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A carbohydrate-linked hypericinic photosensitizing agent

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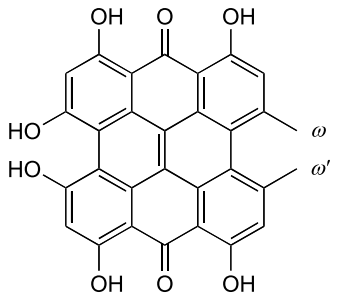
Abstract With respect to an enhanced solubility under physiological conditions, a carbohydrate-containing hypericin-based second-generation photosensitizer was prepared. Its photochemical properties were tested by means of the light-sensitized destruction of bilirubin IXα to be even better than those of the parent compound hypericin. Investigations on binding-interactions with *DNA* showed promising results as well.

Keywords Hypericin; Tetraacetyl-D-glucosamine; Calf-thymus *DNA*; Photodynamic agent; Reactive oxygen species.

Introduction

The compatibility of photosensitizing agents with respect to physiological issues concerning their application in the photodynamic therapy of cancer is an important facet for developing second-generation derivatives of hypericin (**1**) [1]. Having in mind the demands of a ''perfect'' photosensitizer (solubility, bathochromically shifted absorption maxima, and high quantum yields of photosensitizing singlet oxygen and/or reactive oxygen species), we focused on an enhanced solubility in protic solvents, in particular in water. Although hypericin (**1**) itself and other similar phenanthroperylenequinone structures as well are known for their interaction behavior with cell ingredients [2], no representive derivatives of hypericin have been reported so far aiming at an increased cell toxicity based on specific binding-interactions with *DNA* combined with an enhanced solubility under aqueous conditions.



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Results and discussion

With respect to the glycosidic occurrence of the ''monomeric'' precursor emodin in the bark of the breaking buckthorn (*Cortex frangulae*) we considered this as a lead in targeting of second-generation hypericinic photosensitizers [1]. However, unlike to the glycosidic natural compounds, which are prone to hydrolysis under physiological conditions, we decided not to substitute anomeric or other hydroxylic positions of the sugar moiety to guarantee hydrolytic stability. Otherwise, phenolic positions of the hypericin chromophore would also represent a rather unfortunate choice because investigations have shown the need of intact phenolic functionalities for the photochemical properties of interest, *i.e.* the sensitization of reactive oxygen species [1]. Therefore, linker chemistry on the ω,ω'-methyl positions of the chromophore of **1** seemed to be the more desirable method.

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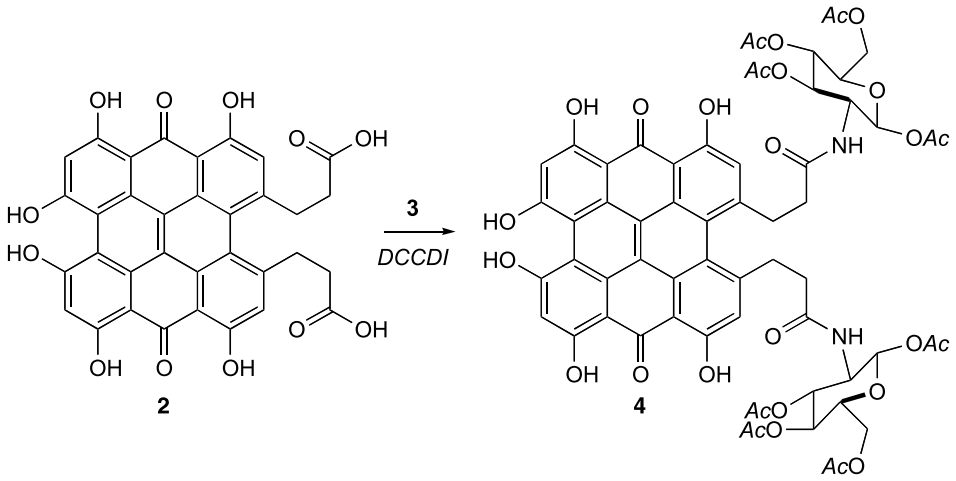

