Langmuir
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Locations and Reorientations of Multi-Ring-Fused 2-Pyridones in Ganglioside G_{M1} Micelles

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Received October 11, 2010. Revised Manuscript Received December 3, 2010

Fluorescent multi-ring-fused 2-pyridones, with chemical resemblance to other biologically active 2-pyridone systems, were solubilized in spherical micelles formed by the gangloiside G_{M1} and studied with respect to their spatial localization and rotational mobility. For this, electronic energy transfer between the multi-ring-fused 2-pyridone (donor) and BODIPY-FL-labeled G_{M1} was determined, as well as their fluorescence depolarization. From the obtained efficiency of energy transfer to the acceptor group (BODIPY-FL), either localized in the polar or in the nonpolar part of the ganglioside, it has been possible to estimate the most likely localization of the multi-ring-fused 2-pyridones. The center of mass of the studied multi-ring-fused 2-pyridones are located at approximately 33 Å from the micellar center of mass, which corresponds to the internal hydrophobic−hydrophilic interfacial region. At this location, the reorienting rates of the multi-ring-fused 2-pyridones are surprisingly slow with typical correlation times of 35−55 ns. No evidence was found for the formation of ground and excited state dimers, even when two monomers were forced to be near each other via a short covalent linker.

Introduction

A recently synthesized series of multi-ring-fused 2-pyridones has been spectroscopically characterized,[1] and their use as cell-staining fluorescent dyes demonstrated.[1a] Ring-fused 2-pyridones are commonly found to have interesting biological activity. Camptothecin[2] and mappicine[3] exemplify substances with anti-tumor and antiviral properties, respectively. Bicyclic 2-pyridones have been reported as antibacterial agents, targeting virulence factors in pathogenic bacteria.[4] Due to the biophysical properties of 2-pyridones and their potential use in drug delivery vehicles, it is of interest to ascertain the precise location of the compounds, for example, within the micellar domain of a block copolymer hydrogel. The use of various lipid phases as drug carriers have been investigated for some time,[5] and polymers that form macromolecular structures have been investigated as drug delivery matrices.[6] If the drug preferentially resides in the micellar core, the loading efficiency is restricted by its small size relative to the micelle and the release is then slower as compared to when the drug is located in the hydrophilic region. This is especially pronounced when the core is in the gel state.[7] Release from the micellar corona on the other hand, which is quite flexible, may be too rapid to monitor.

Detailed knowledge of the location of drug molecules inside a delivery vehicle provides for an added level of understanding, possibly pertaining to drug loading and release processes.[7] In the present study, the location of the multi-ring-fused 2-pyridones was investigated when solubilized in G_{M1} ganglioside micelles. G_{M1} ganglioside micelles were chosen[8] for several reasons, namely: (i) they exhibit a narrow size distribution and their aggregation number is known,[9] (ii) the thickness of the hydrophilic headgroup region (which approximately equals that of the hydrocarbon chain radius) offers a large radial variation in physicochemical properties (e.g., the local dielectric constant, solubility, etc.), and (iii) specifically labeled fluorescent gangliosides exist, which form suitable donor−acceptor pairs with these multi-ring-fused 2-pyridones.[9b]

Materials and Methods

Chemicals. G_{M1} ganglioside was isolated from bovine brain as described by Svennerholm,[10] BODIPY-FL-G_{M1} was synthesized as reported in ref 11, while 581/591-BODIPY-G_{M1} was synthesized as described elsewhere.[9b] Tris(hydroxymethyl)aminomethane hydrochloride and chloroform (spectroscopic grade) were bought from Sigma Aldrich.

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Syntheses of 2-Pyridonebased Compounds. The compounds D-Me, D-H, and D-F (cf. Figure 1) have been synthesized previously.[1a] The bis-polyaromatic 2-pyridones, $(D\text{-}H)_{C2}$ and $(D\text{-}H)_{C3}$, were synthesized as described in the following.

All reactions are run under N_2(g) with anhydrous solvents unless otherwise stated. The ^1H and ^{13}C NMR spectra were recorded at 298 K with a Bruker DRX-400 spectrometer and calibrated using the residual peak of $CHCl_3$ or dimethyl sulfoxide (DMSO) as internal standard ($CHCl_3$: δ_H 7.26 ppm, δ_C 77.16 ppm; DMSO: δ_H 2.50 ppm, δ_C 39.52 ppm). In cases where the diastereomers give different chemical shifts, signals from major and minor diasteromere are indicated with "maj" and "min", respectively. LRMS was conducted on a Micromass ZQ mass spectrometer with ES^+ ionization.

