Photochemistry and Photobiology, 2009, 85: 1201–1206

Cationic Hypericin Derivatives as Novel Agents with Photobactericidal Activity: Synthesis and Photodynamic Inactivation of *Propionibacterium acnes*

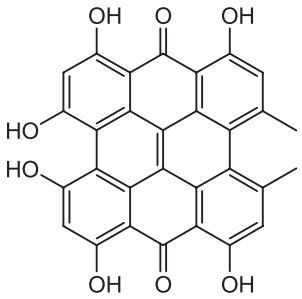
Beate Hager[1], Wolfgang S. L. Strauss[2] and Heinz Falk*[1]

[1]Institute of Organic Chemistry, Johannes Kepler University, Linz, Austria
[2]Institute of Laser Technologies in Medicine and Metrology at the University of Ulm, Ulm, Germany

Received 8 April 2009, accepted 27 April 2009, DOI: 10.1111/j.1751-1097.2009.00587.x

ABSTRACT

The present communication describes for the first time the synthesis and preliminary testing of two cationic hypericin derivatives. Uncharged hypericin derivatives with ω,ω'-attached C_2-linkers leading to a pyridyl or a 4-dimethylaminophenyl residue were prepared and subsequently quaternized by means of iodomethane. Photobactericidal activity was assessed using *Propionibacterium acnes*. The quaternary *N*,*N*,*N*-trimethyl-anilinium derivative displayed a pronounced photodynamic inactivation of the bacteria at low incubation concentrations (< 100 nM) and a short incubation time (1 h) after illumination with yellow light (590 nm, 20 J cm^{-2}), whereas the photobactericidal efficacy of the *N*-methyl-pyridinium derivative was negligible under identical experimental conditions.



1

Formula 1

INTRODUCTION

The powerful photosensitizer hypericin (**1**) occurring in a variety of organisms down to the Jurassic (1,2) is a promising candidate for photodynamic therapy (PDT) of superficial cancers, *e.g.* bladder cancer (3–5), or as a photovirucidal agent (6,7). Although it exhibits high quantum yields for the generation of both the long-lived triplet state and the cytotoxic reactive oxygen species (8,9) widespread application is impeded by its poor solubility in aqueous solvents. Recently, a water soluble formulation of **1** has been successfully applied for fluorescence guided resection of bladder cancer (10).

During the last decade its fundamental chromophore has been derivatized in order to generate second-generation agents (11). These efforts resulted in analogs with redshifted absorption spectra to provide compounds excitable at wavelengths with enhanced penetration depth on the one hand (12–17), and better physicochemical properties, *e.g.* higher solubility under physiological conditions or targeting of specific cellular sites, on the other hand (18–25).

Photodynamic therapy has obtained regulatory approval for the treatment of superficial cancers and precancerous lesions like actinic keratosis and nononcological disease, *e.g.* age-related macular degeneration. In addition, PDT is also suggested as novel therapeutic modality for the treatment of localized microbial infections, *e.g.* infections caused by bacteria (26–28); this topic was initiated by the discovery that photosensitization with compounds positively charged at physiological pH values (29–31) resulted in efficient inactivation of Gram-positive and Gram-negative bacteria. Besides phenothiazines (29,32), compounds of different classes of chromophores well known in PDT, *e.g.* porphyrins (31,33) and phthalocyanines (30), as well as, *e.g.* benzo[*a*]phenoxazinium analogues (34,35) and cationic fullerene C60 derivatives (36), have been synthesized and evaluated with respect to their photobactericidal activity.

Photodynamic therapy is regarded as an alternative modality for the treatment of *Acne vulgaris*. Successful therapy requires disruption of the interchange of the pathogenic factors in acne: hyperkeratinization of the pilosebaceous follicles, proliferation of *Propionibacterium* spp. (in particular, *Propionibacterium acnes* and *Propionibacterium granulosum*), and inflammation (37,38). At present, PDT of acne is clinically performed with topically applied 5-aminolevulinic acid (ALA) or its methyl ester, which are endogenously converted to photosensitizing porphyrins, and illumination in the yellow-red spectral region. Besides lasers (continuous wave and pulsed systems) a variety of different incoherent, broadband light sources (emitting typically between 500 nm and 700 nm) are available for routine clinical application.

Although the role of *Propionibacterium* spp. in the pathogenesis of acne remains unclear, impairment of bacterial proliferation seems to be essential for successful treatment (37,39). Thus, targeted PDT using cationic photosensitizers

*Corresponding author email: heinz.falk@jku.at (Heinz Falk)

might be considered as an alternative treatment concept minimizing damage of pilosebaceous follicles, which has been described for ALA-PDT (40,41).

In the present communication, we describe for the first time the synthesis and preliminary testing of two cationic hypericin derivatives. Attachment of charged substituents at the two ω-methyl groups of **1** provides unperturbed photosensitization of singlet oxygen and/or reactive oxidizing species as assessed previously (11); as quaternary salt moieties, the *N*,*N*,*N*-trimethyl-anilinum and the *N*-methyl-pyridinium ions were chosen. Photobactericidal activity was assessed using *P. acnes*.

MATERIALS AND METHODS

General. The products were characterized using m.p. (Kofler microscope, Reichert), ^{1}H NMR (Bruker DPX 200 and DRX 500 MHz—signal assignments were proven by means of 2D spectra) (NOESY, HMBC), IR (Bruker Tensor 27, KBr), MS (Thermofinnigan LCQ Deca XP Plus and Agilent MSD Trap SL), fluorescence (Varian Cary Eclipse fluorescence instrument), and UV-Vis data (Varian Cary 100 Bio). Fluorescence quantum yields were calculated according to the comparative method of Williams *et al*. (42) using hypericin (**1**) as standard sample. The production of singlet oxygen/oxidizing species by **11–14** was monitored by bilirubin-IXα-degradation according to Ref. (43). The long-wavelength "hypericinic" absorption band intensities of **1** and the four compounds were made equal by adjusting concentrations to provide comparable light absorptions for all compounds investigated. Emodin was hydrolytically extracted from *Cortex frangulae* as described in Ref. (44) and **2** was prepared as described in Ref. (45).