Scheme 1

Synthesis

Starting from emodin aldehyde (Scheme 1) *via* a *Wittig*-strategy and reduction to the anthrone with subsequent hydrogenation of the double bond, and finally dimerization according to Ref. [3], the propionic acid double-elongated intermediate **2** intended to serve as the starting point for the hypericin moiety was obtained. For the next step, we envisaged a *N*,*N*′-dicyclohexylcarbodiimide (*DCCDI*) mediated formal substitution of the acid **2** by D-glucosamine using triethylamine for deprotonation and tetrahydrofuran as the solvent. However, these attempts resulted in the formation of the corresponding *O*-acylurea derivative, which then underwent a 1,3-rearrangement to the *N*-acylurea derivative as described recently [4]. This might be explained by the decreased reactivity of the chain-elongated hypericin system on the one side, and the low solubility of the carbohydrate in the organic solvent on the other side. From a series of such preliminary experiments it followed that we had to use the *DCCDI*-activated acid **2** together with the acetyl-protected sugar derivative tetraacetyl-β-D-glucosamine **3** (prepared in analogy to Ref. [5]) to achieve the linking. Under these conditions the hypericin derivative substituted at positions ω,ω' with the acetyl-protected glucosamine moiety **4** was obtained in a reasonable yield of 66%. Its structure was proven using ^{1}H NMR and mass spectroscopy. Experiments to hydrolyze the acetyl groups of **4** using classical methods failed, but however, it turned out that the solubility of **4** in aqueous systems was high enough for the envisaged purpose.

Properties

Beside the demands on a second-generation photosensitizer, namely an increased solubility under physiological conditions and a shift of the absorption maximum in the regions of medical lasers, the possibility to create singlet oxygen and/or reactive oxygen species was investigated in this context. The second-generation derivative **4** proved to be highly effective in the hypericin-sensitized destruction of bilirubin IXα measured according to Ref. [6] (Fig. 1). In comparison to the naturally occurring **1**, the glyco-derivative

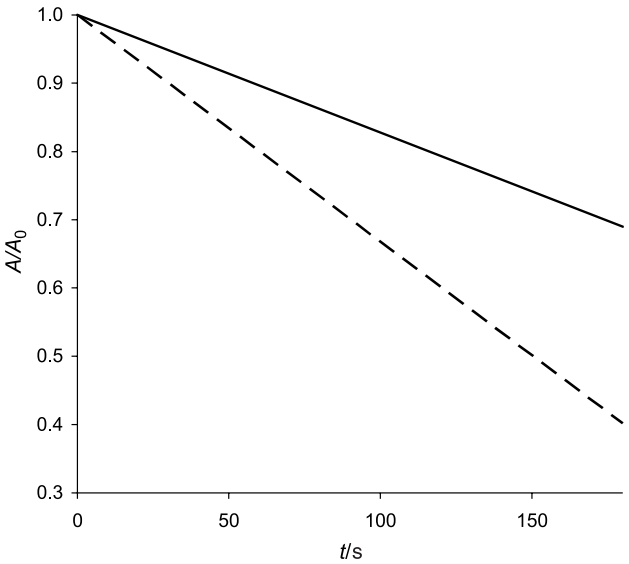

Fig. 1 Hypericin derivative sensitized photooxidation of bilirubin IXα: normalized absorption (A/A_0) *vs.* time curves of solutions of disodium bilirubinate IXα together with the sodium salts of hypericin (**1**, —) and glyco-derivative **4** (– – –)

4 displayed an even better activity to generate singlet oxygen and/or reactive oxygen species.

