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Source: Photochemistry and Photobiology, 74(2):211-215.
Published By: American Society for Photobiology
https://doi.org/10.1562/0031-8655(2001)074<0211:THDPPT>2.0.CO;2
URL: http://www.bioone.org/doi/full/10.1562/0031-8655%282001%29074%3C0211%3ATHDPPT%3E2.0.CO%3B2

Photochemistry and Photobiology, 2001, 74(2): 211–215

Toward Hypericin-derived Potential Photodynamic Therapy Agents¶

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Received 2 November 2000; accepted 6 May 2001

ABSTRACT

To optimize a hypericin derivative as a potential photodynamic therapy agent its light-induced singlet oxygen/superoxide radical formation capability should be enhanced and its long-wavelength absorption band should be bathochromically shifted to better match medicinal lasers. A heavy-atom–substituted derivative was realized by electrophilic iodination of hypericin to yield 2,5-diiodo-hypericin. Using photodestruction of bilirubin IXα this derivative was demonstrated to exhibit an enhanced light-induced singlet oxygen/superoxide radical formation capability as compared to hypericin. With respect to a bathochromically shifted derivative styryl residues were attached to the methyl groups of hypericin by de novo ring synthesis. Although the long-wavelength absorption band of this derivative displayed a bathochromic shift of nearly 40 nm it unfortunately immediately underwent an intramolecular [2 + 2] cycloaddition to yield the corresponding cyclobutane derivative in which the added conjugation system became interrupted.

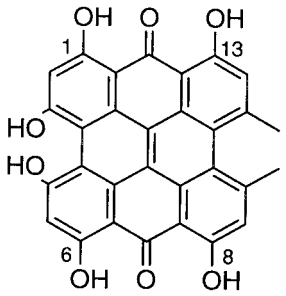
INTRODUCTION

Hypericin (**1**) the photosensitizing pigment from the ubiquitous proliferating weed, St. John's wort (*Hypericum perforatum* L.), constitutes a promising agent for the photodynamic therapy of certain viral or tumoral ailments (for reviews see Kraus *et al.* [1] and Falk [2]). Besides its capability to acidify its surroundings following excitation (3–6) this is thought to be mainly due to its pronounced tendency to sensitize the formation of singlet oxygen and to a smaller extent of superoxide radicals (2). However, the efficiency of **1** for this application might be improved with respect to two issues. Firstly, it might be desirable to enhance the quantum efficiency of the singlet oxygen/superoxide radical formation, and secondly, shift the main absorption band wavelength of hypericin, or more precisely of the 3-hypericinate ion (2) which is slightly below 600 nm, to higher wavelengths in order to match it better with the emission wavelength of common medicinal lasers. It is thought that, as a prerequisite, derivatives of this kind should retain the main functional groups of **1**. Accordingly, synthesis efforts should be restricted to a derivatization of the carbon atoms of **1**.

In principle, the first goal could be reached by heavy-atom substitution of the aromatic protons of **1** because it is known that such substitution would enhance intersystem crossing and concomitantly also enhance, to a certain degree, singlet oxygen sensitization (2). The second goal could be attained by either substituting the aromatic protons by conjugation extending substituents or by enlarging conjugation *via* the two methyl groups in positions 10 and 11.

It might be mentioned that chelation of the *peri*-hydroxyl/carbonyl region of **1** should also result in a bathochromic shift; however, such chelates would be rather unstable under the mainly hydrolytic physiological conditions as experienced for the Al^{3+}, Fe^{3+}, Cu^{2+}, Gd^{3+} and Tb^{3+} chelates of **1** (7). Following a suggestion of Peter de Witte we prepared the mono- and bis-diphenyl-boron chelates of **1** (displaying electrospray mass peaks at $m/z = 667$ and 831 corresponding to the mono- and bis-diphenylboryl complexes), which might have constituted one of the utmost stable chelates to be thought of. Although the main absorption bands of these complexes were found to be shifted to 645 nm they proved to be extremely sensitive with respect to hydrolysis being stable only in aprotic solvents. Accordingly, they were found to be unsuitable as photodynamic therapy agents.

