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PHOTOACTIVABLE FLUOROPHORES. 1. SYNTHESIS AND PHOTOACTIVATION OF *o*-NITROBENZYL-QUENCHED FLUORESCENT CARBAMATES.[1]

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Summary: The synthesis and photoactivation of a new type of fluorescent probe, the photoactivable fluorophore (PAF) are described. The PAFs described in this paper consist of fluorescent aromatic amines coupled to substituted nitrobenzyl quenching chromophores via a carbamate linkage. Photoactivation occurs by an intramolecular photo-redox reaction, converting the o-nitrobenzyl group to a labile *o*-nitrosobenzaldehyde hemiacetal which fragments with loss of CO_2 to liberate the fluorescent amine. Photoactivation quantum yields also are reported.

Fluorescence methods have emerged as indispensible analytical and tracer techniques for a diverse range of biochemical, biophysical and physical studies that depend upon sensitive measurements of the physical environment.[2] Advances in instrumentation have been augmented by the development of new fluorescent probe molecules with specifically tailored characteristics. In this Letter we describe a new type of tracer molecule, the photoactivable fluorophore (PAF). Photoactivable fluorophores are stable non-fluorescent molecules with the latent capacity to become permanently fluorescent.[3,4] PAFs have two distinct advantages over conventional fluorescent probes. They can undergo photoactivation in a specific spatial region, permitting tracer differentiation of molecular species that are otherwise identical, and their movement can be monitored at any time subsequent to photoactivation. These capabilities enable long term molecular surveillance and facilitate measurement of macromolecular transport and diffusion in solution or in complex media by the technique fluorescence photoactivation and dissipation (FPD), developed recently by Ware and us.[5]

Our initial approach to the design of probe molecules with latent, but photoactivable fluorescence involved functionalization of a suitable fluorescent probe with a quencher chromophore that could be cleaved photochemically to liberate the fluorescent molecule from the fluorophore-quencher conjugate. Central to the success of this approach were the incorporation of an effective fluorescence quencher,[6] and the development of efficient photoactivation chemistry. Furthermore, PAFs intended for application to particular problems also would require additional functionalization to impart desirable solubility characteristics and covalent attachment capabilities.

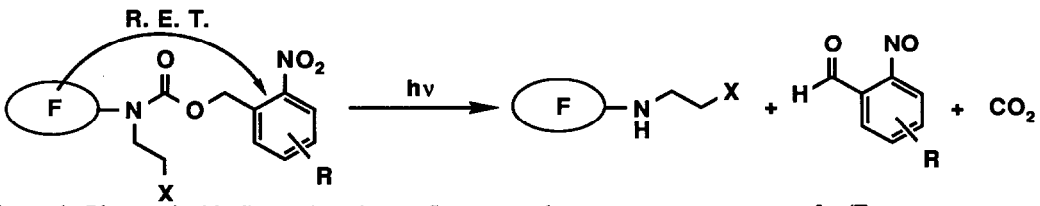
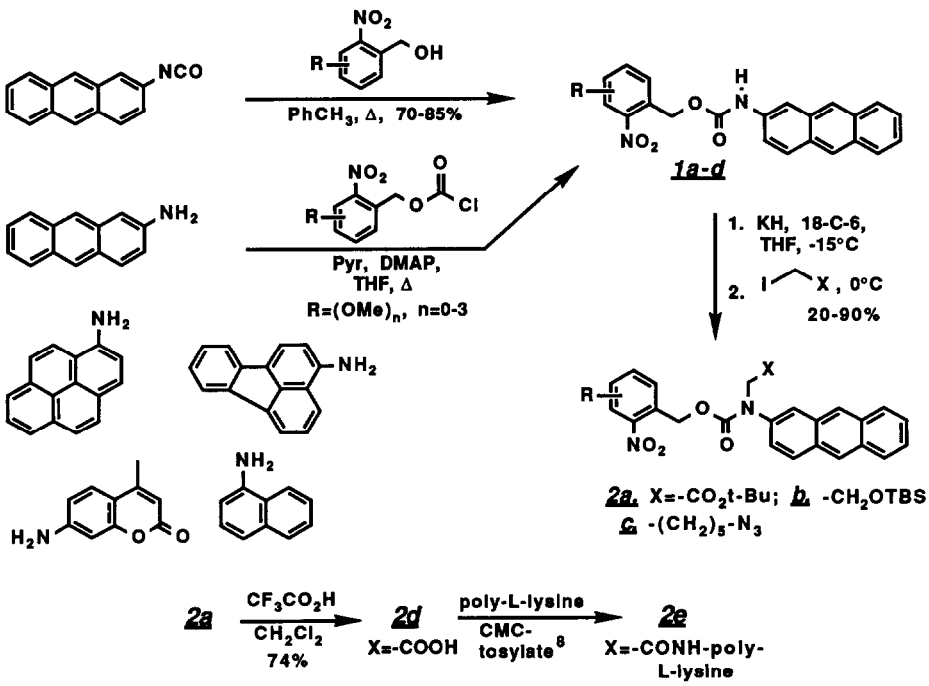