In addition to its high sensitizing activity, we expected **4** to show interactions with cell ingredients, in particular *DNA*. We prepared a 10 μM solution of **4** in water (10% dimethyl sulfoxide) whereby we obtained its characteristic absorption spectrum. This absorption spectrum of **4** dissolved in the aqueous solvent system was indicative of strong intermolecular homointeractions of the pigment (*cf.* the spectroscopic consequences of homoassociation of hypericin (**1**) in form of stacked H-aggregates [7]). Then this solution was titrated with increasing molar ratios of hypericin derivative and calf-thymus *DNA* leaving the concentration of **4** constant. Higher molar concentrations up to 400 μM of calf-thymus *DNA* were observed to go hand in hand with a diminished stacking of the phenanthroperylene quinone skeleton of **4** due to the fact that more and more molecules of **4** became heteroassociated with the double helix, whereby an increased slightly hypsochromically shifted absorption maximum with a significant disproportionality of the two vibrational bands typical of monomeric hypericin chromophores was obtained (*cf.* Fig. 2 for starting and end point of this titration). So we concluded that the effect of lessened stacking is dominant in comparison to the expected decrease of absorption as a result of reduced concentration of non-bonded photosensitizer molecules. It should be mentioned, that a similar behavior of the absorption spectra has been observed for the binding of **1** to serum albumine [7].

In conclusion, we present the highly water-soluble and most efficiently photosensitizing **4** as a second-generation hypericin photosensitizer and thus, a potential candidate for photodynamic therapy concerning its interaction or intercalation with polynucleotides, in particular with *DNA*.

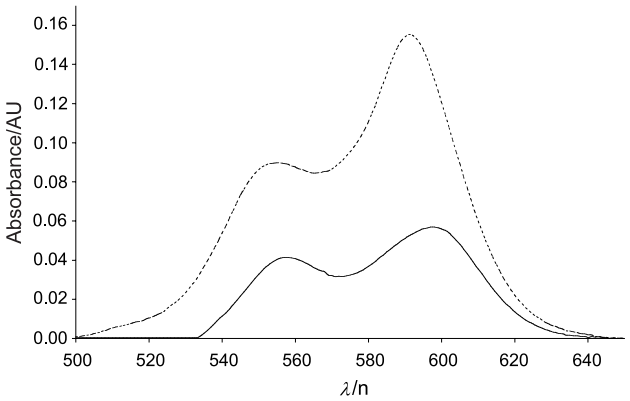

Fig. 2 UV-Vis spectra showing the interaction behavior of **4** with calf thymus *DNA*: *a*) **4** (10 μM) in aqueous solution containing 10% dimethyl sulfoxide (–); *b*) **4** (10 μM) in aqueous solution containing 10% dimethyl sulfoxide and calf thymus *DNA* (400 μM) (. . .)

Experimental

The characterizations of the products were established by means of m.p. (*Kofler* microscope, Reichert), NMR (Bruker DPX 200 MHz or Bruker 500 MHz spectrometer using a TXI cryoprobe with *z*-gradient coil using standard pulse sequences as provided by the manufacturer. Typical 90° hard pulse durations were 9.6 μs (^{1}H) and 18.4 μs (^{1}H), for a 180° pulse, 90° pulses in decoupling experiments were set to 80 μs. HSQC and HMBC experiments were optimized for decoupling constants of 145 Hz for single quantum correlations and 7 Hz for multi-bond correlations. The N*O*ESY mixing time was set to 400 ms), IR (Bruker Tensor 27, KBr), MS (Thermofinnigan LCQ Deca XP Plus), fluorescence (Varian Carey 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.* [8] using hypericin (**1**) as standard sample. The production of singlet oxygen/oxidizing species by **1** and **4** was monitored by bilirubin-IXα-degradation according to Ref. [6]. The long-wavelength absorption band intensities of the two compounds were made equal by adjusting concentrations to provide comparable light absorptions for the two compounds. *N*,*N*′-Dicyclohexylcarbodiimide was obtained from Merck and used as obtained. Compound **2** was prepared according to Ref. [3]. The per-acetylated D-glucosamine hydrochloride was synthesized in analogy to Ref. [5] using benzaldehyde as the imide-protecting reagent during the acetylation process followed by the subsequent hydrolysis to the tetra-*O*-acetyl-protected β-D-glucosamine hydrochloride **3**.

