR}$), remained unvaried up to [*ZZ*-BR]/[HSA]=1, but $\Phi_{ZZ\rightarrow ZE}$ decreased while $\Phi_{ZZ\rightarrow LR}$ increased along with a further increase of [*ZZ*-BR]/[HSA]. These results are explained by the existence of two binding sites, a first-class site and a second-class site, for bilirubin binding to HSA; $\Phi_{ZZ\rightarrow ZE}$ in the second-class site (=0.035) was as low as 1/3 of that in the first-class site (=0.11), but $\Phi_{ZZ\rightarrow LR}$ in the second-class site ($=4.2\times10^{-3}$) was nearly two-times higher than that in the first-class site ($=2.4\times10^{-3}$).

We have already reported that the quantum yields of the photoisomerization of (4*Z*,15*Z*)-bilirubin IXα (*ZZ*-BR) were greatly influenced by the properties of both the solvent and the media.[1,2] As shown in Scheme 1, *ZZ*-BR comprizes two pyrromethenone moieties which form intramolecular hydrogen bonds with a propionic acid side chain.[1—16] Upon irradiation with visible light, *ZZ*-BR underwent isomerization to *E* isomers (*ZE*-, *EZ*, and *EE*-BR) and a cyclized product lumirubin (LR) by breaking the intramolecular hydrogen bonds.[1—20] For example, the intramolecular hydrogen bonds between the second pyrromethenone group (2-PY in Scheme 1) and the propionic acid group at C-8 in the first pyrromethenone group (1-PY in Scheme 1) were broken in the process of *ZZ*→*ZE* isomerization.

In a series of previous results it had become clear that the greater is the intramolecular hydrogen bonding of *ZZ*-BR between the pyrrole or pyrromethenone NH and the carboxyl group broken by the solvent, the greater is the efficiency of the isomerization that takes place.[1—3] Among the solvents and media used, an aqueous solution containing human serum albumin was found to accelerate the *ZZ*→*ZE* isomerization and formation of a cyclized product, LR.

We have also reported on the effect of serum albumins from different animals on the photoisomerization of BR, showing that only human serum albumin (HSA) exhibits a remarkable acceleration of the *ZZ*→*ZE* isomerization and *ZZ*→LR formation.[1,2]