On the one hand we report here attempts to synthesize a heavy-atom–substituted derivative of **1** to check whether this leads to enhanced singlet oxygen production, and on the other hand the preparation of a conjugation-enhanced derivative to test to what extension the absorption band of **1** could be bathochromically shifted by this means.



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MATERIALS AND METHODS

Chemicals and syntheses. Hypericin was prepared and purified according to Falk *et al.* (8) and Kapinus *et al.* (9). Bilirubin IXα was

¶Posted on the website on 8 June 2001.

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†*Abbreviations:* NMR, nuclear magnetic resonance.

of commercial origin (Sigma Chemical Co., Vienna, Austria). Solvents and reagents of highest quality were obtained from commercial suppliers. 1,6,8-Trimethoxy-anthraquinon-3-yl-methyl-triphenylphosphonium bromide (**3**) was prepared according to Falk and Tran (10).

*Pyridinium-2,5-diiodo-1,4,6,8,13-pentahydroxy-10,11-dimethylphenanthro[1,10,9,8-opqra]perylene-7,14-dione-3-olate (**2**·pyridine; $C_{35}H_{19}I_2NO_8$).* To a solution of 35 mg **1** (0.105 mmol) in 12 cm^3 absolute pyridine, 1.2 cm^3 of a solution of I_2 in absolute pyridine (c = 0.4 mol·dm^{-3}) was added under an argon atmosphere and under protection from light. After stirring for 2 h pyridine was distilled off on a rotavapor and the residue was washed twice with a 20% aqueous solution of $Na_2S_2O_3$, three times with H_2O and dried overnight at high vacuum over P_4O_{10}. It should be noted that the pyridinium ion could not be removed by means of acidification of a solution of **2**·pyridine because as deduced by electrospray mass spectrum and ^{1}H nuclear magnetic resonance (^{1}H NMR†) spectra **2** became partially deiodinated by this operation. Yield: 76.4 mg (87%); m.p.: >300°C; ^{1}H NMR (200 MHz, δ, dimethyl sulfoxide-d_6): 15.76 (s, OH-1,6), 13.71 (s, OH-8,13), 8.88 (m, H_{pyr}-2,6), 8.50 (m, H_{pyr}-4), 7.98 (m, H_{pyr}-3,5), 7.33 (s, H-9,12), 2.69 (s, CH_3-10,11) ppm; ^{13}C NMR (50 MHz, δ, dimethyl sulfoxide-d_6): 182.7 (O=C-1,14), 170.4, 166.0, 161.3, 145.6, 143.8, 142.7, 127.0, 125.7, 125.14, 120.6, 120.2, 118.8, 115.6, 107.4, 101.5, 82.4 (C_{ar}), 23.9 (CH_3-10,11) ppm; electrospray mass spectrum: m/z = 755 ([M − H]$^-$); UV–Vis (pyridine; $c = 1 \times 10^{-5}$ mol·dm^{-3}): λ = 604 (50 900), 559 (14 000), 390 (12 080), 345 (33 950), 303 (37 000) nm (ε); fluorescence (pyridine; $c = 1 \times 10^{-7}$ mol·dm^{-3}, λ_{exc} = 550 nm): λ_{em} = 615 (1), 656 (0.6) nm (relative intensity); Φ_f = 0.02.