All experiments involving calf-thymus *DNA* (Sigma) were performed with MilliQ (18 MΩ) water. According to Refs. [9, 10], calf-thymus *DNA* solutions were prepared by dispersing the desired amount of *DNA* in water (MilliQ 18 MΩ) with stirring over night at temperatures below 4°C. The concentration was expressed as the concentration of nucleotides and was calculated by using an average molecular weight of 338 for a nucleotide and an extinction coefficient of 6600 M^{-1} cm^{-1} at 260 nm.

N-Benzylidene-β-D-glucosamine ($C_{13}H_{17}NO_5$)
To a stirred solution of 2.0 g β-D-glucosamine hydrochloride (9.28 mmol) and 1.0 g (11.90 mmol) $NaHCO_3$ in 12 cm^3 distilled water 1.1 cm^3 benzaldehyde, diluted with 10 cm^3 methanol, were added. The mixture was kept at room temperature for 3 h. After evaporation of the solvent, the crude product was suspended in CH_2Cl_2, washed with a small amount of water and 50 cm^3 cold diethyl ether. The *Schiff* base was obtained in a 40% yield. ESI-MS (positive ion mode): $m/z = 268$ ($[M + H]^+$).

N-Benzylidene-O^1,O^3,O^4,O^6-tetraacetyl-β-D-glucosamine ($C_{21}H_{25}NO_9$)
After dissolving 1.0 g of the above *Schiff* base (3.74 mmol) in 5 cm^3 pyridine, the mixture was cooled to 0°C and 4 cm^3 acetic

anhydride (40 mmol) were added. The solution was stirred for 5 h and for further 10 h at room temperature, then poured onto ice/water, and filtered to yield 1.13 g (69%) of the product as a white solid. ^{1}H NMR (500 MHz, *DMSO*-d_6, 30°C): $\delta = 8.39$ (s, –N=C*H*–), 7.71 (m, ar-H2′ and ar-H6′), 7.47 (m, ar-H3′, ar-H4′, and ar-H5′), 6.10 (d, $J = 7$ Hz, 1-C*H*), 5.48 (t, $J = 10$ Hz, 3-C*H*), 4.99 (t, $J = 10$ Hz, 5-C*H*), 4.25 (m, 6-CH_2), 4.03 (d, $J = 12$ Hz, 4-C*H*), 3.52 (t, $J = 10$ Hz, 2-C*H*), 2.03 (s, 6-OA*c*), 1.99 (s, 1-OA*c* and 4-OA*c*), 1.83 (s, 3-OA*c*) ppm; ^{13}C NMR (125 MHz, *DMSO*-d_6, 30°C): $\delta = 170.6$ (6-CH_3*C*=O), 170.0 (1-CH_3*C*=O or 4-CH_3*C*=O), 169.5 (4-CH_3*C*=O or 1-CH_3*C*=O), 169.1 (3-CH_3*C*=O), 165.9 (–N=*C*H–), 136.0 (C1′), 132.0 (C3′, C4′, or C5′), 129.3 (C3′, C4′, or C5′), 128.8 (C3′, C4′, or C5′), 93.0 (C1), 72.8 (C3), 72.1 (C2), 68.4 (C5), 62.2 (C6), 21.1 (6-CO*C*H_3), 21.0 (4-CO*C*H_3 or 1-CO*C*H_3), 20.8 (3-CO*C*H_3) ppm; HMBC (*DMSO*-d_6): –N=C*H*– ↔ –N=*C*H–, *C*3′, *C*2′, *C*6′, *C*1′, and *C*2, 1-C*H*– ↔ *C*1 and *C*2, 2-C*H*– ↔ *C*1, *C*3, and –N=*C*H–, 6-CO*C*H_3 ↔ 6-*C*O*C*H_3, and *C*6, 3-CO*C*H_3 ↔ 3-*C*O*C*H_3 and *C*3; HSQC data were according to structure; ESI-MS (positive ion mode): $m/z = 436$ ($[M + H]^+$).