Compound $(D\text{-}H)_{OH}$ (*cf. Scheme 1*). A total of 1.05 mL of 0.5 M LiOH (aq) was slowly added to 194 mg (0.501 mmol) of D-H[1a] in 5.0 mL of tetrahydrofuran and 5.0 mL of methanol. The mixture was stirred at room temperature overnight and then concentrated under reduced pressure. The residue was taken up in 10 mL of acetic acid and 10 mL of chloroform, and approximately 3 mL of Amberlite IR-120 (H^+) was added. After 3 h of shaking, the resulting solution was filtrated and concentrated to afford 173 mg (93%) of compound $(D\text{-}H)_{OH}$. ^1H NMR (DMSO-d_6): δ 13.54 (1H, bs), 8.93 (1H, s), 8.18 (1H, d, J = 8.0 Hz), 7.87 (1H, d, J = 8.1 Hz), 7.63−7.35 (8H, m), 5.70 (1H, d, J = 8.3 Hz), 3.83 (1H, dd, J = 11.6 Hz, 8.3 Hz), 3.52 (1H, d, J = 11.6 Hz). ^{13}C NMR (DMSO-d_6): δ 169.9, 160.1, 139.2, 136.3, 135.1, 133.6, 130.3 (3C), 129.2 (2C, splitted), 129.1, 128.8, 128.6, 128.2, 127.7, 125.9, 122.4, 120.7, 110.5, 62.7, 31.1. LRMS (ES^+) calcd for $C_{22}H_{16}NO_3S$ [M + H], 374; found, 374.

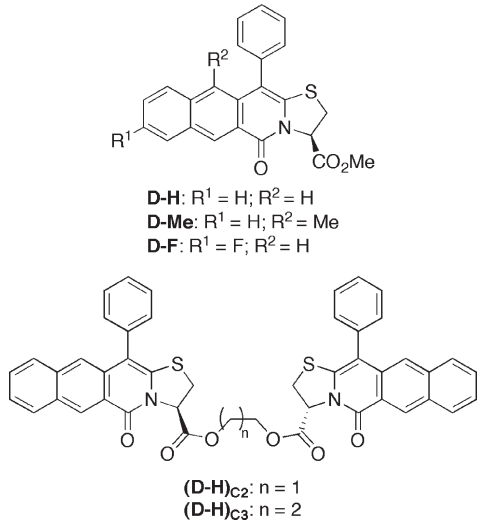


Figure 1. Structures of the studied multi-ring-fused 2-pyridones.

Dimerization of $(D\text{-}H)_{OH}$. A total of 37 mg (0.099 mmol) of $(D\text{-}H)_{OH}$ and 2 mg (0.016 mmol) of 4-dimethylaminopyridine was taken up in 0.5 mL of tetrahydrofuran. Then 0.50 mL (0.050 mmol) of 0.10 M of the appropriate diol in tetrahydrofuran was added followed by 29 mg (0.151 mmol) of *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride. The slurry was stirred at room temperature overnight. An amount of 0.5 mL of CH_2Cl_2 was added, and the mixture was stirred another 7 h and then concentrated under reduced pressure. The residue was taken up in CH_2Cl_2 and washed with 1 M HCl (aq). The aqueous phase was extracted with CH_2Cl_2, and the combined organic phases were then dried over anhydrous Na_2SO_4, filtrated, and concentrated under reduced pressure. The crude product was purified with silica gel chromatography using heptane/ethyl acetate 2:1 → 1:1 as a mobile phase.