*(Z)- and (E)-6-(2-(Pyridin-4-yl)vinyl)-1,3,8-trimethoxy-anthraquinone (**3**)*. A suspension of **2** (0.50 g; 0.77 mM), dried K_2CO_3 (0.20 g; 1.45 mM), and 18-crown-6 (0.15 g; 0.57 mM) in 30 mL dry CH_2Cl_2 was refluxed under argon for 15 min. To this dark-blue ylide solution pyridine-4-carbaldehyde (1.66 g; 15.5 mM) dissolved in 30 mL dry CH_2Cl_2 was added drop-wise in three portions with 40 min reflux intermissions in between. After refluxing for further 30 min, the reaction mixture was filtered, diluted with 50 mL CH_2Cl_2, and extracted with brine. Re-extraction with CH_2Cl_2, drying of the combined organic layers over Na_2SO_4, and evaporation resulted in a yellow colored oil, which crystallized upon addition of diethyl ether yielding 0.27 g (87%) of **3**, $C_{24}H_{19}NO_5$: TLC (silica 60; $CHCl_3$:MeOH = 15:1 (*v:v*)): R_f = 0.51 (*E*)-isomer, 0.46 (*Z*)-isomer; m.p. 176-179°C; ^{1}H NMR (500 MHz, DMSO-d$_6$, 30°C): (*E*)-isomer: δ = 8.61 (d, J = 5.8 Hz, *Py*-H2,6), 7.94 (s, ar-H5), 7.77 (s, ar-H7), 7.72 (d, J = 16.5 Hz, 6-C*H* = CH-), 7.64 (d, J = 5.8 Hz, *Py*-H3,5), 7.57 (d, J = 16.5 Hz, 6-CH = C*H*-), 7.20 (d, J = 2.3 Hz, ar-H4), 7.00 (d, J = 2.3 Hz, ar-H2), 3.99 (s, ar-OCH$_3$), 3.95 (s, ar-OCH$_3$), 3.91 (s, ar-OCH$_3$) ppm; (*Z*)-isomer: δ = 8.51 (d, J = 5.8 Hz, *Py*-H2,6), 7.50 (s, ar-H5), 7.29 (s, ar-H7), 7.24 (d, J = 5.8 Hz, *Py*-H3,5), 7.14 (d, J = 2.3 Hz, ar-H4), 6.98 (d, J = 2.3 Hz, ar-H2), 6.96 (d, J = 12.3 Hz, 6-C*H* = CH-), 6.86 (d, J = 12.3 Hz, 6-CH = C*H*-), 3.92 (s, ar-OCH$_3$), 3.89 (s, ar-OCH$_3$), 3.70 (s, 8-OCH$_3$) ppm; UV-Vis (MeOH + 10% $CHCl_3$): λ_{max} (rel. intensity) = 300 (100), 415 (30) nm; MS (ESI-MS; MeOH:$CHCl_3$ = 1:1 + 1% HCOOH; $\gamma \sim 1$ mg·cm^{-3}; positive ion mode): m/z = 402 $(M+H)^+$.

*(E)-6-(4-(Dimethylamino)styryl)-1,3,8-trimethoxyanthraquinone (**4**)*. Preparation according to the procedure given above by reacting **2** with 4-dimethylamino-benzaldehyde (1.15 g; 7.7 mM) provided 90 mg (28%) of **4**, $C_{27}H_{25}NO_5$: Properties were found to be identical with those described in Ref. (46).

*6-(2-(Pyridin-4-yl)ethyl)-1,3,8-trimethoxy-anthraquinone (**5**)*. Compound **3** (100 mg; 0.25 mM) was dissolved in 250 mL methanol. Thereafter, 25 mL $CHCl_3$ and 30 mg Pd/C (10%) were added. After purging with argon for 15 min the solution was stirred under H_2 and ambient conditions for 20 h. After filtration and evaporation 97 mg (96%) of **5** was obtained, $C_{24}H_{21}NO_5$: m.p. 149-153°C; ^{1}H NMR (500 MHz, DMSO-d$_6$, 30°C): δ = 8.80 (d, J = 4.9 Hz, Py-H2,6), 7.94 (d, J = 4.9 Hz, Py-H3,5), 7.57 (s, ar-H5), 7.45 (s, ar-H7), 7.16 (d, J = 2.2 Hz, ar-H4), 6.98 (d, J = 2.2 Hz, ar-H2), 3.93 (s, 3-OCH$_3$), 3.894 (s, 1-OCH$_3$), 3.889 (s, 8-OCH$_3$), 3.27 (t, J = 7.8 Hz, 6-CH$_2$-C*H*$_2$-), 3.14 (t, J = 7.8 Hz, 6-C*H*$_2$-CH$_2$-) ppm; ^{13}C NMR (125 MHz, δ, DMSO-d$_6$, 30°C): 183.2 (C10), 179.8 (C9), 163.4 (C3), 161.1 (C1), 160.7 (*Py*-C4), 159.0 (C8), 146.9 (C6), 141.9 (*Py*-C2,6), 135.5 (C4a), 133.9 (C10a), 126.8 (*Py*-C3,5), 121.7 (C8a), 119.2 (C7), 118.2 (C5), 117.5 (C9a), 105.0 (C2), 102.3 (C4), 56.4 (1-OCH$_3$ or 8-OCH$_3$), 56.3 (8-OCH$_3$ or 1-OCH$_3$), 55.9 (3-OCH$_3$), 35.7 (6-CH$_2$-C*H*$_2$-), 35.0 (6-C*H*$_2$-CH$_2$-) ppm; IR (KBr): $\bar{\nu}$ = 3377, 3215, 3076, 2925, 2843, 1663, 1634, 1601, 1561, 1501, 1465, 1351, 1327, 1243, 1207, 1130, 1073, 1019, 944, 875, 848, 756 cm^{-1}; UV-Vis (MeOH + 10% $CHCl_3$): λ_{max} (rel. intensity) = 256 (94), 262 (97), 276 (100), 405 (24) nm; MS (ESI-MS; MeOH:$CHCl_3$ = 1:1 + 1% HCOOH; $\gamma \sim 1$ mg·cm^{-3}; positive ion mode): m/z = 404 $(M+H)^+$.

