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VERSATILE FLUORESCENCE LABELING METHOD USING ACTIVATED ESTERS ON SOLID SUPPORT

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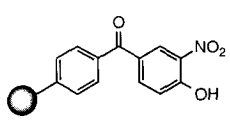
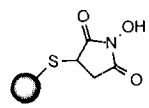
Abstract: A versatile fluorescence labeling method employing support bound nitrophenyl, N-hydroxysuccinimidyl, 1-hydroxybenzotriazoyl and oxime active esters is described. The relative kinetic properties of different esters were systematically compared using an environment-independent benzoquinoline dye.

Fluorescence labeling of biologically active compounds is a useful technique for preparing biochemical probes for *in vivo* and *in vitro* studies. Conventional labeling methods rely upon reaction of amine or thiol nucleophiles with activated dyes, conditions under which side products may form. In most cases, it is necessary to isolate the desired product from unreacted reagents and side products. The preparation of activated dye, moreover, can be difficult given their sensitivity to moisture and various nucleophiles. Thus, a procedure that allows facile preparation of the activated dye and labeling of biologically interesting compounds without chromatographic steps would be a useful tool.

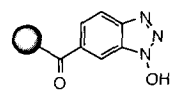
Several resin bound active esters derived from nitrophenol (**1a**)[1], N-hydroxysuccinimide (**1b**)[2], 1-hydroxybenzotriazole (HOBt) (**1c**)[3-5] and Kaiser oxime (**1d**)[6-10] have been developed for peptide or small molecule library synthesis. Very recently, the first example of resin-bound labeling reagent using N-hydroxysuccinimide (**1b**) was reported.[11] We sought to determine the relative reactivities and efficiencies with which active esters derived from these polystyrene resins acylate various amine nucleophiles.



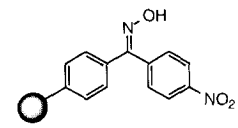
1a



1b



1c



1d

Nitrophenol resin **1a** was prepared by a slightly modified literature procedure (0.87 mmol/g).[12] N-hydroxysuccinimide resin **1b** was synthesized by coupling of commercially available thiol 4-methoxytrityl resin with N-hydroxymaleimide in a manner similar to that described in the literature (0.60 mmol/g).[2] Resins **1c** and **1d** were commercially available and used as purchased from Nova Biochem (1.45 and 1.19 mmol/g respectively).

4-Acetamino-1,8-naphthalimide derivatives were used in the labeling study as these dyes have a high quantum yield (0.7-1.0) in a variety of solvents including water.[13,14] 4-Amino-1,8-naphthalic anhydride (**2**) was acetylated using acetic anhydride at elevated temperature to give 4-acetamido-1,8-naphthalic anhydride **3**. After purification by recrystallization, compound **3** was reacted with 6-aminocaproic acid to give 4-acetamido-

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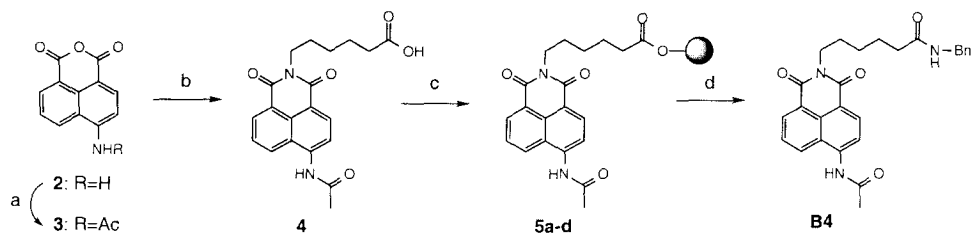
1,8-naphthalimide-N-caproic acid (**4**), which was previously synthesized using a different synthetic pathway (λ_{ex} = 360 nm, ε= 6326, λ_{em} = 340-600 nm).[15] Compound **4** (1.1 eq.) was used for coupling with resins **1a-d** to give solid phase reagents **5a-d** (Scheme). As DCC generates an insoluble urea by-product,[12] diisopropylcarbodiimide (DIC) was used as a coupling reagent in the presence of catalytic 4-dimethylaminopyridine (DMAP). Reaction of **5a-d** with benzylamine (**A4**, 0.1-0.2 eq.) in dichloromethane or THF, followed by simple filtration gave benzyl 4-acetamino-1,8-naphthalimido-N-hexanoic amide (**B4**). When excess benzylamine was used, unreacted amine was removed by washes with aqueous acid (10% $NaHSO_4$) or by incubation with sulfonic acid resin (Dowex 50x-100) and subsequent filtration. Without further purification, this procedure produced high purity **B4** as judged by ^{1}H-NMR (λ_{ex} = 360 nm, ε = 1.22 x 10^4, λ_{em} = 465 nm, in MeOH).[16] The loading level of resin **5a** was determined by mass balance to be 0.50 mmol/g.