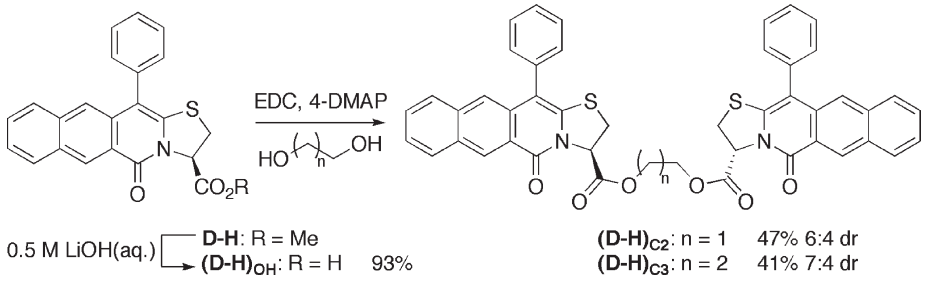
Compound $(D\text{-}H)_{C2}$. By following the procedure for dimerization of $(D\text{-}H)_{OH}$, using ethylene glycol as diol, 18 mg (47%) of $(D\text{-}H)_{C2}$ was obtained as a mixture of diasteromeres in an approximately 6:4 ratio according to ^1H NMR. ^1H NMR ($CDCl_3$): δ 8.98 (2H maj, s), 8.97 (2H min, s), 8.02−7.95 (2H, m), 7.71 (2H, d, J = 8.1 Hz), 7.60 (2H, s), 7.58−7.39 (14H, m), 5.78 (2H maj, dd, J = 8.0 Hz, 2.4 Hz), 5.72−5.67 (2H min, m), 4.63−4.40 (4H, m), 3.63−3.53 (2H, m), 3.50−3.42 (2H, m). ^{13}C NMR ($CDCl_3$): δ 168.4 (2C maj), 168.3 (2C min), 161.6 (2C), 137.7 (2C), 136.7 (2C), 135.8 (2C), 135.1 (2C, split), 131.0 (2C), 130.8 (2C), 130.4 (2C), 129.6 (2C), 129.5 (2C), 129.2 (2C, split), 129.0 (2C), 128.5 (2C), 128.4 (2C, split), 128.0 (2C), 126.0 (2C), 122.7 (2C), 121.9 (2C), 112.8 (2C), 63.7 (2C maj), 63.6 (2C min), 63.0 (2C maj), 62.9 (2C min), 31.4 (2C, broad). LRMS (ES^+) calcd for $C_{46}H_{33}N_2O_6S_2$ [M+H], 773; found, 773.

Compound $(D\text{-}H)_{C3}$. By following the general procedure for dimerization of $(D\text{-}H)_{OH}$, using 1,3- propanediol as diol, 16 mg (41%) of $(D\text{-}H)_{C3}$ was obtained as a mixture of diasteromeres in an approximately 7:4 ratio according to ^1H NMR. ^1H NMR ($CDCl_3$): δ 8.99 (2H, s), 8.05−7.95 (2H, m), 7.76−7.68 (2H, m), 7.63−7.35 (18H, m), 5.79−5.70 (2H, m), 4.44−4.21 (4H, m), 3.65−3.53 (2H, m), 3.44−3.38 (2H min, m), 3.32 (2H maj, dd, J = 11.6 Hz, 2.2 Hz). ^{13}C NMR ($CDCl_3$): δ 168.5 (2C), 161.6 (2C), 137.6 (2C, split), 136.7 (2C), 135.8 (2C), 134.1 (2C), 131.0 (2C), 130.8 (2C), 130.4 (2C), 129.7 (2C, split), 129.5 (2C), 129.3 (2C), 129.0 (2C), 128.5 (2C), 128.4 (2C), 128.0 (2C), 126.0 (2C), 122.7 (2C, split), 121.9 (2C), 112.8 (2C, split), 63.0 (2C), 62.4 (2C) 31.4 (2C), 27.8 (2C maj) 27.7 (2C min). LRMS (ES^+) calcd for $C_{47}H_{35}N_2O_6S_2$ [M+H], 787; found, 787.

Preparation of G_{M1} Micelles. Appropriate amounts of G_{M1} were dissolved in a chloroform/methanol mixture (2:1, v/v), to which the desired amount of fluorophore was added. After evaporation of the organic solvents by a continuous flow of Ar(g), the sample was dried under high vacuum for 3 h. The obtained lipid film was hydrated to 0.16 mM by adding a TRIS-HCl buffer (pH 7.4) containing 150 mM NaCl.

Absorption and Fluorescence Spectra. The absorption spectra were recorded on a Varian Cary 5000 UV−vis spectrometer. The fluorescence spectra were transcribed by means of a

Scheme 1. Synthesis of the Multi-Ring-Fused 2-Pyridone (D-H) Dimers[a]



[a] EDC = *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride; 4-DMAP = 4-dimethylaminopyridine. The diastereomeric ratio (dr) was estimated from ^1H-NMR spectra.

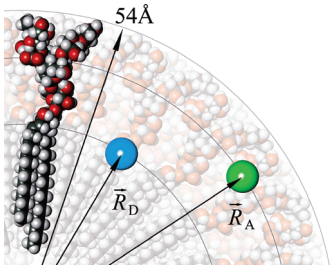


Figure 2. Schematic of a spherical micelle; also shown is a space filling model of the G_{M1} ganglioside. Each G_{M1} lipid is illustrated in a hypothetical conformation, rather than being in the true liquid state. The vector end points indicate the center of mass of a donor molecule ($\vec{R}_D$) and an acceptor ($\vec{R}_A$) group. The donor position is expressed in spherical coordinates $\vec{R}_D = R_D(\cos\alpha \sin\beta, \sin\alpha \sin\beta, \cos\beta)$.