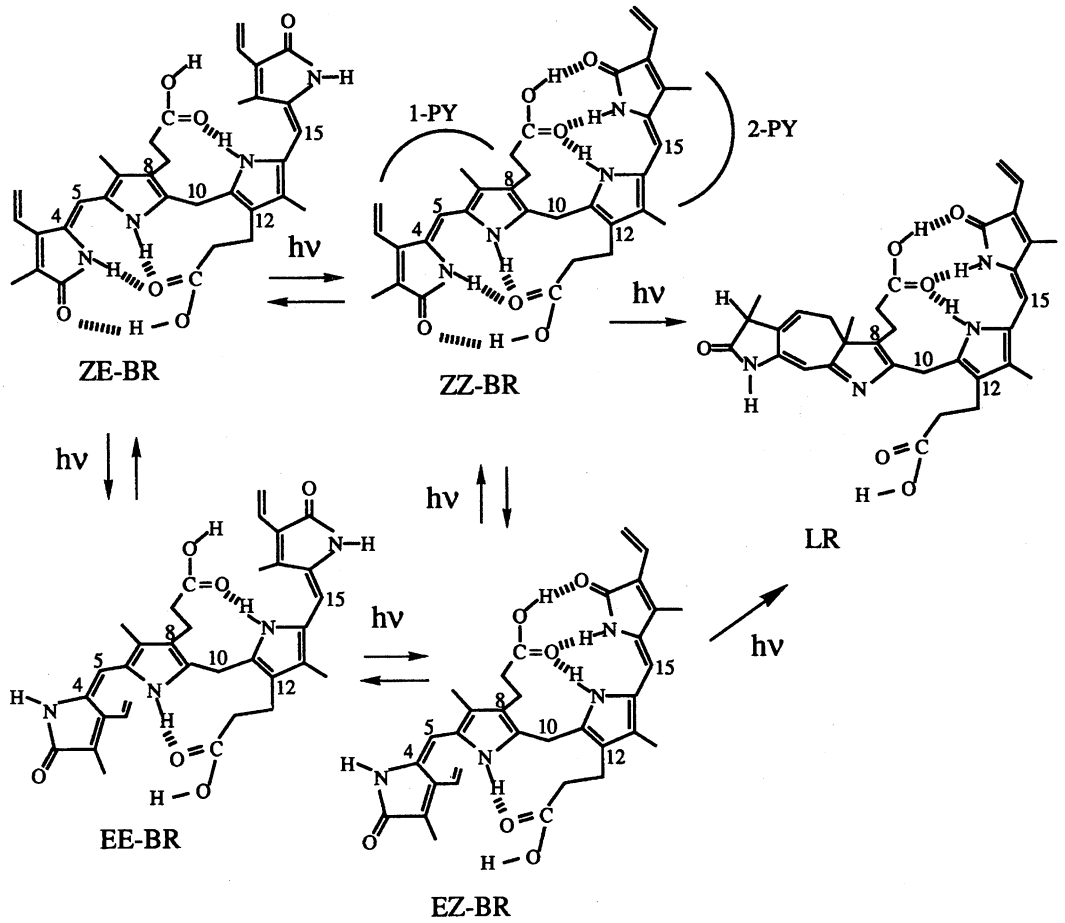
Two binding sites of HSA were usually assumed to be available for bilirubin binding to HSA. A previous determination of the quantum yield of the isomerization was performed in the presence of equimolar amounts of HSA and *ZZ*-BR; under this condition, almost all of the *ZZ*-BR is bound to the first-class site, because of the big difference in the binding constants between the two binding sites: the first-class site (K_1) and the second-class site (K_2), 1.0×10^8 and 3.0×10^6 M^{-1}, respectively.[21,22] It was recently reported that upon irradiation with a blue-white lamp emitting at broad wavelengths the time development of the photoproducts was affected by the molar ratio between *ZZ*-BR and HSA.[10] However, since the light-absorption properties of the production vary along with the irradiation wavelength, it would be desirable to work with monochromatic light in order to reveal the effect of the binding site of HSA on the photochemical behavior of BR. In this respect, we determined the quantum yields of the isomerization at varying molar ratios of *ZZ*-BR and HSA upon 436 nm irradiation in order to reveal the above-mentioned point.

Experimental

Reagents and Solutions. A typical procedure for sample preparation was as follows. Fifty-eight milligrams of *ZZ*-BR (Sigma Chemical Co.)[1,2,23] were dissolved in 25 ml of 0.1 M (1 M=1 mol dm^{-3}) NaOH; 2 ml of this solution was immediately added to each of the 50 ml portions of a 0.05 M phosphate buffer (pH 7.4) containing HSA (fatty acid free, Sigma A3782) at nine different [*ZZ*-BR]/[HSA] ratios.

HPLC Analysis. A high-performance liquid-chromatograph (HPLC, Waters) was constructed using an M600 multisolvent delivery system and an M490 programable multiwavelength detector equipped with an M740 data module. Before HPLC analysis, sample solutions were diluted 10 times with a mobile phase in order to precipitate and to remove HSA by filtration. The analysis was performed in triplicate using an isocratic reversed-phase ion-pair system with 0.1 M dioctylammonium acetate in methanol[1,2,13—17,24,25] as the eluent at a flow rate of 0.7 ml min^{-1} through a C_{18} column (Chemcosorb 5-ODS-H, 4.6×250 mm). The absorbance was monitored at 450 nm to determine the concentration of BR isomers. The concentrations were determined by using the sensitivity ratios between the isomers (*ZZ*: *ZE*: *EZ*: LR=1.0 : 0.70 : 0.67 : 0.43).[1,2,24]

Absorption Spectra. The absorption spectra of *ZZ*-BR (1.50×10^{-5} M) in HSA solutions were measured in a 1 cm quartz cuvette in a spectrophotometer (JASCO 660).



Scheme 1.

The absorbance of the solutions at 436 nm for photochemical measurements was measured in a 1 mm quartz cuvette both before and after irradiation.

