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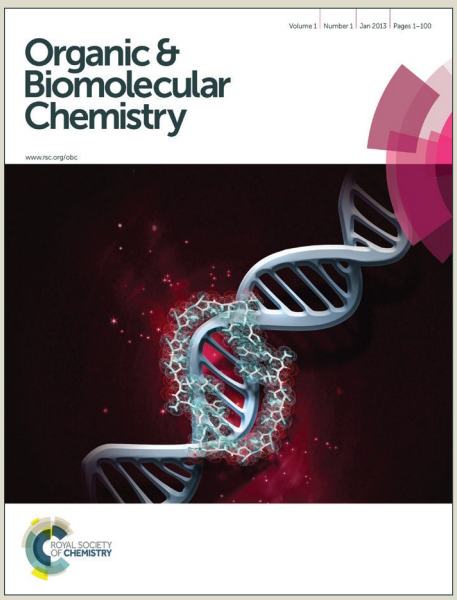

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Fluorescent PET probes based on perylene-3,4,9,10-tetracarboxylic tetraesters

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Perylene-3,4,9,10-tetracarboxylic tetraester-based fluorescent PET probes with aniline receptors attached either at the peri- or the bay-postions have been synthesized. By attaching aniline receptors at the bay position, pH-sensitive "light-up" probes, with fluorescence quantum yields ΦF> 0.75 and fluorescent enhancements FE > 500 in ethanol, have been obtained.

In view of the outstanding photophysical properties of perylene-3,4,9,10-tetracarboxylic acid derivatives,[1] notably fluorescence quantum yields approaching unity and excellent stability,[2] molecular fluorescent probes[3] based on these fluorophores should exhibit superior properties. So far, numerous fluorescent intensity modulating "light-up" probes have been reported, based on the perylene-3,4,9,10-tetracarboxylic acid bisimide (PBI) fluorophore.[4] These probes are quenched by photoinduced electron transfer (PET)[5],[6] or by probe aggregation, generally referred to as aggregation caused quenching (ACQ).[7] Much effort has been invested in the development of probes that detect biologically relevant analytes in aqueous systems[8],[9] but due to the poor intrinsic solubility of the PBI fluorophore,[10] PBI-based probes that are readily soluble in polar solvents are rare. And because PBI probes employed in aqueous solvents are generally close to their solubility limit, quenching of the PBI fluorophore upon analyte binding or detachment[11] often takes place by aggregation caused quenching (ACQ).[12] This may even be the case for probes that have been designed according to the classical "fluorophore-spacer – receptor" PET design.[5a],[13] Therefore, in order to fully exploit the inherent advantageous properties of the perylene fluorophore for application in polar solvents, it is highly desirable to develop truly molecularly dissolved perylene-based probes that are quenched by the PET mechanism.

During the last decades, perylene-3,4,9,10-tetracarboxylic acid derivatives with ester functionality at the peri positions, such as perylene tetraesters (PTEs)[14] and perylene monoimide diesters (PMDEs),[15] have been developed. These dyes have blue-shifted absorption and emission spectra, are synthetically accessible and have retained the high brightness, the high fluorescence quantum yield and the excellent stability of PBIs. The real advantage of these compounds is a strongly improved solubility in organic solvents, in particular the polar solvents. This good solubility is in marked contrast with PBIs, which are inherently soluble at very low concentrations in highly polarizable apolar solvents only.[10]

In this communication we will exploit the strongly enhanced solubility of the PTE chromophore to develop the molecularly dissolved perylene-3,4,9,10-tetracarboxylic ester -based PET probes **1**-**3** and demonstrate the superior performance of these probes in the polar protic solvent ethanol. In the design of the probes **1**-**3** we have chosen to use the "standard" perylene tetrabutyl ester fluorophore and have applied the simplest scheme possible; pH sensitive molecular PET probes designed by employing the "fluorophore-spacer–receptor" format. Aniline was chosen as the proton receptor because of synthetic simplicity. In addition, aniline can be easily modified to form receptors for other cations.[16] We demonstrate herein that PTEs can be selectively substituted. Using this novel approach the aniline receptor units in probes **1**-**3** are either attached at the peri- or the bay-position. Hence, the effect of receptor placement on probe performance, i.e. the *regioselectivity* of photo induced electron transfer, will be investigated in a systematic manner.