Fluorolog -3 spectrometer (Jobin Yvon) equipped with Glan-Thompson polarizers. The bandwidth of the excitation and emission light was 3 and 2 nm, respectively. All fluorescence spectra were corrected. For the study of the donors' location inside the micelles, the emission intensity was monitored at either 470 nm (FL-BODIPY-G_{M1} acceptor) or at 505 nm (581/591-BODIPY-G_{M1} acceptor) (cf. Figure 3).

Fluorescence Lifetime Measurements. The time-resolved fluorescence decays were measured by means of the time-correlated single photon-counting technique[12] by using a Fluorolog-TCSPC (Horiba) spectrometer. The fluorescence decays were collected over 2048 channels, with a resolution of 100 ps/ch, with at least 8000 photons in the peak maximum for the lifetime experiments, which were performed with the emission polarizer set to magic angle (54.7°) with respect to the excitation polarizer. For the time-dependent anisotropy experiments, the fluorescence decays were collected with a 10 000-count difference in peak maxima between the decays collected with parallel and perpendicular polarizer settings. For the pulsed excitation, a NanoLED 280 nm and a 404 nm diode laser were used in combination with an interference filter centered at 280 and 404 nm (HBW 10 = nm), respectively. The emission monochromator (IBH system, U.K.) was used in combination with an interference filter centered at 470 nm (HBW 10 = nm). The fluorescence lifetime was calculated by means of a deconvolution method,[13] which is based on the Levenberg−Marquardt algorithm.[14]

Theoretical Prerequisites

Important theoretical work on donor−acceptor energy transfer in restricted geometries has been published by several research groups (Levitz and co-workers[15]). Based on the formalism developed by the Blumen and Klafter groups,[15a,16] Yekta et al.[17] have derived a general equation for energy transfer from donors to acceptors which exhibits different concentration profiles in spherically symmetric systems. The general equation derived by Yekta et al. is rather complex, but it can be simplified for certain concentration profiles. In this paper, we assume that donors (D) and acceptors (A) are randomly distributed about the micellar

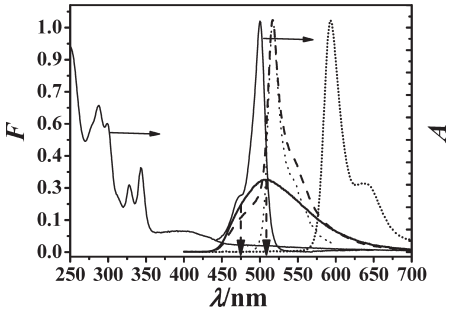


Figure 3. Corrected fluorescence spectra of the D-F variant (with unknown position within a micelle) (solid), the acceptor FL-NP (dotted), the acceptor 581/591-NP (densely dotted) (acceptor's positions are known), and D-F in the presence of FL-NP acceptor (dashed). On the very left, the absorption spectrum of D-F is displayed (solid) followed by the absorption spectrum of the acceptor FL-NP (solid). The arrows pointing downward indicate the wavelength selected for monitoring the fluorescence intensity.

center of mass at the different effective radii R_D and R_A, respectively. (cf. Figure 2). For such a D−A arrangement, Uhlik et al.[18] have derived an equation for the mean energy transfer rate which has been used for the analyses of the steady-state fluorescence data obtained here. The average rate of energy transfer (ω) depends on the distribution of distances $|\vec{R}| = |\vec{R}_D - \vec{R}_A|$, which is random, due to the spherical symmetry of the micelle. The transfer rate also depends on the number of acceptors N_A. The transfer rate can be written as

$$\omega = \frac{{R_0}^6 N_A}{\tau_D} \int_{R_{min}}^{R_{max}} \frac{P(R)\, dR}{R^6} \qquad (1)$$

In eq 1, the donor−acceptor distance (R) depends on the spherical polar orientation angle, β, according to $R = ({R_D}^2 + {R_A}^2 - 2R_D R_A \cos\beta)$. Here $P(R)$ is the normalized number density of acceptors at the distance R from a donor (= probability that an acceptor is found at the distance R from a donor) and is given by $P(R) = R/(2R_A R_D)$.[18] Then the total energy transfer rate (ω_t) which is given by $\rho_D(R_D)\omega$ is proportional to the following expression:

$$\omega_t \propto \rho_D(R_D) N_A \frac{{R_A}^2 + {R_D}^2}{\left({R_A}^2 - {R_D}^2\right)^4} \qquad (2)$$