*(E)-1,3,8-Trimethoxy-6-styryl-anthraquinone (**4**; $C_{25}H_{20}O_5$).* A mixture of 512 mg **3** (0.78 mmol), 222 mg dry and freshly powdered K_2CO_3 (1.61 mmol), 149 mg 18-crown-6 (0.56 mmol) and 30 cm^3 CH_2Cl_2 (p.a., absolute) was refluxed for 15 min. To the resulting dark blue ylide solution 835 mg freshly distilled benzaldehyde (7.87 mmol) dissolved in 30 cm^3 CH_2Cl_2 (p.a., absolute) was added dropwise in three portions whereby after each addition the mixture was refluxed for 40 min. After refluxing the reaction mixture for an additional 30 min it was cooled to room temperature, diluted with 350 cm^3 CH_2Cl_2, filtered and extracted with saturated NaCl solution. The organic phase was dried with anhydrous Na_2SO_4, filtered and the solvent evaporated on a rotavapor. The resulting brown oil was triturated with 150 cm^3 ether, the yellow product was filtered, thoroughly washed with ether and dried on high vacuum. Yield: 306 mg (98%); m.p.: 183–188°C; ^{1}H NMR (200 MHz, δ, acetone-d_6): 7.93–6.86 (m, 9 H_{ar} + CH=CH), 4.02 (s, OCH_3), 3.99 (s, OCH_3), 3.96 (s, OCH_3) ppm; ^{13}C NMR (50 MHz, δ, acetone-d_6): 184.5 (C=O), 181.7 (C=O), 164.0, 162.0, 160.4 (3 C_{ar}–O), 142.9, 136.5, 133.7, 136.5, 129.1, 128.84, 128.80, 128.6, 127.2, 122.8, 120.2, 118.7, 117.1, 105.6, 102.2, (C=C + C_{ar}), 56.7 (OCH_3), 56.1 (OCH_3) ppm; UV–Vis (methanol; $c = 1 \times 10^{-5}$ mol·dm^{-3}): λ = 419 (7100), 293 (18 700), 225 (30 900) 201 (47 400) nm (ε).

*(E)-1,3,8-Trihydroxy-6-styryl-10H-anthracene-9-one (**5**; $C_{22}H_{16}O_4$).* A mixture of 104 mg **4** (0.26 mmol), 2.11 g $SnCl_2 \cdot 2H_2O$, 100 cm^3 HBr (47%) and 400 cm^3 glacial acetic acid (p.a.) was refluxed for 4 h. The reaction mixture was then cooled to room temperature, poured into 1500 cm^3 H_2O and centrifuged. The residue was washed twice with H_2O and dried over P_4O_{10} in high vacuum. Yield: 67 mg (75%); m.p.: 225–229°C; ^{1}H NMR (500 MHz, δ, acetone-d_6): 12.45 (s, OH-1), 12.32 (s, OH-8), 9.85 (s, OH-3), 7.65 (AA′-part of the AA′MM′X-system, H_{ph}-2,6), 7.44 (d, J = 16.3 Hz, H–C=C), 7.40 (M,M′-part, H_{ph}-3,5), 7.32 (X-part, H_{ph}-4), 7.21 (d, J = 16.3 Hz, C=C–H), 7.16 (s, H-5), 7.02 (s, H-7), 6.49 (m, H-4), 6.30 (m, H-2), 4.37 (s, CH_2) ppm; ^{13}C NMR (50 MHz, δ, acetone-d_6): 192.6 (C=O), 166.6, 165.8, 163.9 (3 C_{ar}–O), 146.1, 145.8, 143.4, 137.8, 133.6, 129.8, 129.4, 128.6, 128.3, 115.6, 113.4, 110.3, 110.1, 108.3, 102.2 (C=C + C_{ar}), 33.6 (CH_2) ppm; UV–Vis (methanol; $c = 1 \times 10^{-5}$ mol·dm^{-3}): λ = 388 (32 500) nm (ε).