The synthesis of probe **1b**, which bears a single amino group at an ester located at the peri-position, commences by reacting perylene dibutylester monoanhydride **4**[17] with a functional alcohol and excess butylbromide in the presence of the strong base DBU, see Scheme 1. To the best of our knowledge, this is the first time that

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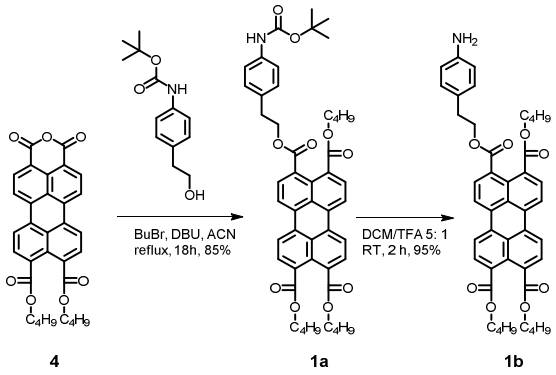
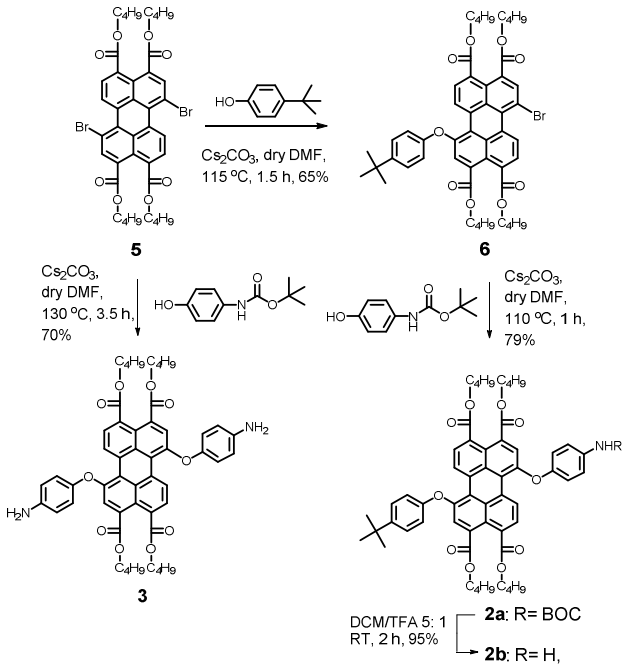
this straightforward synthetic procedure, which allows for the incorporation of a single functionality at the peri-position of PTEs, has been reported. For the synthesis of **1b**, BOC protected 4-amino-2-hydroxyethyl benzene was required, in order to prevent imidization of the anhydride by the primary amino functionality of the aniline. The reaction of compound **4** with BOC protected aniline yielded the BOC protected compound **1a**, which is conveniently deprotected to yield probe molecule **1b**. The overall yield of **1b** was 80% after column chromatography. The identity of the novel monofunctional PTEs **1a** and **1b** has been deduced from their ^{1}H NMR spectra in a straightforward fashion. For **1b**, the 8 aromatic perylene protons give rise to 6 distinguishable doublets. Likewise, the 4 methylene units attached to the ester oxygen atoms, give rise to 3 different triplets in the ^{1}H NMR spectrum, see Figure S4.



Scheme 1. Synthesis of compound **1b**.

The synthesis of bay-substituted PTEs **2b** and **3**, is depicted in Scheme 2 and starts from 1,7-dibromoperylene-3,4,9,10-tetracarboxylic tetrabutyl ester **5**, a synthon that is readily available in isomerically pure form.[18] In our initial attempts to synthesize compound **3**, we reacted compound **5** with 4-aminophenol under standard conditions. This resulted in the isolation of a mixture containing substantial amounts of monodebrominated product **7** and only a small amount of the desired compound **3**, see Scheme S1 and Figure S1. By using BOC-protected aminophenol, compound **3** was readily obtained in 70% yield after column chromatography. A separate deprotection step to remove the BOC-group was not required, because a spontaneous deprotection had taken place at the elevated temperature used for this reaction. For PBIs it has been reported that substitution of the bromine atoms at the bay positions with two different substituents can be achieved by subsequent substitution reactions.[19] With the synthesis of compound **2b**, we demonstrate that this approach can be applied for PTEs as well. In the first step 4-*tert*-butylphenol was attached to dibromo compound **5**, and the mono substituted compound **6** was obtained in 65% yield. Subsequent substitution of compound **6** with BOC-protected aminophenol, followed by a standard deprotection step, yielded the desired compound **2b** in 75% yield, after purification by column chromatography.



Scheme 2. Synthesis of compounds **2b** and **3**.