Since the distribution of acceptors around any donor is identical, the fluorescence decays monoexponentially with a rate constant = $1/\tau_D + \omega$. The randomly distributed acceptors surrounding the micellar center of mass can be considered as one effective acceptor which quenches donor fluorescence at a transfer rate = ω_t. The ratio between the fluorescence intensity of the donor in the absence (F_D^0) and in the presence (F_D) of acceptors can then be related to the total energy transfer rate in a similar way as is done for a D−A pair:[19]

$$\frac{F_D^0}{F_D} = 1 + \omega_t \tau_D \qquad (3)$$

Thus, the energy transfer causes a decrease of fluorescence intensity of a donor. Since the donor and acceptor molecules

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Table 1. Radial Distances between Various 2-Pyridiones and the Micellar Center[a]

donor	acceptor	$\langle n \rangle$	P_0	$[(F_D(NP)/F_D(P)]_{exp}$	R_A (Å)[b]	R_D (Å)
D-F	FL-NP[c]	0.75	0.47	1.15	22.7	33.5
	FL-P[c]	0.77	0.46	1.15	41.6	33.5
D-H	FL-NP[c]	0.71	0.49	1.06	22.7	32.8
	FL-P[c]	0.76	0.47	1.06	41.6	32.8
D-H	581/591-NP[d]	1.27	0.28	1.03	22.7	31.0
D-Me	581/591-P[d]	1.36	0.26	1.07	41.6	32.8
	FL-NP[c]	0.73	0.48	1.07	22.7	32.8
	FL-P[c]	0.77	0.46	1.07	41.6	32.8

[a] The concentration of G_{M1} ganglioside was 0.16 mM, for which the micellar aggregation number is 168[21]. P_0 denotes the fraction of micelles which is not containing any acceptors. The average number of acceptor molecules per micelle $\langle n \rangle = N_A/N_{mic}$. [b] Corresponds to a fully extended G_{M1} molecule. [c] R_0 = 49.5 Å. [d] R_0 = 38.4 Å.

are distributed among the micelles, one needs to account for the probability that micelles do not contain any acceptor. By assuming a Poisson distribution,[20] this fraction of micelles (P_0) can be calculated from the relation $P_0 = \exp(-\langle N_A/N_{mic} \rangle)$. The ratio within the angled brackets represents the average occupation number of acceptors per micelle, which can be calculated from the known values of the number of acceptor molecules (N_A) and micelles (N_{mic}) in the studied system. For calculating the latter value, the previously determined value of the micellar aggregation number of G_{M1} gangliosides has been used (N_{agg} = 168).[21] Hence, it is possible to correct the experimental donor fluorescence intensities ($F_D^{0,exp}$ and F_D^{exp}) with respect to micelles lacking acceptors according to the relations $F_D^0 = F_D^{0,exp}(1-P_0)$ and $F_D = F_D^{exp} - F_D^{0,exp}P_0$.

From fluorescence depolarisation experiments, the steady-state and the time-resolved anisotropy were calculated. In the analyses, the time-resolved anisotropy was modeled according to

$$r(t) = \sum_k r_k(0) \exp(-t/\phi_k) \qquad (4)$$

In eq 4, ϕ_k denotes rotational correlation times and $r(0) = \Sigma_k r_k(0) \leq r_0$, where r_0 stands for the *fundamental* anisotropy.[22]

Results

Depths of Fluorescent Molecules Solubilized in G_{M1} Micelles. For positioning the studied multi-ring-fused 2-pyridones within G_{M1} micelles, the Förster mechanism of electronic energy transfer[23] has been applied. The studied donor molecules are polyaromatic 2-pyridones, which are hereafter referred to as D-Me, D-H, and D-F (cf. Figure 1). Four different BODIPY modified G_{M1} molecules constitute the acceptors: FL-BODIPY-G_{M1}, in which the acceptor group is specifically attached in either the nonpolar (FL-NP) or in the polar (FL-P) region of the G_{M1} micelle; also 581/591-BODIPY-G_{M1} was used for labeling the polar (581/591-P) and nonpolar (581/591-NP) region. The positions of the acceptor groups in G_{M1} micelles refer to the fully extended lipid molecule (cf. Table 1). The surface density values of the donors appearing in eqs 1 and 2 are not known. This can be circumvented if the donor concentration is identical in each of two experiments performed with same acceptor group, localized at different positions in the G_{M1} ganglioside. By forming the ratio between the corrected experimental ratios (cf. eq 2) obtained with the acceptor pairs FL-P and FL-NP or 581/591-P and 581/591-NP, the explicit value of $\rho_D(R_D)$ is not needed. Thereby, the question of determining possible values of R_D distances is defined by finding the minima of the following expression:

$$\left[\frac{F_D^0(NP) - F_D(NP)}{F_D^0(P) - F_D(P)}\left(\frac{F_D(P)}{F_D(NP)}\right) - \frac{\omega_t(NP)}{\omega_t(P)}\right]^2 = 0 \qquad (5)$$