*(E,E)-1,3,4,6,8,15-Hexahydroxy-10,13-distyryl-dibenzo[a,o]perylene-7,16-dione (**6**; $C_{44}H_{26}O_8$).* A mixture of 108.1 mg **5** (0.314 mmol), 4.4 mg $FeSO_4 \cdot 7H_2O$ (0.016 mmol), 162.2 mg pyridine-*N*-oxide (1.71 mmol), 1.6 cm^3 pyridine and 160 mm^3 piperidine were stirred under argon with protection from light for 1 h at 105°C. After cooling down to room temperature the dark blue reaction mixture was poured into 12 cm^3 HCl (c = 2 mol·dm^{-3}) and stirred for 30 min at room temperature in the dark. The precipitate was centrifuged, washed three times with 3% HCl, three times with H_2O and dried over P_4O_{10} under high vacuum. Yield: 106.5 mg (100%); due to its sensitivity it could be characterized only by its electrospray mass spectrum: m/z = 681 ([M − H]$^-$) and its qualitative UV–Vis: λ = 599 (33), 565 (38), 403 (59), 334 (100) nm (relative intensity).

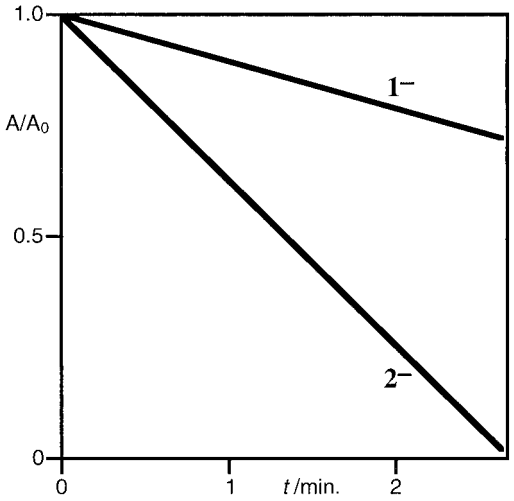


Figure 1. Normalized absorption changes (A/A_0) at λ = 457 nm with time of aerated solutions of disodium bilirubinate IXα (1×10^{-5} mol·dm^{-3}) containing $\mathbf{1}^-$ or $\mathbf{2}^-$ (3×10^{-6} mol·dm^3) in 80% aqueous ethanol upon irradiation at λ > 570 nm.

*(E,E)-1,3,4,6,8,13-Hexahydroxy-10,11-distyryl-phenanthro[1,10,9,8-opqra]perylene-7,14-dione (**7**; $C_{44}H_{24}O_8$).* When a solution of **6** in acetone was irradiated under bubbling with air using the usual method of preparation of **1** (8) a primary, but intermediate, product absorbing at 632 nm could be observed. However, because of its pronounced photosensibility we were not able to isolate it in a pure form. Therefore, the final product **8** from the intramolecular two-step photoreaction comprising **7** as its intermediate was prepared and characterized as described below.

*(E,E)-1,3,4,6,8,13-Hexahydroxy-10,11-diphenyl-9b,11a-dihydro-dibenzo[bcef]cyclobuta[m]coronene-7,14-dione (**8**; $C_{44}H_{24}O_8$).* A solution of 72 mg **6** in 1.8 dm^3 acetone (p.a.) (**6** was dissolved by means of sonication) was bubbled with air and then irradiated for 1.5 h by means of a 700 W tungsten lamp. The solvent of the resulting red solution was evaporated on a rotavapor and the residue separated by means of preparative thin layer chromatography (silica, 20 × 20 × 0.2 cm) using tetrahydrofuran/acetic acid = 10/1 as the eluent. Yield: 14.2 mg (20%); ^{1}H NMR (500 MHz, δ, dimethyl sulfoxide-d_6): 18.2 (br. s, OH), 14.5 (br. s, 4 OH), 7.7–6.6 (m, 14 H_{ar}), 4.13 (m, AB-part of ABCD-system, 2 $CH_{cyclobutane}$), 3.55 (m, CD-part of ABCD-system, 2 $CH_{cyclobutane}$) ppm; ^{13}C NMR (50 MHz, δ, dimethyl sulfoxide-d_6): 186.1 (C=O), 174.2 (C_{bay}–O), 167.0, 155.0, 145.7, 142.7, 141.1, 141.0, 140.6, 131.7, 131.6, 128.7, 127.4, 127.3, 125.5, 120.4, 114.6, 110.3, 67.4 (2 $CH_{cyclobutane}$), 38.4 (2 $CH_{cyclobutane}$) ppm; 2D C–H correlation cross peak between the ^{13}C and ^{1}H signals at 67.4 and 4.13 ppm and 38.4 and 3.55 ppm, respectively, electrospray mass spectrum: m/z = 679 ([M − H]$^-$); UV–Vis (acetone): λ = 585 (100), 543 (49), 505 (21), 470 (29), 342 (79) nm (relative intensity); fluorescence (acetone, λ_{exc} = 550 nm): λ_{em} = 590.4 (100), 637 (32) nm (relative intensity).