The good solubility of probes **1**-**3**, which are readily soluble in organic solvents ranging from toluene to methanol, is representative for non-bay and bay-substituted perylene tetra butylesters. In ethanol the solubilities of **1b**, **2b** and **3** are $2.0*10^{-4}$ M (0.14 g/l), $7.7*10^{-3}$ M (7.0 g/l) and $5.8*10^{-4}$ M (0.5 g/l), respectively. A significantly increased solubility is observed for the bay substituted compounds **2b** and **3**. Also the pronounced positive effect of the t-butylphenol substituent on the solubility of **2b** is clearly visible. All compounds are soluble in ethanol water mixtures as well, but their solubility strongly decreases with increasing the water contents. Spectroscopic characterization of all probes has been performed in pure ethanol at 3-5 μmolar concentrations. This is 2-3 orders of magnitude below the solubility limits of the probes, and therefore it is safe to assume that the spectroscopic data reported here refer to the molecularly dissolved probe molecules, and not to aggregates.

Compound **1b** exhibits a low fluorescence in ethanol; to the naked eye this compound appears to be non-fluorescent. After addition of one drop of HCl, upon formation of **1bH$^+$**, the absorption does not change at all, but the fluorescence intensity increases strongly, see Figure 1 (top). The fluorescence quantum yield Φ_F[20] increases from 0.019 to 0.89[21] resulting in a fluorescence enhancement of 47. Thus, by attaching the strong aniline electron donor at the peri-position of the PTE fluorophore, a pH probe with high fluorescence enhancement and a high fluorescence quantum yield in its protonated state has been constructed. Compound **2b** contains the same aniline donor moiety that is now attached to the bay position of the PTE core. The absorption and emission of **2b** in ethanol are shown in Figure 1 (bottom). Upon formation of **2bH$^+$**, the

absorption spectrum (in blue) has shifted 11 nm to the blue. This shift is indicative of electronic conjugation between the perylene chromophore and the aminophenol unit, which becomes more electron deficient upon protonation. More importantly, a strong green fluorescence appears upon protonation. The fluorescence quantum yield Φ_F increases from ~1.2 10^{-3} before protonation, to 0.87 after protonation. This results in a huge fluorescence enhancement of approximately 700.[22] This observation clearly indicates that by attaching the aniline quencher moiety at the bay- instead of the peri-position a fluorescent probe with a substantially higher fluorescence enhancement has been constructed.

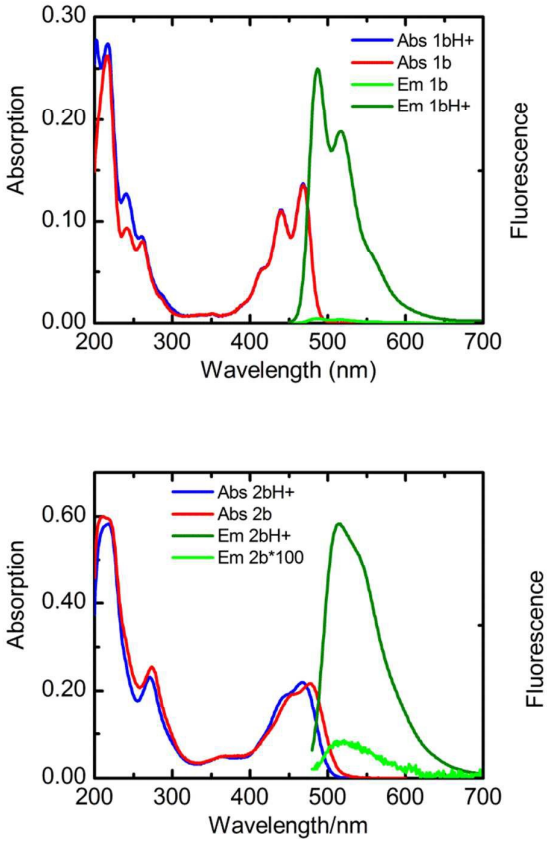


Figure 1. Absorption and emission spectra of **1b** and **1bH$^+$** (top, 3.5 μmol/l) and **2b** and **2bH$^+$** (bottom, 5.0 μmol/l) in ethanol. Compounds **1bH$^+$** and **2bH$^+$** were formed by the addition of HCl. The fluorescence intensity of **2b** was magnified by a factor 100.

For compound **3**, bearing two aniline receptors at the bay-positions, similar observations have been made, see Figure S2. Upon formation of **3H$_2^{2+}$**, the absorption spectrum (in blue) has shifted 20 nm to the blue. The fluorescence quantum yield Φ_F increases from ~4*10^{-4} to 0.75 resulting in a fluorescence enhancement of around 1800. In order to see whether probes **1**-**3** are potentially useful for pH probing in aqueous solvents, we tested compound **3** in 1:1 ethanol/water mixtures. The result, illustrated by Figure S2, is similar to that obtained in pure ethanol. Strong fluorescence is observed in acid media, virtually no fluorescence is obtained in base, and the fluorescence enhancement is well above 500. This experiment clearly demonstrates that PTE probes like **3** are applicable in aqueous media as well.