In eq 5, P and NP refer to the acceptor groups linked to the polar and nonpolar region of the G_{M1} lipid, respectively. The values of ω_t depend on R_D according to eq 2. An iterative calculation method yields a single solution for $R_D \in \langle 0;54 \rangle$Å, that is, for a physically relevant value of R_D (Table 1), which must not exceed the known value of the micellar radius (= 54 Å).[21] The obtained values of $R_D \approx 33$ Å for all the studied multi-ring-fused 2-pyridones imply that these compounds are localized in the interfacial region of G_{M1} micelles. A similar localization was found, for example, for the aromatic probe PRODAN when solubilized in PCL-PEO vesicles.[24] PRODAN is frequently used for monitoring solvent/environmental relaxation caused by an instantaneous electronic perturbation (vide infra). However, the majority of PRODAN molecules are easily released from the vesicles into the bulk phase by the addition of small amounts of THF (approximately 10% by volume). Similarly, the BODIPY and NBD probes preferentially reside at the interface of lipid membranes,[24] while other probes, such as 2,5,8,11-tetra-*tert*-butylperylene, prefer to solubilize in the interior of lipid bilayers.[25] For the compounds studied here, however, the substitution of hydrogen by fluorine or by a methyl group has no influence on their effective distance from the micellar center of mass (cf. Table 1). This is in agreement with a recent study where the substitution of four hydrogen atoms by four methyl groups in the aromatic core of BODIPY connected to phospholipid did not prevent the chromophore from residing close to the lipid−water interface.[26]

Considering the chemical structure of the acceptors, one might question the obtained donor positions, which are calculated within the assumption of a fully extended G_{M1} molecule. These results (Table 1) are based on the assumption that the acceptor groups reside effectively at the same distance from the micellar center of mass in the nonpolar (FL-NP) as well as in the polar (FL-P) positions of a fully extended lipid (cf. Figure 2). Consequently, one could also question the above estimations of donor depths (R_D). There is a certain degree of spatial freedom of the BODIPY group, since it is attached to the lipid chain via a linker, which allows for displacements toward the micellar center of mass as well as the bulk phase. This positional uncertainty influences the *absolute* values of R_D, which range between 19 Å (for FL-NP and FL-P pointing toward the center) and 35 Å (for FL-NP and FL-P pointing toward the bulk phase). However, the analysis yields a relative position with respect to the acceptors, which is given by $[R_D(P) - R_A(NP)]/[R_A(P) - R_A(NP)]$ ranging between 0.57 and 0.60. Thus, this relatively small range means that the donor molecules reside at the nonpolar/polar interface at approximately

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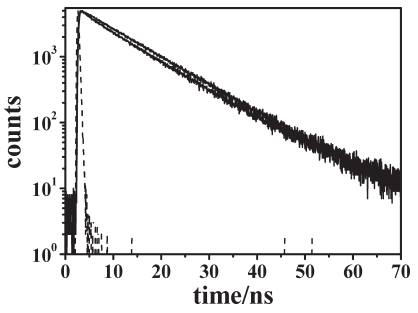

Figure 4. Time-resolved fluorescence decay for D-H in G_{M1} micelles occurring on the nanosecond time scale. Emission has been recorded at 470 and 550 nm (upper curve).

half the distance between P-acceptor and NP-acceptor, while still somewhat shifted toward to the P-acceptor.