Scheme. Synthesis of solid phase dye reagent and labeling of amine.



a. Ac_2O, NMP, 150 °C (56%). b. 6-aminohexanoic acid, NMP, 150 °C (62%). c. **1a-d**, DIC, DMAP, NMP, rt. d. $BnNH_2$, THF or CH_2Cl_2, rt.

Measurement of the fluorescence spectra of a sequential dilution of **B4** in methanol showed a good linear correlation in the 5 nM – 5μM range (data not shown). The relative fluorescence intensity of **B4** varied only slightly in a variety of solvents: DMSO (0.6), H_2O (0.8), EtOH (0.9), NMP (0.9), MeOH (1.0), DMF (1.0), THF (1.1), CH_3CN (1.2), EtOAc (1.2), acetone (1.2), and CH_2Cl_2 (1.2). The presence of up to 1mM p-toluenesulfonic acid (pTSA) or benzylamine did not significantly affect the fluororescence of **B4** in MeOH. At a 10 mM concentration of benzylamine or pTSA, the fluorescence intensity of **B4** decreased by 2% and 16%, respectively. Higher concentrations of pTSA seemed to destroy the dye structure generating green colored material (not studied further).

Fluorescence measurements of the amination product, **B4**, were used to determine the reaction rate constant for the different resins. Resins **5a-d** were reacted with different concentrations of benzylamine (100 μM – 100 mM), and the initial rates were determined by monitoring fluorescence intensity as a function of time (Table). The reaction rates of hydrolysis or methanolysis were measured in a similar fashion in the presence and absence of base. The relative rates of reaction were: **5c** >> **5b** > **5a** >> **5d**. The selectivity of aminolysis over hydrolysis and alcoholysis was more than a million fold for **5a** and **5b**, and about 60,000 fold for **5c**. Resin **5d** was not studied further due to its slow reaction rate, which requires longer reaction time and elevated temperature. The presence of 1% diisopropylethylamine (DIEA) increased the hydrolysis/alcoholysis rate by

approximately one hundred fold, but these reactions are still much slower than the aminolysis reaction. The selectivity for reaction with amines over water and alcohol suggest that this solid phase labeling strategy might be applied to aqueous reaction conditions if combined with appropriate water-compatible resins such as PEG or macropore.