O^1,O^3,O^4,O^6,-Tetraacetyl-β-D-glucosamine hydrochloride
(**3**, $C_{14}H_{22}ClNO_9$)
To a refluxing solution of 500 mg (1.87 mmol) of the above product in 2.5 cm^3 acetone 265 mm^3 conc. HCl were added. The product precipitated spontaneously whereupon the mixture was cooled to 0°C, filtered, and washed with diethyl ether yielding 73%. ^{1}H NMR (500 MHz, *DMSO*-d_6, 30°C): $\delta = 8.69$ (s, –NH_2), 5.90 (d, $J = 9$ Hz, 1-C*H*), 5.35 (t, $J = 10$ Hz, 3-C*H*), 4.94 (t, $J = 10$ Hz, 4-C*H*), 4.19 (m, 6-CH_2), 4.07 (m, 5-C*H*), 4.01 (m, 6-CH_2), 3.58 (t, $J = 10$ Hz, 2-C*H*), 2.17 (s, 1-OA*c*), 2.04 (s, 1-OA*c*), 2.00 (s, 6-OA*c*), 1.98 (s, 4-OA*c*) ppm; ^{13}C NMR (125 MHz, *DMSO*-d_6, 30°C): $\delta = 171.0$ (3-CH_3*C*=O), 170.8 (4-CH_3*C*=O and 6-CH_3*C*=O), 170.3 (1-CH_3*C*=O), 91.5 (C1), 73.7 (C5), 71.8 (C3), 69.3 (C4), 63.3 (C6), 53.9 (C2), 22.3 (1-CO*C*H_3 and 3-CO*C*H_3), 21.8 (4-CO*C*H_3 and 6-CO*C*H_3) ppm; HMBC (*DMSO*-d_6): *C*1 ↔ 2-C*H*, 1-C*H*, and 1-CO*C*H_3, *C*2 ↔ 3-C*H* and 1-C*H*, *C*3 ↔ 1-C*H*, 4-C*H*, and 3-CO*C*H_3, *C*6 ↔ 6-CH_2 and 5-C*H*, *C*4 ↔ 5-C*H* and 3-C*H*; HSQC data were according to structure; ESI-MS (positive ion mode): $m/z = 385$ ($[M + H]^+$).