Photoisomerization. Photoisomerization of *ZZ*-BR (1.00×10^{-4} M) was carried out under a N_2 atmosphere in an aqueous phosphate buffer solution (pH 7.4) containing HSA at nine different concentrations ([*ZZ*-BR]/[HSA]=0.50, 0.70, 0.80, 0.90, 1.00, 1.25, 1.50, 1.75, and 2.00). Photoisomerization was performed in the presence of varying concentrations of HSA (9.4×10^{-5}, 1.6×10^{-4}, and 3×10^{-4} M) with a molar ratio of [*ZZ*-BR]/[HSA]=1 as well. The irradiation was performed using a merry-go-round type apparatus with 436 nm light from a 400 W high-pressure mercury lamp through a solution filter (44 g dm^{-3} $CuSO_4\cdot5H_2O$+2.7 M NH_3+75 g dm^{-3} $NaNO_2$ in water).

The quantum yields of the isomerization were determined at the early stage at which the concentration of the resulting photoisomers increased linearly with the irradiation time. The $\Phi_{ZZ\rightarrow LR}$ values were calculated by assuming that LR was formed directly by the photoexcitation of *ZZ*-BR. In this case, the fraction of light absorbed by *ZZ*-BR in solution was calculated from the fraction of *ZZ*-BR in photoequilibrating mixtures and the absorbance of the sample solutions at 436 nm both before and after irradiation.[1,2,14] The light intensity was measured by using potassium tris(oxalato)ferrate(III) actinometry.

The photostationary state isomer compositions were measured in various molar ratios of the BR-HSA complex in a buffered solution (pH 7.4) by irradiating *ZZ*-BR under conditions similar to those employed in the quantum-yield measurement.

Results and Discussion

Effects of [*ZZ*-BR]/[HSA] on the Absorption of *ZZ*-BR. The absorption spectra of *ZZ*-BR (1.50×10^{-5} M) were measured in a 0.05 M phosphate buffered solution (pH 7.4) containing HSA at [*ZZ*-BR]/[HSA] from 0.5 to 2 (Fig. 1). With an increase in [*ZZ*-BR]/[HSA], the maximum wavelength of the absorption spectrum was slightly red shifted from 455 to 456.5 nm, accompanied by an increase in the molar-extinction coefficient in the 400—550 nm region.

Effects of [*ZZ*-BR]/[HSA] on the Photochemistry of *ZZ*-BR. Figure 2 shows the time development of BR isomers at varying [*ZZ*-BR]/[HSA] upon 436 nm irradiation. The photostationary state was reached after ca. 20 min of irradiation.

The quantum yields of isomerization and the photostationary state isomer compositions were independent of the HSA concentration from 9.4×10^{-5} to 3×10^{-4} M at [*ZZ*-BR]/[HSA]=1, as well as the aging time after sample preparation. However, they are dependent on the molar ratio, [*ZZ*-BR]/[HSA]. Figure 3 summarizes the effect of the molar ratio, [*ZZ*-BR]/[HSA], on the

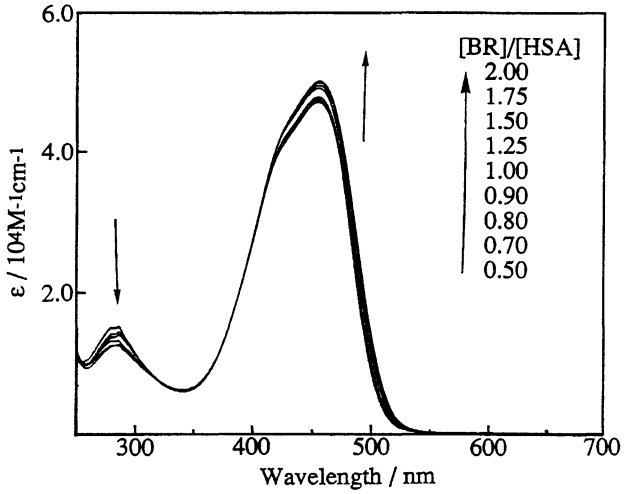


Fig. 1. Absorption spectra of *ZZ*-BR in aqueous buffered solution in the presence of HSA at varying [*ZZ*-BR]/[HSA].

quantum yields of the isomerization and the photostationary state isomer compositions at the initial concentration of *ZZ*-BR=1.0×10^{-4} M.