Photodestruction of bilirubin IXα. Following Hagenbuchner and Falk (11) irradiation of the thermostated (20.0 ± 0.1°C) aerated solutions of sodium bilirubinate, together with $\mathbf{1}^-$ or $\mathbf{2}^-$ in 80% aqueous ethanol contained in SiO_2 cuvettes (d = 1 cm), were performed by means of a 300 W tungsten lamp and a cut-off filter blocking light below 570 nm and measuring the UV–Vis spectra at intervals of 20 s (Fig. 1).

General. Melting points were measured by means of a Kofler hot stage microscope (Reichert, Vienna, Austria)—they are uncorrected. ^{1}H and ^{13}C NMR spectra were measured on the Bruker DPX 200 and DRX 500 spectrometers at 20°C using the solvent proton signal

as internal reference. Two-dimensional C–H correlation was measured using the pulse program INVIEAGSSI, Bruker XWINNMR 2.60, of the spectrometers standard software. UV–Vis spectra were recorded by means of a Hewlett–Packard 8453 instrument. Fluorescence was recorded by means of a Hitachi F-4010 spectrometer the quantum yield of $\mathbf{2}^-$ was derived using a fluorescence quantum yield estimate of $\mathbf{1}^-$ of $\Phi_f = 0.2$ (2) as comparison. Electrospray mass spectra were measured with a Hewlett–Packard 89987 quadrupole instrument.

RESULTS AND DISCUSSION

Heavy-atom substitution

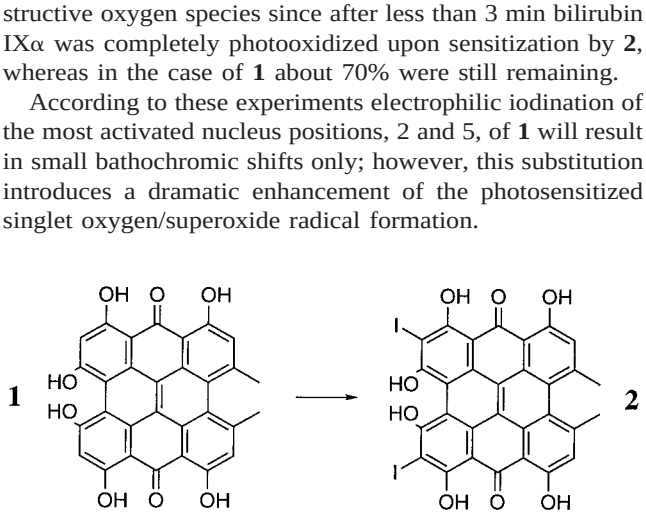
Because it is commonly known that heavy-atom substitution enhances intersystem crossing and thus also enhances singlet oxygen/superoxide radical formation, bromine has been hitherto introduced by means of electrophilic substitution to the hypericin nucleus to yield the 2,5-di-, 2,5,9-tri- and 2,5,9,12-tetrabromo-hypericins (12). In addition to lowered fluorescence quantum yields as compared to **1** these derivatives also showed enhanced light-mediated antiviral properties (13). Following this lead we now explored iodine substitution of **1**.

It turned out that upon reaction of **1** with I_2 in pyridine the electrophilic disubstitution product **2** was obtained as its pyridinium salt **2**·pyridine in high yield. To explore other substitution degrees iodination under changed conditions was applied. However, by using one equivalent of I_2 only a mixture of the mono- and di-iodination product of the reaction was obtained, which could not be separated satisfactorily. In contrast, a high molecular excess (1:40) of iodine did not result in any tri- or tetra-substituted derivatives.