Fluorescence Lifetime and Depolarization Studies of Multi-Ring-Fused 2-Pyridones in G_{M1} Micelles. Previous fluorescence lifetime experiments performed on the monomeric multi-ring-fused 2-pyridones dissolved in CH_2Cl_2 revealed a single-exponential relaxation.[1b] The fluorescence lifetimes of the bis-forms $(D\text{-}H)_{C2}$ and $(D\text{-}H)_{C3}$ in $CHCl_3$ are 13.1 and 13.2 ns, respectively. These values are very similar to 13.4 ns, as was previously obtained for D-H dissolved in CH_2Cl_2,[1b] which suggests negligible intramolecular quenching. Interestingly, the obtained relative fluorescence quantum yield (Φ_f) of D-H and $(D\text{-}H)_{C2}$, that is, $\Phi_{f,(D\text{-}H)2}/\Phi_{f,D\text{-}H}$, is 0.85. This reveals an influence of static quenching. Moreover, the molar absorptivity of the bis-form is twice that of the monomer. Furthermore, this is supported by the almost identical fluorescence and absorption spectra as compared to the monomeric form. Sterically however, the linker groups ($-CH_2CH_2-$ and $-CH_2CH_2CH_2-$) could very likely allow for the formation of intramolecular ground state dimers, as well as excited state dimers. When residing in G_{M1} micelles, the fluorescence relaxation of $(D\text{-}H)_{C2}$ and $(D\text{-}H)_{C3}$ are more complex than in $CHCl_3$, and can be adequately described by a sum of two or three exponential functions. The corresponding average lifetimes are 18.9 and 18.2 ns, respectively. These values are similar to the monoexponential fluorescence (τ_f = 17 ns) previously reported for DH in glycerol.[1b] The formation of excited state dimers is unlikely, since no additional emission bands were observed. Also the presence of intramolecular ground state dimers is negligible, since the shapes of the absorption spectra were practically identical to that measured for D-H dissolved in G_{M1} micelles. Neither absorption nor emission spectra differ between D-Me, D-H, and D-F when solubilized in G_{M1} micelles. For these solubilized compounds a complex fluorescence relaxation was observed, which is similar to that observed for $(D\text{-}H)_{C2}$ and $(D\text{-}H)_{C3}$. A slight lifetime dependence on the selected wavelength region of emission was also found. The decay of DH is biexponential with an average fluorescence lifetime of 19.6 ns at 470 nm; the decay is reasonably well described by a single lifetime τ_f = 20.5 ns at 550 nm (cf. Figure 4). The wavelength dependency is compatible with a weak solvent relaxation effect, which is expected for polar molecules whose permanent dipole moment changes upon electronic excitation, but its impact on lifetime and fluorescence spectra is more strongly pronounced in electronic charge transfer processes.[27] A slow relaxation rate of the nearby molecular surrounding of an excited fluorophore means that the process occurs on a time scale comparable to the fluorescence relaxation and reorienting rate. This is investigated through studying the fluorescence anisotropy of D-H, $(D\text{-}H)_{C2}$, and $(D\text{-}H)_{C3}$ when solubilized in G_{M1} micelles. The micelles' rotational correlation time is estimated to be approximately 200 ns, which implies a negligible influence on the fluorescence anisotropy. Interestingly, the average fluorescence lifetimes (~18−19 ns) for all compounds are very similar, and so are the rotational correlation times; that is, all compounds reveal both a short (~1−3 ns) and a much longer (35−55 ns) correlation time. For $(D\text{-}H)_{C2}$ and $(D\text{-}H)_{C3}$, the reorientations are very similar; that is, the fast and slow correlation times are ~2.7 and ~55 ns, respectively. The corresponding correlation times for D-H are ~1.6 and ~35 ns, respectively. The overall faster reorienting motions obtained for D-H can be expected, since its molecular volume is close to one-half of that for a dimer. A likely explanation for the lower reorientation rate stems from slow local reorienting motions of the G_{M1} molecules in the region between the nonpolar and polar parts inside of the micelle. Thus, an influence similar to solvent relaxation can be expected.