The BR isomer compositions at the photostationary state were constant at [*ZZ*-BR]/[HSA]=0.5—0.7. The *ZZ*-BR composition at the photostationary state increased from 58 to 75% along with increasing [*ZZ*-BR]/[HSA] from 0.8 to 2.0. On the other hand, the *ZE*-BR composition at the photostationary state decreased from 39 to 22% along with increasing [*ZZ*-BR]/[HSA] from 0.8 to 2, while the *EZ*-BR composition was very low and almost constant at any [*ZZ*-BR]/[HSA] examined.

The quantum yields of the isomerization were almost constant at [*ZZ*-BR]/[HSA]=0.5—1. However, along with an increase in [*ZZ*-BR]/[HSA] from 1.00 to 2.00, the quantum yield of the *ZE*-BR formation ($\Phi_{ZZ\rightarrow ZE}$) decreased, but that of the LR formation ($\Phi_{ZZ\rightarrow LR}$) increased. The $\Phi_{ZZ\rightarrow EZ}$ value was very low (<0.02), and seemed to be almost constant. The *EZ*-BR composition at the photostationary state was as low as 3% at any [*ZZ*-BR]/[HSA] examined. It was therefore difficult to accurately determine the $\Phi_{ZZ\rightarrow EZ}$ values; the highest limit of $\Phi_{ZZ\rightarrow EZ}$ was estimated from the photostationary state isomer composition as being <0.02. The change in the *ZZ*- and *ZE*-BR compositions seemed to be qualitatively in accordance with the change in $\Phi_{ZZ\rightarrow ZE}$ with [*ZZ*-BR]/[HSA], and to indicate that the quantum yields of *ZE*→*ZZ* isomerization ($\Phi_{ZE\rightarrow ZZ}$) are less sensitive to [*ZZ*-BR]/[HSA]. However, we can not discuss the relation between the quantum yields and the isomer compositions in a quantitative manner, because of the difficulty to determine accurate values of $\Phi_{ZE\rightarrow ZZ}$, due to the instability of the *ZE* isomer in the purification procedure. In any case, the quantum yields of the isomerization, as well as the isomer compositions at the photostationary state, depend on [*ZZ*-BR]/[HSA].