Figure 1. Photoactivable fluorophore is non-fluorescent due to resonant energy transfer (Forster energy transfer)[6] to quenching chromophore. Photoactivation cleaves the quencher and restores fluorescence. **X** is a reactive functional group for covalent linking to macromolecules. **R** can be a polar or lipophilic group to alter PAF solubility.

A simple prototype which accomodated these design criteria was a carbamate consisting of a fluorescent amine and a photocleavable *o*-nitrobenzyl group[7] with matched spectral characteristics to insure efficient quenching of the fluorophore excited state, as illustrated in Figure 1. The carbamate nitrogen also would provide the capability to attach a covalent linking group.

Carbamate synthesis was straightforward and could be accomplished by either of the two routes outlined in Scheme I.[8] The fluorescent aromatic amines were converted to the corresponding isocyanates by treatment with phosgene in refluxing toluene,[9] which reacted with a variety of substituted *o*-nitrobenzyl alcohols to generate the carbamates in excellent yields. Alternatively, the amines could be treated with *o*-nitrobenzyl chloroformates in the presence of DMAP and pyridine, though yields for these acylations were lower (40-50%) for several of the poorly nucleophilic amines. Covalent linking appendages could be attached via the acetic acid group, which was introduced by alkylation with t-butyl iodoacetate and subsequent ester cleavage. Alkylations with other functionalized alkyl iodides also were carried out, but the acetic acid group was most versatile and could be incorporated efficiently and on a large scale. This acetic acid appendage (**2d**) was utilized to covalently label poly-L-lysine (47,000 MW) via an amide linkage (3% labeling of available sites), permitting macromolecular diffusion studies.

SCHEME I



Several of the purified carbamates did exhibit low levels of residual fluorescence, depending upon the nature of the fluorophore and the quencher chromophore. In general, fluorophores with longer wavelength emission such as 1-aminopyrene and 3-aminofluoranthene were quenched less efficiently than fluorophores that emitted at lower wavelengths, perhaps as a result of poorer spectral overlap with the *o*-nitrobenzyl quenching chromophores.

The most effective quenching chromophore was the 4,5-dimethoxy-2-nitrobenzyl group, which has a strong lower energy absorption (λ_{max} = 353 nm) into which energy can be efficiently transferred. This absorption band is not present in the spectrum of the parent 2-nitrobenzyl group, and is significantly attenuated in the spectrum of the 3,4,5-trimethoxy-2-nitrobenzyl group, resulting in higher residual fluorescence by the carbamate PAFs containing these chromophores. Figure 2 illustrates the emission spectra for the quenched 4,5-dimethoxy-2-nitrobenzyl carbamate of 2-aminoanthracene, 2-aminoanthracene and the O-methyl carbamate of 2-aminoanthracene,[9] which more closely represents the actual fluorescent chromophore that undergoes quenching by o-nitrobenzyl chromophores.

Photoactivation of the *o*-nitrobenzyl carbamates is a unimolecular process, occurring via formation of an *o*-nitrosobenzaldehyde hemiacetal,[10] which is labile and decomposes with loss of CO_2 to liberate the free fluorescent amine. The quantum yields for photoactivation of the 2-aminoanthracene carbamates and a variety of substituted *o*-nitrobenzyl alcohols were measured[11] (Table I) in order to determine the optimal substitution of the *o*-nitrobenzyl chromophores. Methoxyl groups lowered the quantum efficiency, but enhanced the extinction coefficient at 350 nm so that the 4,5-dimethoxy-2-nitrobenzyl carbamate photoactivated most rapidly. A nitro group in the 6-position enhanced the quantum efficiency, but lowered the absorptivity at 350 nm. The 3,4,5-trimethoxy-substituted derivative was virtually unreactive when irradiated, due to lower absorptivity at 350 nm, and to sterically induced conformational effects of the three methoxyl groups in which the 3-methoxy group forces the adjacent nitro group out of the plane of the aromatic ring, thereby diminishing its capability to abstract a benzylic hydrogen atom. The quantum yields for the carbamates are significantly lower than for the parent alcohols. Two factors contribute to this phenomenon: 1) the resonance electron withdrawing effect of the carbamate carbonyl lowers the efficiency of hydrogen atom abstraction, and 2) the aromatic amine absorbs a large fraction of the incident irradiation.