*6-(4-(Dimethylamino)phenethyl)-1,3,8-trimethoxy-anthraquinone (**6**)*. Prepared by hydrogenation of **4** in analogy to **5** in 95% yield, $C_{27}H_{27}NO_5$: m.p. 81-84°C; ^{1}H NMR (500 MHz, $CDCl_3$, 30°C): δ = 7.65 (d, J = 6.5 Hz, *Ph*-H3,5), 7.60 (s, ar-H5), 7.31 (m, 3H, *Ph*-H2,6 and ar-H7), 7.00 (s, ar-H4), 6.78 (s, ar-H2), 3.98 (s, 1-OCH$_3$ or 3-OCH$_3$), 3.97 (s, 3-OCH$_3$ or 1-OCH$_3$), 3.94 (s, 8-OCH$_3$), 3.14 (s, 6H, N-CH$_3$), 3.03 (s, 4H, 6-CH$_2$-CH$_2$-) ppm; ^{13}C NMR (125 MHz, δ, $CDCl_3$, 30°C): 184.3 (C10), 181.7 (C9), 164.0 (C3), 161.9 (C1), 160.0 (C8), 147.2 (*Ph*-C1), 143.4 (C6), 141.3 (C4a), 136.5 (C10a), 134.8 (*Ph*-C4), 130.6 (C7), 122.4 (C8a), 118.5 (C9a), 120.9 (*Ph*-C3,5) 119.0 (C5), 118.6 (C4), 105.4 (C2), 102.2 (*Ph*-C2,6), 56.7 (1-OCH$_3$ od. 3-OCH$_3$), 56.6 (3-OCH$_3$ od. 1-OCH$_3$), 56.0 (8-OCH$_3$), 46.7 (N-CH$_3$), 37.8 (6-CH$_2$-C*H*$_2$-), 36.5 (6-C*H*$_2$-CH$_2$-) ppm; UV-Vis (MeOH + 10% $CHCl_3$): λ_{max} (rel. intensity) = 228 (100), 270 (98), 404 (14) nm; IR (KBr): $\bar{\nu}$ = 3375, 3215, 3074, 2926, 2854, 1668, 1597, 1515, 1459, 1325, 1244, 1203, 1162, 1130, 1070, 1018, 946, 754 cm^{-1}; MS (ESI-MS; MeOH; $\gamma \sim 1$ mg·cm^{-3}; positive ion mode): m/z = 447 $(M+H)^+$.

*1,3,8-Trihydroxy-6-(2-(pyridin-4-yl)ethyl)-10H-anthracen-9-one (**7**)*. To a refluxing solution of **5** (70 mg; 0.17 mM) in 10 mL glacial acetic acid $SnCl_2$x2H_2O (307 mg) dissolved in 5 mL aqueous HBr solution (47%) was added under argon. The reaction mixture was further refluxed for 1 h, cooled to room temperature, and poured into ice. The suspension was centrifuged at 5000 ***g***, the residue washed with distilled H_2O (3x), and dried under vacuum, which yielded 50.1 mg (85%) of **7**, $C_{21}H_{17}NO_4$: ^{1}H NMR (500 MHz, DMSO-d$_6$, 30°C): δ = 12.35 (s, 1-OH or 3-OH), 12.22 (s, 8-OH or 1-OH), 10.82 (s, 8-OH), 8.54 (d, J = 4.1 Hz, *Py*-H2,6), 7.43 (d, J = 4.1 Hz, *Py*-H3,5), 6.86 (s, ar-H5), 6.76 (s, ar-H7), 6.44 (s, ar-H4), 6.24 (s, ar-H2), 4.31 (s, -CH$_2$-), 3.00 (d, J = 7.2 Hz, 6-CH$_2$-C*H*$_2$-), 2.97 (d, J = 7.2 Hz, 6-C*H*$_2$-CH$_2$-) ppm; UV-Vis (DMSO): λ_{max} (rel. intensity) = 261 (75), 358 nm (100); MS (ESI-MS; MeOH:$CHCl_3$ = 1:1 + 1% HCOOH; $\gamma \sim 1$ mg·cm^{-3}; positive ion mode): m/z = 348 $(M+H)^+$.

*2-(4-(Dimethylamino)phenethyl)-4,5,7-trihydroxy-anthracen-10(9H)-one (**8**)*. Prepared in analogy to **7** in 79% yield, $C_{24}H_{23}NO_4$): ^{1}H NMR (200 MHz, DMSO-d$_6$, 30°C): δ = 12.37 (s, 1-OH or 3-OH), 12.20 (s, 3-OH or 1-OH), 10.80 (s, 8-OH), 7.06 (d, J = 8.4 Hz, *Ph*-H3,5), 6.85 (s, ar-H5), 6.72 (s, ar-H7), 6.65 (d, J = 8.4 Hz, *Ph*-H2,6), 6.43 (s, ar-H4), 6.23 (s, ar-H2), 4.32 (s, -CH$_2$-), 2.84 (m, 10H, 6-CH$_2$-CH$_2$- + -CH$_3$) ppm; UV-Vis (DMSO): λ_{max} (rel. intensity) = 262 (100), 362 nm (65); MS (ESI-MS; MeOH; $\gamma \sim 1$ mg·cm^{-3}; positive ion mode): m/z = 390 $(M+H)^+$.

*1,3,4,6,8,15-Hexahydroxy-10,13-bis(2-(pyridin-4-yl)ethyl)-dibenzo[ao]perylen-7,16-dione (**9**)*. A mixture of **7** (172 mg; 0.50 mM), $FeSO_4 \times 7H_2O$ (6.4 mg; 0.02 mM), pyridine-*N*-oxide (254 mg; 2.67 mmol), 2.5 mL abs. pyridine, and 0.25 mL piperidine was stirred for 1 h at 115°C under argon and light protection. After cooling 8.4 mL 2*M* HCl was added and the mixture stirred for 30 min at room temperature. The precipitate was centrifuged at 5000 ***g***, washed with 3% HCl (3x) and distilled H_2O (3x), and dried under vacuum over P_2O_5 and exclusion of light resulting in **9**, $C_{42}H_{28}N_2O_8$: UV-Vis (methanol): λ_{max} (rel. intensity) = 375 (100), 545 (76), 582 nm (70). Due to its light sensitivity this intermediate was immediately photocyclized without further characterization.

*10,13-bis(2-(4-(Dimethylamino)phenethyl)-1,3,4,6,8,15-hexahydroxy-dibenzo[ao]perylen-7,16-dione (**10**)*. Prepared in analogy to **9,** $C_{48}H_{40}N_2O_8$: UV-Vis (methanol): λ_{max} (rel. intensity) = 375 (94), 550 (99), 575 nm (100). Due to its light sensitivity this product was immediately photocyclized.