The constitution of **2** as the 2,5-diiodo-derivative of **1** followed immediately from its ^{1}H NMR and mass spectra. The UV–Vis main absorption band of the pyridinium salt of **2** became only slightly bathochromically shifted to 604 nm as compared to the absorption band of the pyridinium salt of **1** at 602 nm (14). Compared to **1** the fluorescence quantum yield of **2** dropped to about 0.02.

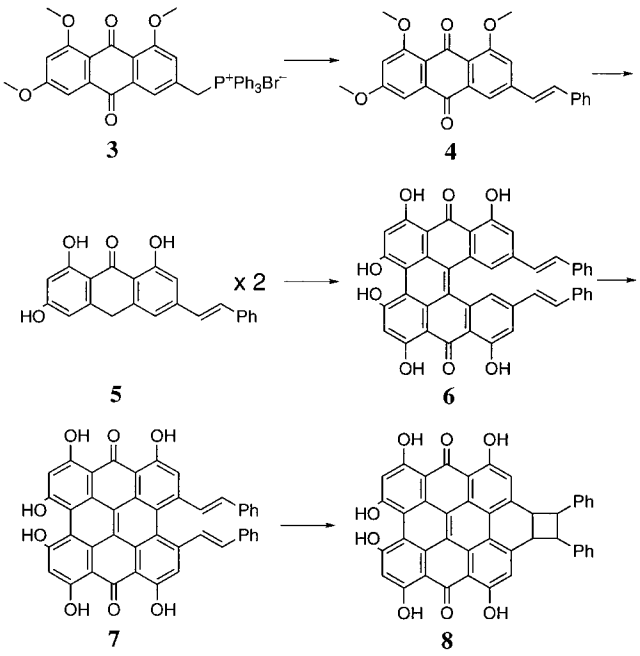
To test for an enhanced photochemical singlet oxygen/superoxide radical formation of **2** in comparison to **1** we used the recently introduced photosensitized bilirubin IXα destruction method (11). As shown in Fig. 1 this experiment proved that indeed there is enhanced formation of the destructive oxygen species since after less than 3 min bilirubin IXα was completely photooxidized upon sensitization by **2**, whereas in the case of **1** about 70% were still remaining.

According to these experiments electrophilic iodination of the most activated nucleus positions, 2 and 5, of **1** will result in small bathochromic shifts only; however, this substitution introduces a dramatic enhancement of the photosensitized singlet oxygen/superoxide radical formation.



Extension of conjugation

Extension of conjugation for derivatives of **1** with retention of the oxo and hydroxyl functionalities could be reached in principle by either attaching substituents to the free ring positions or by extending the conjugation of the hypericin moiety at the two methyl groups. In the first case, the additional substituent would become sterically congested by its neighboring functional groups, and therefore the resulting conjugation extension would become greatly diminished by torsional deformation unless the conjugation was achieved *via* an acetylene bond. In this case no problems from an interaction with the neighboring groups could occur. However, our attempts to prepare such derivatives by means of coupling of acetylenes with halo-hypericin or halo-emodin derivatives were so far in vain.



Therefore, we set out to prepare a stilbene analogous hypericin derivative in which the styryl groups were attached to the two ω,ω′-sites of the methyl groups. They were chosen because molecular modeling predicted only small torsional deformation at the styryl residues thus providing effective conjugation between the styryl and hypericin moieties. To achieve this goal we started with the emodin-derived phosphonium salt **3**, which recently had allowed for the preparation of an ω,ω′-appended 18 carbon chains hypericin derivative (10). Wittig reaction of the ylid derived from **3** with benzaldehyde provided the styryl-quinone **4** in nearly quantitative yield. Compound **4** was then regioselectively reduced and deprotected in an one-pot reaction using $SnCl_2$–acetic acid–HBr to yield about 75% of the styryl-anthrone **5**. Following the preparation of **1** described in detail by Falk *et al.* (8) the anthrone **5** was oxidatively dimerized to yield the protohypericin analogous compound **6**. The configurations (*E*) of **4**, **5** and **6** at the exocyclic double bonds were inferred from the analysis of the high-resolution ^{1}H NMR spectrum of **5** in which the vinyl and aromatic proton signals could