At this stage it appears that an anticipated faster electron transfer from an aniline attached to the bay position causes the larger fluorescence enhancement of **2b** as compared to **1b**. In order to validate this hypothesis we have collected the photo physical data from compounds **1b**, **2b** and **3** and their protonated analogues. These data, obtained from steady state and time-resolved optical measurements, are compiled in Table 1. Analysis, using Eqs 1a-d, yields rates of fluorescence k_F and rates of non-radiative decay k_{nr}.

$$\Phi_F = \frac{k_F}{k_F + k_{nr}} \qquad \tau_F = \frac{1}{k_F + k_{nr}} \qquad k_F = \frac{\Phi_F}{\tau_F} \qquad k_{nr} = \frac{k_F}{\Phi_F} - k_F \qquad \text{Eq. 1a-d}$$

Table 1. Photophysical data of compounds **1**-**3** in ethanol. Protonation was achieved by adding HCl.

Comp	λ_{abs} (nm)	λ_{em} (nm)	Φ_F	FE	τ_F (ns)	k_F [b]	K_{nr} [b]
1b	468	488	0.019		- [a]	2.1 [c]	110
1bH$^+$	468	488	0.89	47	4.16	2.1	0.26
2b	478	518	1.2*10^{-3}		- [a]	1.7 [c]	1400
2bH$^+$	467	513	0.87	700	5.25	1.7	0.24
3	482	527	4.2*10^{-4}		- [a]	1.6 [c]	3900
3H$_2^{2+}$	462	509	0.75	1800	4.61	1.6	0.54

a: lifetimes too short to be measured accurately. b: rates in 10^8 s^{-1}. c: taken the same as for protonated compound, assuming that k_F is not influenced by protonation.

For the peri substituted compound **1bH$^+$**, a rate of fluorescence k_F of 2.1* 10^8 s^{-1} has been determined. For the bay-substituted compounds **2bH$^+$** and **3H$_2^{2+}$**, the rate of fluorescence, 1.6-1.7* 10^8 s^{-1}, is 20% lower. Fluorescence quenching by non-radiative decay takes place for all compounds. The protonated compounds (**1bH$^+$**, **2bH$^+$** and **3H$_2^{2+}$**) have quenching rates k_{nr} that are around one order of magnitude below their rates of fluorescence, typically 2-5* 10^7 s^{-1} For this reason all protonated probes are highly fluorescent compounds. Fluorescence quenching in the non-protonated compounds, most likely caused by electron transfer from the free amine to the excited PTE, is much faster. By assuming that the rate of fluorescence does not change upon protonation and that the additional quenching observed upon deprotonation is due to electron transfer only, rates of electron transfer k_{ET} have been determined: 1.1*10^{10} s^{-1} for **1b** , ~1.4*10^{11} s^{-1} for **2b** and ~3.9*10^{11} s^{-1} for **3**. These data clearly reveal that quenching by the aniline moiety is much more effective from the bay position; transferring the aniline moiety from the peri- to the bay-position results in an increase of the quenching rate by one order of magnitude. The increase in fluorescence enhancement induced by transferring the receptor from the peri- to the bay-position is even larger because the rate of fluorescence k_F of bay-substituted compounds is substantially lower.

In conclusion, we have exploited the potential of the perylene fluorophore with the developments of the highly soluble bay-substituted perylene tetraester-based PET probes **2b** and **3**. These pH probes exhibit fluorescence quantum yields close to unity and extremely high fluorescent enhancements with values well above 500. The peri-substituted PTE-based probe **1b** has a high fluorescence quantum yield as well, but exhibits a modest fluorescence enhancement of 47. Photophysical experiments revealed that the large increase in FEs, that is achieved by receptor attachment at the bay position, is caused by a faster electron transfer from the aniline substituent at the bay position along with the lower inherent rate of fluorescence of bay substituted PTEs. Thus, attachment of spacer receptor units at the bay positions is the preferred strategy for developing highly sensitive perylene based fluorescent probes. Our current research is focused on the development of water-soluble PTE-based fluorescent probes for probing in pure water.[23]

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Keywords: fluorescent probes • perylenes • photoinduced electron transfer • fluorescence spectroscopy • synthetic chemistry

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