*1,3,4,6,8,13-Hexahydroxy-10,11-bis(2-(pyridin-4-yl)ethyl)-phenanthro[1,10,9,8-opqra]perylen-7,14-dione (**11**)*. Protohypericin derivative **9** was dissolved in 2 L methanol (p. a.) and irradiated with a 700 W Hg high-pressure lamp and air admission for 1 h. The wine-red solution was filtered over Celite and evaporated. The residue was chromatographed over Sephadex LH20 with methanol as eluent providing 81.5 mg **11** in 48% yield (based on **7**), $C_{42}H_{26}N_2O_8$: m.p. >350°C; ^{1}H NMR (500 MHz, MeOH-d_4, 30°C): δ = 7.58 (s, ar-H9 and ar-H12), 7,52 (d, J = 4.0 Hz, 2*Py*-H2,6), 6.79 (s, ar-H2 and ar-H5), 6.63 (d, J = 4.0 Hz, 2*Py*-H3,5), 3.11 (m, 4H, 10-C*H*$_2$-CH$_2$- and 11-C*H*$_2$-CH$_2$-), 2.46 (m, 4H, 10-CH$_2$-C*H*$_2$- and 11-CH$_2$-C*H*$_2$-) ppm (OH signals were not found due to H/D exchange); IR (KBr): $\bar{\nu}$ = 3418, 3075, 2947, 2834, 1600, 1504, 1464, 1427, 1250, 1188, 1115, 1029, 848, 802 cm^{-1}; UV-Vis (methanol; $c = 2.20\cdot10^{-4}$ M): λ_{max} (ε / L mol^{-1} cm^{-1}) = 329 (2278), 381 (1128), 446 (1028), 475 (1110), 511 (841), 548 (1737), 592 nm (3147); fluorescence (methanol; $c = 1.03\cdot10^{-5}$ M, λ_{ex} = 550 nm): λ_{em} (rel. intensity) = 595 (100), 644 nm (30) / Φ_f = 0.1; MS (ESI-MS; MeOH; $\gamma \sim 1$ mg·cm^{-3}; negative ion mode): m/z = 685 ([M-H])$^-$.

*3,4-bis(4-(Dimethylamino)phenethyl)-1,6,8,10,11,13-hexa-hydroxyphenanthro[1,10,9,8-opqra]perylen-7,14-dione (**12**)*. Prepared from **10** in analogy to **11** and obtained in 27% yield (based on **10**), $C_{48}H_{38}N_2O_8$: m.p. >350°C; ^{1}H NMR (500 MHz, MeOH-d_4, 30°C): δ = 7.58 (s, ar-H2 and ar-H5), 6.81 (s, ar-H9 and ar-H12), 6.30 (d, J = 8.1 Hz, *Ph*-H3,5), 6.15 (d, J = 8.1 Hz, *Ph*-H2,6), 2.95 (m, 3-C*H*$_2$-CH$_2$- and 4-C*H*$_2$-CH$_2$-), 2.38 m, (4-CH$_2$-C*H*$_2$- and 3-CH$_2$-C*H*$_2$-), 2.29 (s, -N-CH$_3$) ppm (OH signals were not found due to H/D exchange); IR (KBr): $\bar{\nu}$ = 3385, 2925, 1600, 1465, 1425, 1252, 1185, 1113, 994, 847 cm^{-1}; UV-Vis (methanol; $c = 1.44\cdot10^{-5}$ M): λ_{max} (ε / L mol^{-1} cm^{-1}) = 325 (15069), 388 (7222), 442 (7569), 511 (5417), 548 (10069), 591 nm (16597); fluorescence (methanol; $c = 1.44\cdot10^{-5}$ M, λ_{ex} = 550 nm): λ_{em} (rel. intensity) = 596 (100), 643 (35) / Φ_f = 0.01; MS (ESI-MS; MeOH; $\gamma \sim 1$ mg·cm^{-3}; negative ion mode): m/z = 769 ([M-H])$^-$.

*4,4′-(2,2′-(1,6,8,10,11,13-Hexahydroxy-7,14-dioxo-7,14-dihydrophenanthro[1,10,9,8-opqra]perylen-3,4-diyl)bis(ethan-2,1-diyl))bis(1-methylpyridinium)iodide (**13**)*. Compound **11** (15.0 mg, 0.02 mM) was dissolved in 10 mL methanol p.a. and 3 mL CH_3I were added. Thereafter, the mixture was stirred for 30 min and refluxed for 4 h. After evaporation 20.0 mg (100%) of the quaternary ammonium salt **13** were obtained, $C_{44}H_{32}I_2N_2O_8$: m.p. >350°C; ^{1}H NMR (500 MHz, MeOH-d_4, 30°C): δ = 7.60 (s, ar-H2 and ar-H5), 7.56 (d, J = 6.0 Hz, 2*Py*-H2,6), 6.80 (s, ar-H9 and ar-H12), 6.70 (d, J = 6.0 Hz, 2*Py*-H3,5), 2.53 (m, 3-CH$_2$-C*H*$_2$- and 4-CH$_2$-C*H*$_2$-) ppm (OH signals were not found due to H/D exchange and the 3-CH$_2$-CH$_2$ and 4-CH$_2$-CH$_2$- signals were covered by the solvent signal; IR (KBr): $\bar{\nu}$ = 3446, 2929, 2856, 1622, 1543, 1464, 1424, 1379, 1250, 1186, 1114, 796 cm^{-1}; UV-Vis (methanol; $c = 1.01\cdot10^{-3}$ M): λ_{max} (ε / L mol^{-1}·cm^{-1}) = 358 (1255), 548 (145), 592 nm (267); fluorescence (methanol; $c = 1.01\cdot10^{-4}$ M, λ_{ex} = 550 nm): λ_{em} (rel. intensity) = 596 (100), 644 nm (30) / Φ_f = 0.08; no mass spectrum could be obtained using various ionization methods.