10,11-Bis(N-(D-tetrahydro-2,4,5-triacetoxy-6-(acetoxymethyl)-2H-pyran-3-yl)carbamoylethyl)-1,3,4,6,8,13-hexahydroxy-phenanthro[1,10,9,8-opqra]perylene-7,14-dione
(**4**, $C_{62}H_{58}N_2O_{28}$)
An argon-flushed mixture of 42 mg **2** (0.12 mmol) and 750 mm^3 triethyl-amine in 50 cm^3 dry *THF* was heated to 40°C under stirring. After the addition of 40 mg **3** (0.06 mmol), the suspension was stirred for further 30 min. An amount of 26 mg *N*,*N*′-dicyclohexylcarbodiimide (0.12 mmol) was dissolved in 5 cm^3 dry *THF*. The reaction mixture and the activating diimide solution were combined and stirred for 23 h under Ar. Upon subsequent evaporation of the solvent under reduced pressure and extraction with ethyl acetate/water a black solid was obtained. Purification by means of column chromatography with chloroform/methanol = 3/1 yielded 51 mg (66%) of the product. Mp >350°C; TLC: $R_f = 0.56$ ($CHCl_3$:*Me*OH = 3:1); ^{1}H NMR (500 MHz, *DMSO*-d_6, 30°C): $\delta = 18.48$ (s, 3-OH, 4-OH), 14.72 (s, 1-OH, 6-OH), 14.08 (s, 8-OH, 13-OH), 7.79 (s, –CON*H*–), 7.77 (s, –CON*H*–), 7.45 (s, ar-H9, ar-H12), 6.58 (s, ar-H2, ar-H5), 5.48 (m, H2′), 3.54 (m, ar-CH_2–CH_2–), 2.26 (m, ar–CH_2–CH_2–), 2.05 (s, 1′-OA*c* or 3′-OA*c*) ppm; ^{13}C NMR data were partially derived by 2D-experiments; a total characterization of the sugar moiety was not possible; ^{13}C NMR (125 MHz, *DMSO*-d_6, 30°C): $\delta =$ 183.8 (C14, C15), 174.1 (C3, C4), 169.1 (–CONH–), 168.5 (C1, C6), 162.3 (C8, C13), 119.7 (C3b, C4b), 119.0 (C11b, C10b), 117.6 (C9, C12), 108.2 (C6a, C14a), 104.9 (C2a, C5a), 101.9 (C3a, C4a), 51.7 (ar–CH_2–*C*H_2–), 49.6 (C2′), 36.4 (1-COCH_3 or 3-COCH_3), 31.6 (ar–*C*H_2–CH_2–) ppm; N*O*ESY (*DMSO*-d_6): ar-*H*9 and ar-*H*12 ↔ ar-C*H*$_2$–CH_2–, ar–CH_2–C*H*$_2$–, and 1-COC*H*$_3$ or 3-COC*H*$_3$, –CON*H*– ↔ *H*2′ and ar–CH_2–C*H*$_2$–, ar–C*H*$_2$–CH_2– ↔ ar–CH_2–C*H*$_2$–, ar-*H*9, ar-*H*12, and 1-COC*H*$_3$ or 3-COC*H*$_3$, (–CON*H*–) ↔ (m, ar–C*H*$_2$–CH_2–); HMBC (*DMSO*-d_6): ar–C*H*$_2$–CH_2– ↔ *C*7, *C*14, *C*8, *C*13, *C*11b, *C*10b, *C*6a, *C*14a, and ar–C*H*$_2$–CH_2–, ar-*H*2, ar-*H*5 ↔ *C*3, *C*4, *C*1, *C*6, *C*3b, *C*4b, *C*2a, *C*5a, and *C*3a, *C*4a, –CON*H*– ↔ *C*2′ and ar–CH_2–*C*H_2–; HSQC data were according to structure; IR (KBr): $\bar{\nu} = 3420$, 2927, 2854, 1718, 1559, 1465, 1244, 1114, 1035, 847, 669 cm^{-1}; UV-Vis (80% *Et*OH, $c = 2.0 \cdot 10^{-6}$ mol · dm^{-3}): λ_{max} (ε) = 555 (10000), 600 (60000) nm ($dm^3 \cdot mol^{-1} \cdot cm^{-1}$); UV-Vis (*DMSO*, $c = 2.0 \cdot 10^{-5}$ mol · dm^{-3}): λ_{max} (ε) = 555 (10850), 600 (2600) nm ($dm^3 \cdot mol^{-1} \cdot cm^{-1}$); fluorescence (80% *Et*OH, $c = 1.0 \cdot 10^{-7}$ mol · dm^{-3}, $\lambda_{ex} = 550$ nm): λ_{em} (rel. int.) = 597 (100), 644 (10) nm; $\Phi = 0.17$; fluorescence (*DMSO*, $c = 1.0 \cdot 10^{-7}$ mol · dm^{-3}, $\lambda_{ex} = 550$ nm): λ_{em} (rel. int.) = 604 (100), 656 (3) nm, $\Phi = 0.11$.

Acknowledgements

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