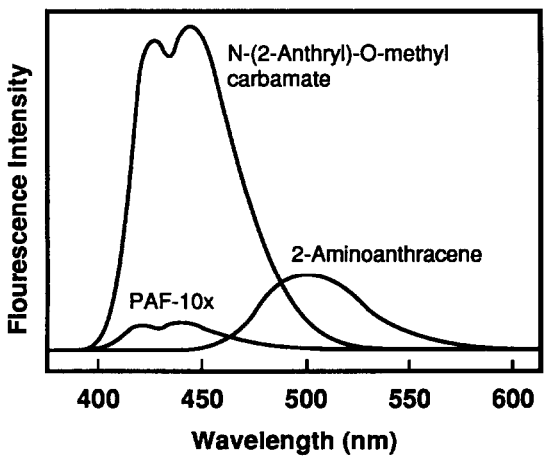
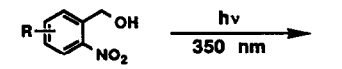


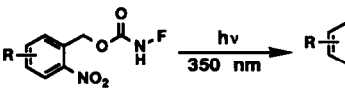
Figure 2. Emission spectra (λ_{ex}=375 nm) of 2-aminoanthracene (1 μM), its O-methyl carbamate (1μM) and its O-(4,5-dimethoxy-2-nitrobenzyl) carbamate (1 μM) in 1:1 (v/v) THF:H_2O. Residual PAF emission is negligible at the 2-aminoanthracene emission λ_{max} (495 nm).

TABLE I

PHOTOACTIVATION QUANTUM YIELDS



R	Φ
H	0.45
6-NO_2	0.59
4,5-$(OMe)_2$	0.07
4,5-(OCH_2O)-	0.12
3,4,5-$(OMe)_3$	< 0.005



R	F	Φ
H	2-Anthryl	0.013
6-NO_2	2-Anthryl	decomp.
4,5-$(OMe)_2$	2-Anthryl	0.05
3,4,5-$(OMe)_3$	2-Anthryl	< 0.001
H	Cyclohexyl	0.07
H	Phenyl	0.043
4,5-$(OMe)_2$	Cyclohexyl	0.06
4,5-$(OMe)_2$	Phenyl	0.045

Comparison of quantum yields for the O-(4,5-dimethoxy-2-nitrobenzyl)-carbamates of aminocyclohexane and 2-aminoanthracene reveals very little decrease as a result of non-productive absorption by the aminoanthracene chromophore. This indicates that energy transfer from the anthryl chromophore to the 4,5-dimethoxy-2-nitrobenzyl chromophore is quite efficient. In the analogous O-(2-nitrobenzyl)-carbamates, a significant drop in quantum yield is observed, indicating that energy transfer between chromophores is less efficient. This lower efficiency of energy transfer is also manifest in the poorer fluorescence quenching by this unsubstituted 2-nitrobenzyl chromophore.

Although the photoactivation quantum efficiencies are less than 10% for these carbamate PAFs, useful levels of fluorescence can be generated by short (50-100 msec) laser irradiation, permitting studies of macromolecular transport and diffusion. These studies are in progress and will be described elsewhere.

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8. All new compounds gave satifactory spectral data. Reported yields correspond to purified, isolated materials. CMC-tosylate -- 1-Cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluenesulfonate, a DCC analog.

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11. Quantum yields (with accuracy to ± 10%) were determined by HPLC quantitation of remaining carbamate after irradiation of 100 μM PAF solutions at 350 nm for 20-300 sec. in a Rayonet photochemical reactor configured with a merry-go-round apparatus. Actinometry involved the photoaquation of Reinecke's salt (Wegner, E. E.; Adamson, A. W. *J. Amer. Chem. Soc.* **1966**, 88, 394-404). Quantum yields for several *o*-nitrobenzyl analogs have been measured. Recent values (Zhu, Q. Q.; Schnabel, W.; Schupp, H. *J. Photochemistry* **1987**, 39, 317-332) for several analogs are in good agreement. Earlier values (Baryzynski, H.; Sanger, D. *Makromol. Chem.* **1981**, 93, 131-137) are too high, since these were obtained at very high concentrations.

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