*4,4′-(2,2′-(1,6,8,10,11,13-Hexahydroxy-7,14-dioxo-7,14-dihydrophenanthro-[1,10,9,8-opqra]perylen-3,4-diyl)bis(ethan-2,1-diyl))bis(N,N,N-trimethyl-benzenaminium)iodide (**14**)*. The quaternary salt **14** was quantitatively obtained from **12** in analogy to **13**. $C_{50}H_{44}I_2N_2O_8$: m.p. >350°C; ^{1}H NMR (500 MHz, MeOH-d_4, 30°C): δ = 7.73 (m, *Ph*-H2,6), 7.63 (s, ar-H2 and ar-H5), 6.82 (s, ar-H9 and ar-H12), 6.40 (m, *Ph*-H3,5), 3.72 (s, -N-CH$_3$) ppm (OH signals were not found due to H/D exchange the 3-CH$_2$-CH$_2$ and 4-CH$_2$-CH$_2$- signals were covered by the solvent signal), UV-Vis (methanol; $c = 2.97\cdot10^{-4}$ mol·dm^{-3}): λ_{max} (ε / L mol^{-1}·cm^{-1}) = 376 (9378), 549 (505), 592 nm (875); fluorescence (methanol; $c = 2.97\cdot10^{-5}$ M, λ_{ex} = 550 nm): λ_{em} (rel. intensity) = 597 (100), 645 nm (26)/Φ_f = 0.04; IR (KBr): $\bar{\nu}$ = 3446, 2926, 2855, 1621, 1463, 1402, 1250, 1187, 1120, 1005, 796, 618 cm^{-1}; no mass spectrum could be obtained using various ionization methods.

Photodynamic inactivation. To determine the photodynamic efficacy of **11–14**, a culture of *Propionibacterium acnes* (ATCC-No. 6919, DSM-No. 1897; provided by the Institute of Medical Microbiology and Hygiene, University of Ulm, Ulm, Germany) was prepared (McFarland 0.5, *p*H 7, BBL™ Thioglycolate medium from Becton Dickinson) and incubated for 24 h under anaerobic condition. Thereafter, a second culture was prepared (McFarland 0.5, *p*H 7; 7.5 mL) and subdivided into three equal aliquots, which were incubated with 100 nM of **1** or the corresponding derivatives **11–14** at 37°C for 1 h (anaerobic condition was not attained within the incubation period). From each of these cultures two aliquots of 1.1 mL were transferred to a 4-well plate. One well was irradiated with yellow light (LED source, WaveLight EN001C, 590 nm, 110 mW cm^{-2}) at room temperature in ambient atmosphere and under subdued light, whereas the other one served as dark control. The aliquots were illuminated for different times, so that light doses up to 40 J cm^{-2} were attained. In each case, 100 μL aliquots were diluted several 10-fold and 10 μL-aliquots of each dilution were plated on Schaedler blood-agar Petri dishes (heipha Dr. Müller) and cultivated under anaerobic condition. After 3 days, cell viability was assessed by counting the colonies. Survival rates were calculated as percentages of the dark controls. In the case of derivative **14**, photodynamic inactivation of *P. acnes* was additionally assessed for incubation concentrations of 50 and 500 nM as described above.

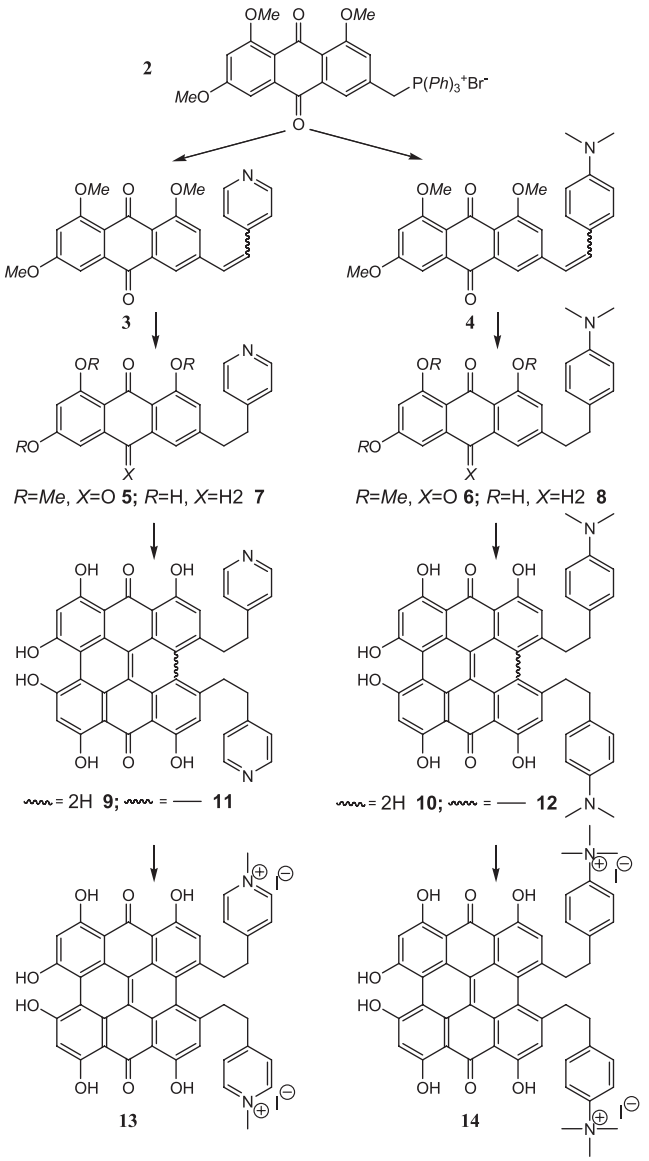
RESULTS AND DISCUSSION

Synthesis

As the starting material for the synthesis of the two envisaged cationic hypericin analogs, the easily available phosphonium salt **2** (45) was chosen and reacted in a Wittig reaction in the presence of 18-crown-6 and potassium carbonate with pyridine-4-carbaldehyde or 4-dimethylamino-benzaldehyde (Ehrlich aldehyde). To avoid a cycloaddition between the two adjacent double bonds in the corresponding hypericin derivatives, the products **3** and **4** thus available in modest yields could be easily and nearly quantitatively hydrogenated using palladium on charcoal as catalyst and methanol as solvent under ambient conditions to **5** and **6**. The latter were deprotected and reduced in acceptable yields to the anthrone derivatives **7** and **8** in the common way (12–17) using $SnCl_2$/HBr in glacial acetic acid. Dimerization by means of $Fe(SO_4)_2$/pyridine-*N*-oxide in pyridine and piperidine as the catalyst (12–17) provided the protohypericin derivatives **9** and **10**, which were immediately photocyclized to the hypericin derivatives **11** and **12**. The overall yields of these steps from anthrone to hypericin derivatives were also rather modest. Finally, methylation of **11** and **12** with iodomethane quantitatively provided the corresponding quaternary ammonium salts **13** and **14**. All precursors **3–10** as well as the hypericin derivatives **11** and **12** could be nicely characterized by means of spectroscopic techniques. In the case of the salts **13** and **14,** no detailed characterization could be achieved. However, quaternization of tertiary amines is regarded as an analytical characterization on its own—the quantitative yields corresponding to the addition of two mol equivalents of iodomethane and the distinctive shifts of the aromatic proton signals in the vicinity of the charges are sufficient evidence for their proper constitutions (Scheme 1).