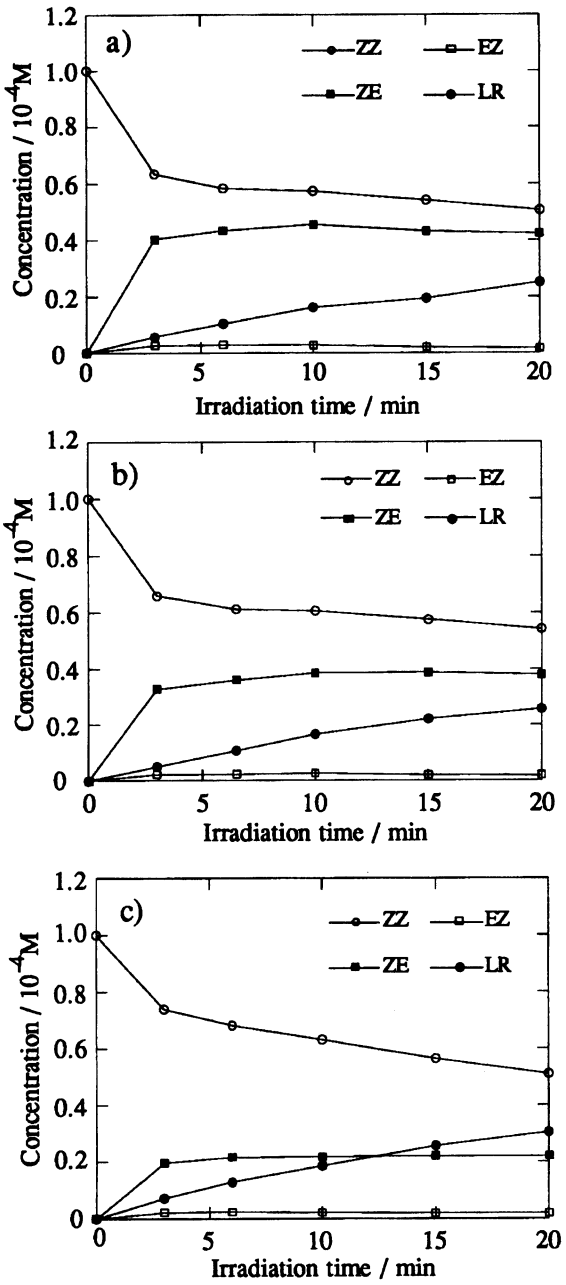


Fig. 2. The time development of the photoproducts starting from *ZZ*-BR on 436 nm irradiation in aqueous buffered solution in the presence of HSA at [*ZZ*-BR]/[HSA]=0.5 (a), 1.0 (b), and 2.0 (c).

Effects of the Binding Site in HSA on the Photochemistry of *ZZ*-BR. No effect of the concentration of HSA on the quantum yields of the isomerization and the photostationary state isomer compositions of BR was observed at a molar ratio of [*ZZ*-BR]/[HSA]=1. Therefore, the results in Fig. 3 indicate the change in the photochemical behavior of BR, depending not on the concentration of HSA, but on the molar ratio of [*ZZ*-BR]/[HSA]. As described above, HSA has two binding sites, first- and second-class binding sites. Since the

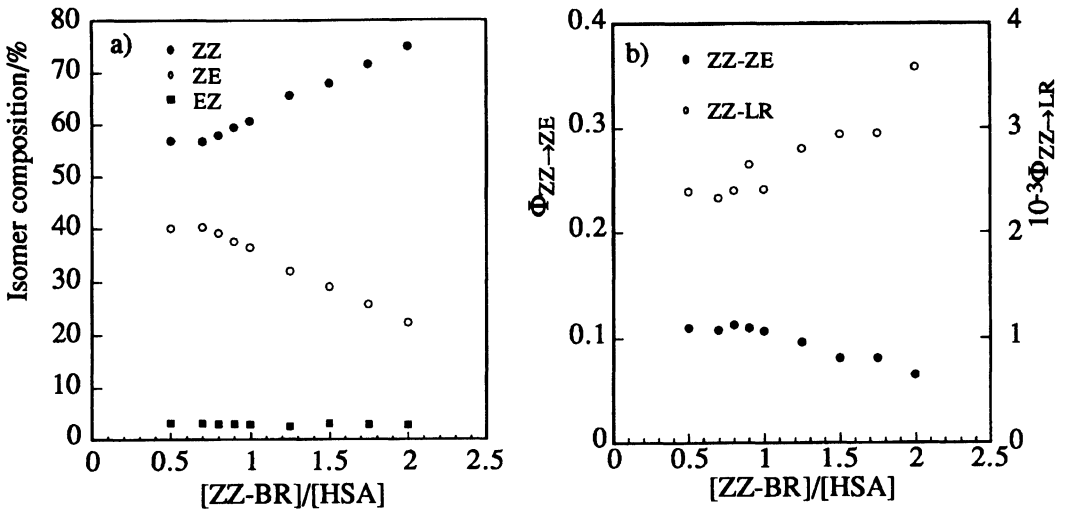


Fig. 3. Effect of [*ZZ*-BR]/[HSA] on the photostationary state isomer composition and the quantum yields of isomerization of bilirubin.

pK_a value of the carboxylic groups of *ZZ*-BR is 4.4, in an aqueous buffered solution at pH 7.4 most of the *ZZ*-BR exist as dianions.[22] Thus, two molecules of *ZZ*-BR dianion can be bound to one molecule of HSA in an aqueous buffered solution (pH 7.4). Because of the large difference in K_1 and K_2, 1.0×10^8 and 3.0×10^6 M^{-1}, respectively, *ZZ*-BR dianion is bound to albumin exclusively in the first-class site at [*ZZ*-BR]/[HSA] up to nearly 1. When [*ZZ*-BR]/[HSA] increases to more than 1, the *ZZ*-BR dianion comes to bind to the second-class binding sites.