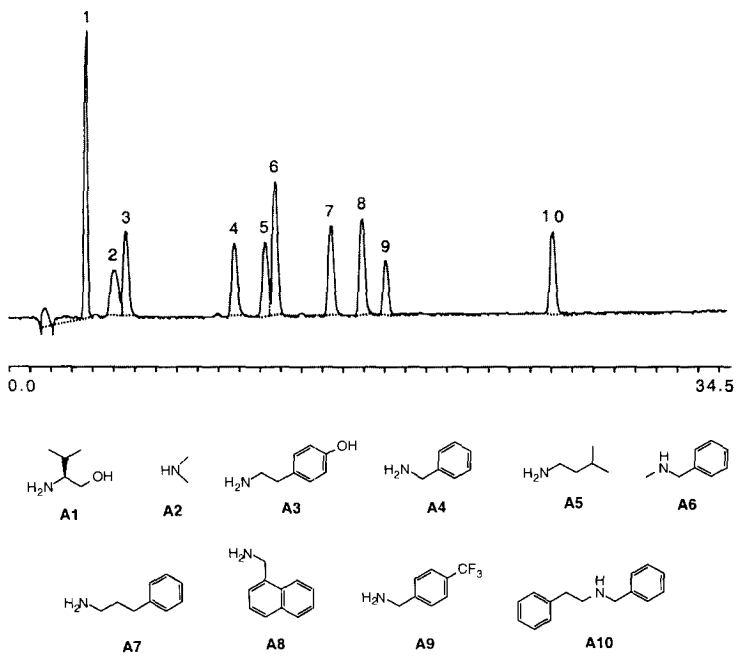
Table. Loading level of solid phase reagents **5a-d** and kinetic data.

	5a	**5b**	**5c**	**5d**
Loading level[a] (mmol/g)	0.56 (0.50)[b]	0.40	0.76	0.68
k ($M^{-1}min^{-1}$) for $BnNH_2$	24.4 (1.0)[c]	36.4 (1.5)	2390 (98)	0.060 (1/400)
k ($M^{-1}min^{-1}$) for H_2O	4.5×10^{-6}	2.1×10^{-5}	0.041	N/D
k ($M^{-1}min^{-1}$) for H_2O[d]	2.8×10^{-4}	1.6×10^{-3}	N/D	N/D
k ($M^{-1}min^{-1}$) for MeOH	1.1×10^{-5}	N/D	N/D	N/D
k ($M^{-1}min^{-1}$) for MeOH[d]	8.6×10^{-4}	N/D	N/D	N/D

[a]By fluorescence measurement. [b]By mass balance method. [c]Relative reactivity. [d]In the presence of 1% DIEA.

Solution phase fluorescence labeling has been used to encode combinatorial libraries.[17,18] The ability to use **5a** for this purpose was tested using primary, secondary and alcohol containing amines (total amine amount was about 0.1 eq. of **5a**). After removal of the resin by filtration, HPLC analysis (C8 analytical column, 35-65 % CH_3CN gradient in H_2O, detection at 360 nm) revealed that every amine in the mixture had been comparably labeled, demonstrating that **5a** can be used to encode libraries (Figure).

Figure. HPLC trace of a variety of amines labeled using **5a**.



In summary, we synthesized and compared four different solid phase activated esters (nitrophenol, n-hydroxysuccinimide, HOBt and Kaiser oxime) and demonstrated their utility as a fluorescence labeling method. This strategy can be used to label biologically active amines for use as molecular probes or for the encoding of small molecule libraries.

Acknowledgement

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16. **B4**: Rf 0.5 (MeOH:CH_2Cl_2 1:10); ^1H NMR (500 MHz, $CDCl_3$ + [D_6]DMSO): δ 1.32-1.38 (m, 2H), 1.57-1.66 (m, 4H), 2.14 (t, J=7.4 Hz, 2H), 2.25 (s, 3H), 4.02 (t, J=7.5 Hz, 2H), 4.24 (d, J=5.7 Hz, 2H), 7.11-7.25 (m, 5H), 7.72 (dd, J=8.6, 7.2 Hz, 1H), 8.02 (t, J=5.7 Hz, 1H), 8.26 (d, J=8.1 Hz, 1H), 8.41 (d, J=8.2 Hz, 1H), 8.47 (d, J=7.2 Hz, 1H), 8.61 (d, J=8.6 Hz, 1H), 10.23 (s, 1H); MS (FAB) m/e 458.1 (MH^+); HRMS (FAB) Calcd for ($C_{27}H_{27}N_3O_4$)H^+: 458.2080, Found 458.2083.
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