In a previous study, the obtained fundamental fluorescence anisotropy[22] of D-H was $r_0 = 0.28 \pm 0.01$. For D-H solubilized in G_{M1} micelles, the initial value of the time-resolved fluorescence anisotropy $r(0) = 0.287$ (cf. eq 4). Thus, unresolved fast reorienting motions of D-H molecules in micelles are not present. The corresponding initial anisotropy of $(D\text{-}H)_{C2}$ and $(D\text{-}H)_{C3}$ is $r(0) = 0.252$ and $r(0) = 0.257$, respectively. A significant lowering of $r(0) < r_0$ for the dimers due to reorienting motions is not expected, since the molecular volume is approximately twice as large. A reasonable explanation for the apparent lowering, however, could be intramolecular energy migration, whereby $r(0)$ would decrease. The only exception to this is the instance when the transition dipoles within a dimer happen to be colinearly oriented, which implies that $r(0) = r_0$. The lowered $r(0)$ values for $(D\text{-}H)_{C2}$ and $(D\text{-}H)_{C3}$ are ascribed to very fast depolarizations caused by fast intramolecular electronic energy migration within the bis-fluorophoric molecules. Since the distance between the D-H groups within a dimer is short, this process will occur on a time scale beyond the time resolution of the equipment used. By using the following relation $^1/_2(3\cos^2\theta - 1) = r(0)/r_0$, the lowered $r(0)$ value for the dimers allows for an estimation of the intramolecular angle (θ) between the $S_0 \leftrightarrow S_1$ transition dipoles of the two D-H groups. For $(D\text{-}H)_{C2}$ and $(D\text{-}H)_{C3}$, the possible solutions obtained are θ_{C2} = 16.6° or 163.4°and θ_{C3} = 15.3° or 164.7°, respectively. For aromatic molecules, the electronic transition dipoles between singlet states are in-plane polarized with the lowest transitions frequently directed along the molecular long axis.[28] Thus, the molecular long axes of the two monomers either tend to be mutually parallel or antiparallel. The latter implies an extended molecule, as is indicated in Figure 1. But what solutions are the most probable? By considering the molecular structure of the D-H molecule, a nonvanishing permanent dipole moment is expected. Furthermore, the structure of $(D\text{-}H)_{C2}$ and $(D\text{-}H)_{C3}$ infers that these dipoles tend to repel each other, so that an extended conformation resembling the structure shown in Figure 1 would be energetically favorable. The extended structure appears to be favorable in vacuum, as is evident from results of computer simulations of the dimer. These were performed based on using an energy minimization program (CS Chem3D Pro version 5.0). In the excited state, the possibility of intramolecular interactions causing excimer formation might exist. However, no

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evidence for this occurrence was found neither in G_{M1} micelles nor in low viscous solvents, for example, chloroform.

Concluding Remarks

Taken together, apart from having unusual fluorescence spectroscopic properties,[1b] the studied multi-ring-fused 2-pyridones are located in the hydrophobic−hydrophilic micellar interface where they undergo slow and restricted tumbling. Furthermore, experimental evidence reveals no tendency for neither ground state nor excited state dimer formation at locally high monomer concentrations, nor indeed even when covalently linked together.

Acknowledgment. We are grateful to M.Sc. Oleg Opanasyuk for preparing Figure 2. Also, this work was financially supported by the Swedish Research Council, the Kempe Foundations, and the Grant Agency of the Czech Republic (P208/10/1090).

List of Abbreviations

FL-P	N-(BODIPY-FL-pentanoyl)-neuraminosyl-ganglioside (G_{M1} = FL-BODIPY-G_{M1} linked covalently to polar headgroup of G_{M1})
FL-NP	FL-BODIPY-G_{M1} = N-(BODIPY-FL-pentanoyl)-ganglioside (G_{M1}=FL-BODIPY-G_{M1} linked covalently to nonpolar headgroup of G_{M1})
581/591-P	*N*-(BODIPY-581/591-pentanoyl)-neuraminosyl-ganglioside (G_M = 581/591-BODIPY-G_{M1} linked covalently to polar headgroup of G_{M1})
581/591-NP	*N*-(BODIPY-581/591-pentanoyl)-ganglioside (G_{M1} = 581/591-BODIPY-C5-nonpolar-G_{M1} = 581/591-BODIPY-G_{M1} linked covalently to nonpolar headgroup of G_{M1})
D-F	Methyl 8-Fluoro-2,3-dihydro-12-phenyl-benzo[*g*]-thiazolo[3,2-*b*]isoquinoline-5-one-3-carboxylate
D-H	Methyl 2,3-Dihydro-12-phenyl-benzo[*g*]thiazolo-[3,2-*b*]isoquinoline-5-one-3-carboxylate
D-Me	Methyl 2,3-Dihydro-11-methyl-12-phenyl-benzo[*g*]thiazolo[3,2-*b*]isoquinoline-5-one-3-carboxylate
$(D\text{-}H)_{C2}$	1,2-Di(2,3-dihydro-12-phenyl-benzo[*g*]thiazolo-[3,2-*b*]isoquinoline-5-one-3-carboxylate) ethane
$(D\text{-}H)_{C3}$	1,3-Di(2,3-dihydro-12-phenyl-benzo[*g*]thiazolo-[3,2-*b*]isoquinoline-5-one-3-carboxylate) propane
G_{M1}	Ganglioside G_{M1}
r_0	Fundamental fluorescence anisotropy
$r(0)$	Initial fluorescence anisotropy
R_0	Förster radius
τ_f	Fluorescence lifetime
ϕ	Rotational correlation time