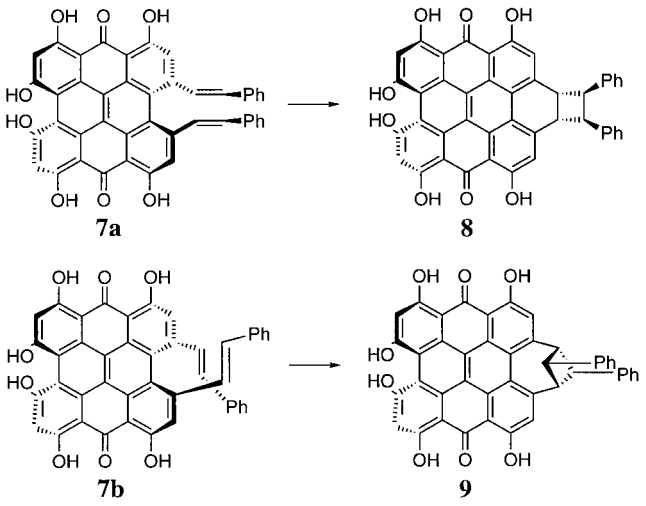


Figure 2. Intramolecular photochemical [2 + 2] cycloaddition of the two conformers **7a** and **7b** to yield the cyclobutane *trans,trans,cis*-**8** and *trans,cis,trans*-**9** diastereomers (arbitrary enantiomers drawn).

be unequivocally separated. Thus, the vinylic protons displayed a coupling constant of the H–C=C–H fragment of about 16 Hz, which is characteristic of an (*E*)-configured diastereomer.

The photocyclization of **6** to yield eventually the desired conjugation extended hypericin **7** provided an unexpected result. Upon irradiation of an aerated acetone solution of **6** an intermediate absorption at 632 nm developed in the absorption spectrum of the withdrawn samples but this was synchronously superseded by an absorption at 585 nm. Attempts to recover or isolate this intermediate to which we assign the constitution **7** and the configuration (*E,E*) failed because of its pronounced photosensitivity. Therefore, the final product of the two-step photoreaction was isolated and its constitution derived by mass and NMR spectroscopy was found to be that of a hypericin with a diphenyl-substituted cyclobutane ring attached at the ω,ω′-sites of the two methyl groups. A cyclobutane ring was formed by a photochemically allowed intramolecular [2 + 2] cycloaddition of the two vinyl groups (15). Nevertheless, this result was surprising because the analogous photochemical [2 + 2] cycloaddition of (*E*)-stilbene to yield mainly the *trans,trans,cis*-diastereomer **I** and to a small extent the *trans,cis,trans*-diastereomer **II** of 1,2,3,4-tetraphenyl-cyclobutane proceeded very sluggishly to yield only about 15% of the first and 2–5% of the latter isomer upon irradiation of stilbene solutions in benzene for up to 2 months (16–18).

With respect to the reaction mechanism, the constitution and stereochemistry of the final product molecular modeling (MM2 force field of ChemDraw) of the educt distyryl–hypericin **7** revealed that, in principle, there are two low-energy conformations, which might serve as starting points of the photoreaction. In the first one the two styryl groups are somewhat twisted against the twisted hypericin moiety as shown in Fig. 2 (**7a**). The other one (**7b**) is characterized by an overlap of the two double bonds within the conjugated system (Fig. 2). Photochemical [2 + 2] cycloaddition in the first case yields **8**, which would be formed as the