Photosenitizing properties

The pyridine and aniline derivatives **11** and **12**, their respective quaternized derivatives **13** and **14**, and hypericin (**1**) were irradiated in the presence of sodium bilirubinate IXα. This is a simple method to determine photosensitized production of singlet oxygen and/or reactive oxygen species from molecular oxygen in aerated solutions by monitoring the oxidative destruction of bilirubin IXα (43). The results displayed in Fig. 1 reveal a slightly better activity of the pyridine derivative **11** as compared with hypericin (**1**), whereas the aniline derivative **12** is more or less nonsensitizing. This latter effect has already been observed with a corresponding π-conjugated ω-(4-dimethylaminobenzal)-hypericin derivative (46) where we suggested that



Scheme 1.

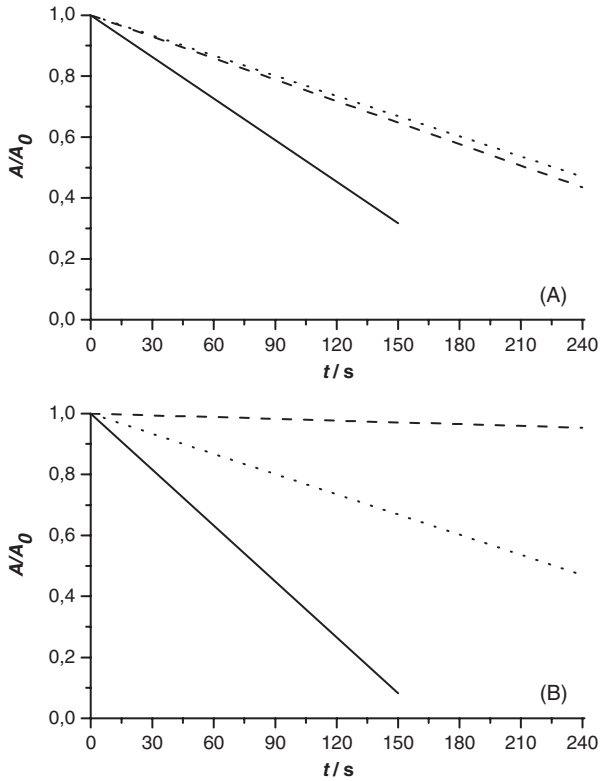


Figure 1. Hypericin derivative sensitized photooxidation of bilirubin IXα: normalized absorption (A/A_0) *vs* time curves of solutions of disodium bilirubinate IXα together with the sodium salts of (A) hypericin (**1**) (…), the hypericin derivative **11** (– – –) and the corresponding quaternary salt **13** (—), and (B) hypericin (**1**) (…), the hypericin derivative **12** (– – –) and the corresponding quaternary salt **14** (—).

this effect might be due to an intramolecular charge transfer as the predominant de-excitation pathway in the excited state. Obviously, lack of π-conjugation in **12** does not block this pathway. The rather small fluorescence quantum yields observed point into the same direction (Φ_f(**11**) = 0.1, Φ_f(**12**) = 0.01, Φ_f(**13**) = 0.08, Φ_f(**14**) = 0.04). Both quaternized derivatives **13** and **14** display an even higher production of singlet oxygen and/or reactive oxygen species than **1**, with the anilinium derivative **14** as the most potent derivative making it a very promising candidate for the use as a PDT agent.

Photodynamic inactivation of *Propionibacterium acnes*

Photodynamic inactivation of *P. acnes* was assessed by incubating the bacteria with 100 nM **1** or **11–14** followed by irradiation with 20 and 40 J cm^{-2} yellow light (590 nm). For all compounds, no inhibition of bacterial growth was found without illumination, *i.e.* hypericin and its derivatives itself were not cytotoxic. Only for **14** a significant inactivation of *P. acnes* was found after irradiation. Cell inactivation depended also on the incubation concentration; inactivation was also observed after photodynamic treatment at 50 nM for 1 h. These results are summarized in Fig. 2. Complete eradication of the bacteria was observed at the 500 nM concentration even at 20 J cm^{-2} (lowest light dose used in this study). It is worthwhile to note that with hypericin (**1**) significant inactivation of *P. acnes* could be achieved only using an incubation concentration of 1 μM and a light dose of >200 J cm^{-2}! Accordingly, the quaternary anilinium derivative **14** is more efficient by orders of magnitude than the parent compound **1**. The mode of association of the cationic substituent with the cell wall of the Gram-positive bacteria seems to be important for its photodynamic efficacy as deduced by comparison of the anilinium derivative **14** with the analogous pyridinium derivative **13**. Although both cationic hypericin derivatives display a similar ability to sensitize singlet oxygen or reactive oxygen species production as revealed above by photodestruction of bilirubin IXα, photodestruction of *P. acnes* was negligible after sensitization with the pyridinium derivative **13**. Different photodynamic efficacies of anilinium and pyridinium analogues have also been reported for *meso*-substituted porphyrins: For *Enterococcus seriolicidia* (Gram-positive) inactivation was higher after photosensitization with *meso*-tetra(4-*N,N,N*-trimethyl-anilinium)porphyrin as compared with *meso*-tetra(4-*N*-methyl-pyridyl)porphyrin, whereas for *Vibrio anguillarum*

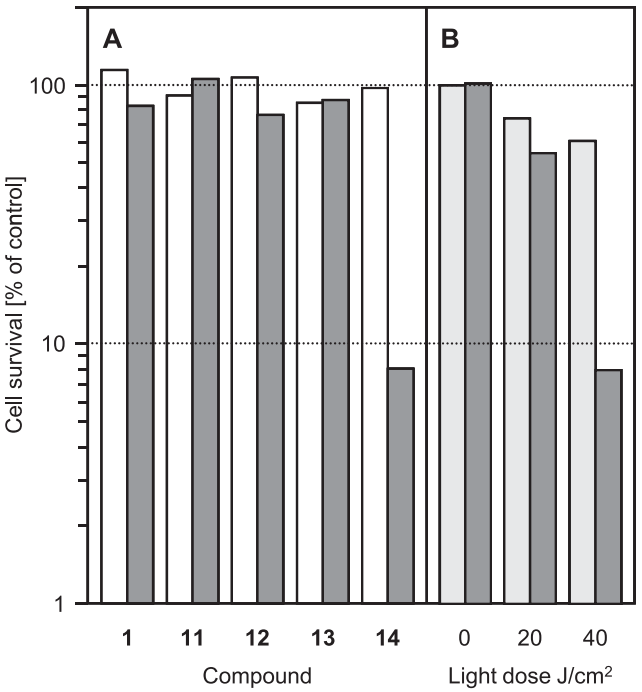


Figure 2. Photodynamic inactivation of *Propionibacterium acnes*: (A) hypericin (**1**) or hypericin derivatives **11**–**14** (100 nM, 1 h) and 0 J cm^{-2} (□) or 40 J cm^{-2} (■); (B) hypericin derivative **14** (50 nM (□) or 100 nM (■), 1 h); irradiation: 590 nm at 110 mW cm^{-2}.