Therefore, the observed effects of the BR/HSA molar ratio on the photochemistry of *ZZ*-BR can be attributed to the effects of binding sites with different properties. The quantum yields for the isomerization of *ZZ*-BR obtained at various molar ratios of [*ZZ*-BR]/[HSA] are expressed in the form of a linear combination of the quantum yields of the isomerization bound to the first- and second-class sites.

One can estimate the quantum yields for the isomerization as well as the molar extinction coefficient of *ZZ*-BR bound to the first- and second-class sites at the excitation wavelength (436 nm) from the observed values in varying molar ratios of [*ZZ*-BR]/[HSA]. In this case, it is assumed that no cooperative effect between the first- and second-class binding sites exists which can affect the behavior of *ZZ*-BR. On the basis of the above assumptions, the observed absorbance at 436 nm (A_{obs}) and the quantum yields (Φ_{obs}) of the isomerization of *ZZ*-BR are described by

$$A_{obs} = \varepsilon_{1st}C_{1st} + \varepsilon_{2nd}C_{2nd} \quad (1)$$

and

$$\Phi_{obs} = \alpha_{1st}\Phi_{1st} + \alpha_{2nd}\Phi_{2nd}, \quad (2)$$

where ε is the molar extinction coefficient, C the concentration of *ZZ*-BR and Φ the quantum yield of isomerization; the subscripts (1st and 2nd) correspond to the first- and second-class binding sites, respectively, and α_{1st} and α_{2nd} are the fractions of the light intensity absorbed by *ZZ*-BR bound to the first- and second-class sites of HSA. In this argument, BR is assumed to be bound to the first-class site up to [*ZZ*-BR]/[HSA]=1. The molar extinction coefficients and quantum yields for isomerization at [BR]/[HSA]=0.5 are used to estimate ε_{1st} (4.34×10^4 $M^{-1}cm^{-1}$) and Φ_{1st} ($\Phi_{ZZ\rightarrow ZE}$= 0.11 and $\Phi_{ZZ\rightarrow LR}=2.4\times10^{-3}$), because BR should be bound exclusively to a first-class site at [BR]/[HSA]= 0.5, as described above.

A plot of A_{obs}-$\varepsilon_{1st}C_{1st}$ against C_{2nd} gives a slope of 4.70×10^4 $M^{-1}cm^{-1}$ as ε_{2nd}. The molar extinction coefficient at 436 nm for *ZZ*-BR bound to the second-class site on HSA is larger than that to the first-class site. Plots of Φ_{obs}-$\alpha_{1st}\Phi_{1st}$ against α_{2nd} for $\Phi_{ZZ\rightarrow ZE}$ and $\Phi_{ZZ\rightarrow LR}$ give slopes of 0.035 and 4.2×10^{-3}, respectively, which are regarded as being $\Phi^{2nd}_{ZZ\text{-}ZE}$ and $\Phi^{2nd}_{ZZ\text{-}LR}$, respectively. Thus, $\Phi_{ZZ\rightarrow ZE}$ in a second-class site (=0.035) is as low as 1/3 of that in a first-class site (=0.11), but $\Phi_{ZZ\rightarrow LR}$ in a second-class site (= 4.2×10^{-3}) is nearly two-times higher than that in a first-class site ($=2.4\times10^{-3}$).

Conclusions

The $\Phi_{ZZ\rightarrow ZE}$ at a first-class site is higher than that at a second-class site, while the inverse holds for the value of $\Phi_{ZZ\rightarrow LR}$. Thus, the environment of the bilirubin binding site of HSA should be different between the first- and the second-class sites; the intramolecular hydrogen bonding as well as the conformation of bilirubin incorporated into HSA might be different in the first- and second-class binding sites.

The quantum yields of *ZZ*→*ZE* and *ZZ*→LR formation of *ZZ*-BR, both in the first- and second-class sites of HSA were higher than that in the other serum albumins or in an organic solvent.[1,2]

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