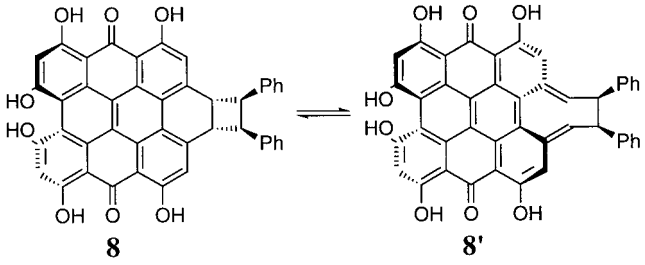


Figure 3. Valence tautomerism between the cyclobuta-cyclohexadiene tautomer **8** and the cyclooctatriene tautomer **8′**.

trans,trans,cis-diastereomer comparable to the configuration of the main product **I** of stilbene dimerization. Photochemical [2 + 2] cycloaddition in the second case would yield the *trans,cis,trans*-diastereomer **9** (Fig. 2) corresponding to the configuration of the minor product **II** of stilbene dimerization.

Now, the constitutional possibility **8** is consistent with the two carbon signals at 67.4 and 38.4 ppm in the ^{13}C NMR spectrum. The chemical shifts of the cyclobutane protons of our final product were observed at about 4.13 and 3.55 ppm as the AB and CD parts of an ABCD system, and they were found to be correlated to the carbon signals at 67.4 and 38.4 ppm. Correlations and symmetry thus proved the constitution **8** for the final product. This structural assignment is further corroborated by the earlier finding (18) that for stilbene dimerization the diastereomer **I**, which corresponds to the stereochemistry of **8**, is the one that is mainly formed.

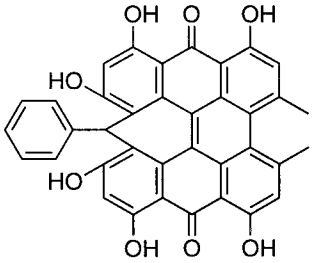
The unusual high chemical shift value for two cyclobutane carbon atoms at 67.4 ppm might be explained by a fast valence tautomerism involving the cyclobuta-cyclohexadiene unit of **8** and the cyclooctatriene system of **8′** as displayed in Fig. 3. Such a fast equilibrium between **8** and **8′** would lead to the signal of two carbon atoms bearing the phenyl substituents within the normal shift region of cyclobutanes at about 40 ppm. The chemical shift of the other two carbon atoms at nearly 70 ppm would then be the result of averaging the shift of an aromatic (120 ppm) and a cyclobutane (40 ppm) carbon type atom. Interestingly enough, molecular modeling showed these two tautomers to be of similar energy.

CONCLUSIONS

The results showed that heavy-atom substitution, *e.g.* by iodine, of hypericin (**1**) at the ring sites provides a good means to enhance the desired photosensitized singlet oxygen/superoxide radical formation. Extending the conjugation of the hypericin system by attaching styryl moieties to the methyl groups of **1** failed in effect because of the very high photoreactivity of the resulting double bonds to undergo a [2 + 2] cycloaddition.

Interestingly enough, this reaction constitutes one of the rare exceptions of hypericin derivatives exhibiting intramolecular photochemistry as exemplified with 3-*O*-benzyl-hypericin and 3,4-di-*O*-benzyl-hypericin. The first derivative resulted in the formation of the blepharismin type compound **10** when irradiated in presence of 1,8-bis-dimethylaminonaphthalene (19). The second compound produced, under the same conditions, a stable radical ion pair (20).

Nevertheless, the experiments described above proved that the long-wavelength absorption band can be considerably bathochromically shifted by conjugation extension with two styryl residues. Attempts are now under way in our laboratory to extend hypericin conjugation by means of condensing additional benzene rings to the methyl bearing sides of the hypericin nucleus or by attaching conjugation extension groups to one methyl group of hypericin only.



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Acknowledgement—Advice of Prof. Dr. N. Müller with regard to NMR interpretations is gratefully acknowledged.

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