(Gram-negative) the pyridinium analogue was more efficient in cell killing (31).

In conclusion, the present study demonstrates a convenient route to cationic hypericin derivatives and reveals that the quaternary anilinium derivative **14** is a very promising lead for further development of hypericin-based bacteriocidal photosensitzers, which might be of interest for PDT of localized infections. Further work is needed to elucidate its spectrum of antimicrobial activity.

Acknowledgements—The authors would like to thank Prof. Dr. Andreas Essig (Institute of Medical Microbiology and Hygiene, University of Ulm, Ulm, Germany) for his helpful discussion on the experimental design of quantitative assessment of photobactericidal activity, and Petra Kruse (Institute of Laser Technologies in Medicine and Metrology at the University of Ulm, Ulm, Germany) for perfect technical assistance.

REFERENCES

1. Falk, H. (1999) From the photosensitizer hypericin to the photoreceptor stentorian—The chemistry of phenanthroperylene quinones. *Angew. Chem. Int. Ed. Engl* **38**, 3116–3136.
2. Wolkenstein, K., J. H. Gross, H. Falk and H. F. Schöler (2006) Preservation of hypericin and related polycyclic quinone pigments in fossil crinoids. *Proc. Biol. Sci.* **273**, 451–456.
3. Vandenbogaerde, W. J., E. M. Delaey, A. M. Vantieghem, B. H. Himpens, W. J. Merlevede and P. A. M. de Witte (1998) Cytotoxicity and antiproliferative effect of hypericin and derivatives after photosensitization. *Photochem. Photobiol.* **67**, 119–125.
4. Kamuhabwa, A. A., T. Roskams, M. A. D'Hallewin, L. Baert, H. van Poppel and P. A. M. de Witte (2003) Whole bladder wall photodynamic therapy of transitional cell carcinoma rat bladder tumors using intravesically administered hypericin. *Int. J. Cancer* **107**, 460–467.
5. Kubin, A., F. Wierrani, U. Burner, G. Alth and W. Gruenberger (2005) Hypericin—The facts about a controversial agent. *Curr. Pharm. Des.* **11**, 233–253.
6. Lavie, G., Y. Mazur, D. Lavie and D. Meruelo D (1995) The chemical and biological properties of hypericin—A compound with a broad spectrum of biological activities. *Med. Res. Rev.* **15**, 111–119.
7. Lavie, G., Y. Mazur, A. M. Prince, D. Pascual, L. Liebes, B. Levin and D. Meruelo (1995) Hypericin as an inactivator of infectious viruses in blood components. *Transfusion* **35**, 392–400.
8. Darmanyan, A. P., L. Burel, D. Eloy and P. Jardon (1994) Singlet oxygen production by hypericin in various solvents. *J. Chem. Phys.* **91**, 1174–1785.
9. Redmond, R. W. and J. N. Gamlin (1999) A compilation of singlet oxygen yields from biologically relevant molecules. *Photochem. Photobiol.* **70**, 391–475.
10. Kubin, A., P. Meissner, F. Wierrani, U. Burner, A. Bodenteich, A. Pytel and N. Schmeller (2008) Fluorescence diagnosis of bladder cancer with new water soluble hypericin bound to polyvinylpyrrolidone: PVP-hypericin. *Photochem. Photobiol.* **84**, 1560–1563.
11. Waser, M. and H. Falk (2007) Towards second generation hypericin based photosensitizers for photodynamic therapy. *Curr. Org. Chem.* **11**, 547–558.
12. Lackner, B., Y. Popova, C. Etzlstorfer, A. A. Smelcerovic, C. W. Klampfl and H. Falk (2005) Syntheses and properties of two heterocyclically substituted hypericin derivatives: 10,11-Dibenzothiazolyl-10,11-didesmethylhypericin and 10,11-dibenzoxazolyl-10,11-didemethylhypericin. *Monatsh. Chem.* **136**, 777–793.
13. Lackner, B., C. Etzlstorfer and H. Falk (2004) Synthesis and properties of 10,11-dibenzimidazolyl-10,11-didemethylhypericin—The first heterocyclically substituted hypericin derivative. *Monatsh. Chem.* **135**, 1157–1166.
14. Waser, M., Y. Popova, C. W. Klampfl and H. Falk (2005) 9,12-Dibenzothiazolylhypericin and 10,11-dibenzothiazolyl-10,11-didemethylhypericin: Photochemical properties of hypericin derivatives depending on the substitution site. *Monatsh. Chem.* **136**, 1791–1797.
15. Waser, M. and H. Falk (2006) Condensed emodin derivatives and their applicability for the synthesis of a fused heterocyclic hypericin derivative. *Eur. J. Org. Chem.* 1200–1206.
16. Lackner, B. and H. Falk (2002) Concerning the diastereomerization of stilbenoid hypericin derivatives. *Monatsh. Chem.* **133**, 717–721.
17. Aigner, S. and H. Falk (2008) On synthesis and properties of hypericin–porphyrin hybrids. *Monatsh. Chem.* **139**, 1513–1518.
18. Uzdensky, A. B., D. E. Bragin, M. S. Kolosoy, A. Kubin, H. G. Loew and J. Moan (2003) Photodynamic effect of hypericin and a water-soluble derivative on isolated crayfish neuron and surrounding glial cells. *J. Photochem. Photobiol. B* **72**, 27–33.
19. Falk, H., A. O. Sarhan, H. T. N. Tran and R. Altmann (1998) Synthesis and properties of hypericins substituted with acidic and basic residues: Hypericin tetrasulfonic acid—A water soluble hypericin derivative. *Monatsh. Chem.* **129**, 309–318.
20. Altmann, R., H. Falk and H. J. Gruber (1998) Synthesis and properties of ionophore conjugated hypericin derivatives. *Monatsh. Chem.* **129**, 235–244.
21. Altmann, R. and H. Falk (1997) The deprotonation and protonation equilibria of a hypericin derivative in aqueous solution. *Monatsh. Chem.* **128**, 571–584.
22. Lackner, B., K. Bretterbauer, C. Schwarzinger and H. Falk (2005) A route to amino functionalized hypericin derivatives and their chemical and photochemical properties pertaining to photodynamic therapy. *Monatsh. Chem.* **136**, 2067–2082.
23. Geißlmeir, D. and H. Falk (2008) ω,ω'-appended nucleo-base derivatives of hypericin. *Monatsh. Chem.* **139**, 1127–1136.
24. Zuschrader, J., G. Reiter and H. Falk (2008) ω,ω'-urea- and dithioacetal-derivatives of hypericin. *Monatsh. Chem.* **139**, 995–998.
25. Zuschrader, J., W. Schöfberger and H. Falk (2008) A carbohydrate-linked hypericinic photosensitizing agent. *Monatsh. Chem*, **139**, 1387–1390.
26. Jori, G., C. Fabris, M. Soncin, S. Ferro, O. Coppellotti, D. Dei, L. Fantetti, G. Chiti and G. Roncucci (2006) Photodynamic therapy in the treatment of microbial infections: Basic principles and perspective applications. *Lasers Surg. Med.* **38**, 468–481.

27. Jori, G. (2006) Photodynamic therapy of microbial infections: State of the art and perspectives. *J. Environ. Pathol. Toxicol. Oncol.* **25**, 505–519.
28. Hamblin, M. R. and T. Hasan (2004) Photodynamic therapy: A new antimicrobial approach to infectious disease? *Photochem. Photobiol. Sci.* **3**, 436–450.
29. Wilson, M., T. Burns, J. Pratten and G. J. Pearson (1995) Bacteria in supragingival plaque samples can be killed by low-power laser light in the presence of a photosensitizer. *J. Appl. Bacteriol.* **78**, 569–574.
30. Minnock, A., D. I. Vernon, J. Schofield, J. Griffiths, J. H. Parish and S. T. Brown (1996) Photoinactivation of bacteria. Use of a cationic water-soluble zinc phthalocyanine to photoinactivate both Gram-negative and Gram-positive bacteria. *J. Photochem. Photobiol. B* **32**, 159–164.
31. Merchat, M., G. Bertolini, P. Giacomini, A. Villanueva and G. Jori (1996) Meso-substituted cationic porphyrins as efficient photosensitizers of Gram-positive and Gram-negative bacteria. *J. Photochem. Photobiol. B* **32**, 153–157.
32. Fimple, J. L., C. R. Fontana, F. Foschi, K. Ruggiero, X. Song, T. C. Pagonis, A. C. Tanner, R. Kent, A. G. Doukas, P. P. Stashenko and N. S. Soukos (2008) Photodynamic treatment of endodontic polymicrobial infection in vitro. *J. Endod.* **34**, 728–734.
33. Maisch, T., C. Bosl, R. M. Szeimies, B. Love and C. Abels (2007) Determination of the antibacterial efficacy of a new porphyrin-based photosensitizer against MRSA ex vivo. *Photochem. Photobiol. Sci.* **6**, 545–551.
34. Foley, J. W., X. Song, T. N. Demidova, F. Jilal and M. R. Hamblin (2006) Synthesis and properties of benzo[*a*]phenoxazinium chalcogen analogues as novel broad-spectrum antimicrobial photosensitizers. *J. Med. Chem.* **49**, 5291–5299.
35. Cincotta, L., J. W. Foley and A. H. Cicotta (1987) Novel red absorbing benzo[*a*]phenoxazinium and benzo[*a*]phenothiazinium photosensitizers: In vitro evaluation. *Photochem. Photobiol.* **46**, 751–758.
36. Spesia, M. B., M. E. Milanesio and E. N. Durantini (2008) Synthesis, properties and photodynamic inactivation of *Escherichia coli* by novel cationic fullerene C60 derivatives. *Eur. J. Med. Chem.* **43**, 853–861.
37. Maisch, T. (2007) Anti-microbial photodynamic therapy: Useful in the future? *Lasers Med. Sci.* **22**, 83–91.
38. Taylor, N. N. and M. L. Gonzales (2009) The practicalities of photodynamic therapy in acne vulgaris. *Br. J. Dermatol.* **160**, 1140–1148.
39. Eady, E. A., J. H. Cove, K. T. Holland and W. J. Cunliffe (1989) Erythromycin resistant propionibacteria in antibiotic treated acne patients: Association with therapeutic failure. *Br. J. Dermatol.* **121**, 51–57.
40. Hongcharu, W., C. R. Taylor, Y. Chang, D. Aghassi, K. Suthamjariya and R. R. Anderson (2000) Topical ALA-photodynamic therapy for the treatment of acne vulgaris. *J. Invest. Dermatol.* **115**, 183–192.
41. Divaris, D. X., J. C. Kennedy and R. H. Pottier (1990) Phototoxic damage to sebaceous glands and hair follicles of mice after systemic administration of 5-aminolevulinic acid correlated with localized protoporphyrin IX fluorescence. *Am. J. Pathol.* **136**, 891–897.
42. Williams, A. T. R., S. A. Winfield and J. N. Miller (1983) Relative fluorescence quantum yields using a computer controlled luminescence spectrometer. *Analyst* **108**, 1067–1071.
43. Hagenbuchner, K. and H. Falk (1999) Concerning the hypericin senitized photooxidation of bilirubin IXα. *Monatsh. Chem.* **130**, 1075–1081.
44. Falk, H., J. Meyer and M. Oberreiter (1993) A convenient semisynthetic route to hypericin. *Monatsh. Chem.* **124**, 339–341.
45. Falk, H. and T. N. H. Tran (1996) Synthesis and properties of an ω,ω'–appended eighteen carbon chains hypericin derivative. *Monatsh. Chem.* **127**, 717–723.
46. Obermueller, R. A. and H. Falk (2002) Concerning the absorption and photochemical properties of an ω-4-dimethylaminobenzal hypericin derivative. *Monatsh. Chem.* **132**, 1519–1526.