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Synthesis and Characterization of Dimaleimide Fluorogens Designed for Specific Labeling of Proteins

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Abstract: A series of aromatic compounds were prepared bearing two maleimide groups attached directly to the fluorescent cores. The resulting derivatives do not fluoresce until the maleimide groups undergo their typical thiol addition reaction, thus removing their ability to quench fluorescence, as shown by kinetic and spectral characterization studies. In this way, the title compounds serve as fluorogens capable of detection of small thiols or appropriately sized dithiols. Recombinant α-helical proteins were then designed to bear two cysteine residues capable of regioselective dithiol addition reaction with the dimaleimide fluorogens, thus acting as spatially encoded substrates that form specifically labeled covalent complexes. The efficiency of this in vitro fluorescent protein-labeling reaction demonstrates the feasibility of the development of a method for the fluorescent labeling of specific recombinant proteins.

Introduction

In this post-genomic era, a huge number of gene products remain to be categorized, and this monumental task requires protein-labeling techniques that will lend precision and speed to the process. The fluorescent labeling of proteins has proven to be a powerful approach to determining the dynamics of their turnover (synthesis and degradation), localization, and protein−protein interactions. Several labeling techniques have been developed that involve the use of fluorescent dyes bearing reactive functional groups, such as succinimidyl esters or maleimides, known to react with amines or thiols; however, these techniques are typically nonspecific; many such functional groups exposed on the surface of any protein may be labeled.[1−3] The genetic fusion of target proteins to fluorescent proteins, such as jellyfish green fluorescent protein (GFP), is the most specific and broadly applied approach to following individual protein dynamics in living cells[4] and even determining cellular localization of proteins at a whole genome scale.[5] However, there are some limitations of this method. For example, the entire sequence of GFP must be properly folded into its 11-stranded β-barrel structure for it to function as a fluorophore, but maturation into soluble, fluorescent protein is not spontaneous and may be problematic, especially at or above room temperature.[6] Moreover, GFP fluorescence is sensitive to the environment of its fusion with test proteins and can be difficult to distinguish from the autofluorescent background of living cells. Protein-engineering approaches have resulted in GFP variants, for which some of these limitations have been overcome; however, one problem that cannot be addressed is that the steric bulk and metabolic stability of a 27 kDa β-barrel protein can significantly perturb the dynamics and localization of test proteins.[4,6]

Tsien and co-workers have developed an alternative genetically encoded protein-labeling strategy, which addresses the steric bulk and stability problem, as well as the limited range of fluorescent characteristics of GFP and its variants.[7] This technique involves the use of a short peptide sequence, optimized to comprise a β-turn sequence,[8] containing four Cys residues positioned to allow their reaction with a biarsenical fluorogenic compound. The resulting biarsenical peptide adduct is much more fluorescent than the initial fluorogen. Furthermore, the probe peptide sequence necessary for reaction with the biarsenical fluorogen is small (16 aa in its typical application[8]), implying a minimal perturbation of the target protein, and presents a minimal 4-Cys motif (CCPGCC) that confers enough specificity for the biarsenical fluorogen that it may be used in vivo.[8] The chief disadvantage of this method is the obvious toxicity of the arsenic derivatives used. Although Tsien has developed a protocol[9] for the administration of antidotes that render the method broadly applicable, alternative methods that do not rely on such toxic metals would offer a distinct advantage.

Over the past few years, we have synthesized and characterized several fluorogens that contain maleimide groups.[10,11]

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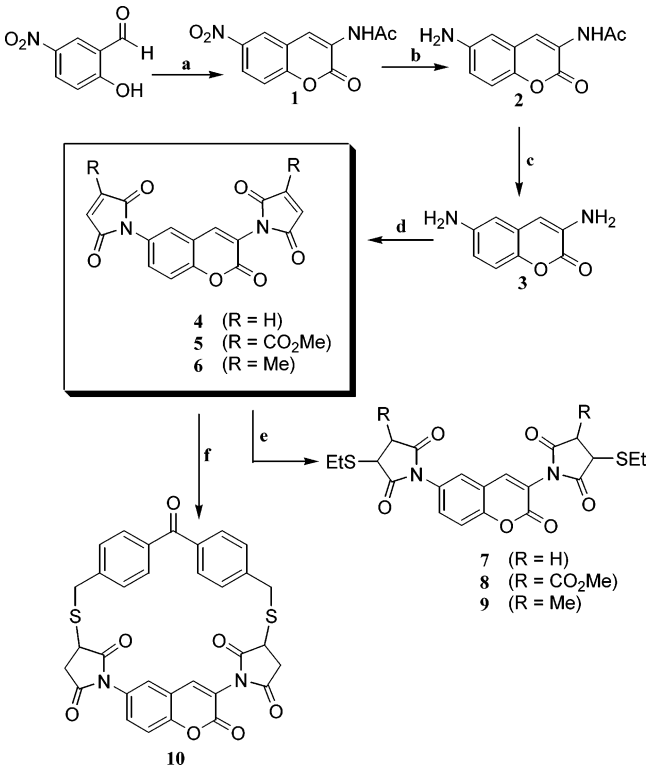
Maleimide groups are known to react fairly selectively with thiols via addition reactions involving their C=C double bond. They are also known to quench fluorescence in their conjugated form, but not as their thiol adduct products. These properties were demonstrated in the characterization of a naphthopyranone derivative bearing a maleimide group whose fluorescence increased dramatically upon reaction with glutathione.[12,13] We reasoned that if a fluorogen was prepared bearing *two* maleimide groups, then its latent fluorescence would only be realized upon its reaction with *2 equiv* of thiol. Furthermore, if the positioning of maleimide groups was such that they were separated by a precise distance, then the resulting fluorogen should react rapidly and specifically with compounds presenting two sulfhydryl groups separated by the appropriate distance. While similar bifunctional thiol-reactive fluorogens, such as dibromobimane, have been used previously to label proteins,[14,15] the selectivity of maleimide groups for reaction with thiols, rather than several different nucleophiles, would appear to be advantageous for the ultimate application to in vivo labeling experiments. Herein, we present the synthesis of a series of such dimaleimide fluorogens, the characterization of their reactions with thiols and dithiols, and the changes in their fluorescent properties accompanying these reactions. Furthermore, we demonstrate our ability to specifically label the leucine-zipper-forming peptide of the transcription factor Fos in which two cysteine residues have been substituted into the sequence in positions that ensure spatially specific reaction with the dimaleimide fluorogens when the peptide is in an α-helical conformation. Finally, we discuss the potential of the application of these fluorogens for the specific labeling of protein targets.

Results and Discussion

Synthesis. Shown in Schemes 1 and 2 are the routes taken to prepare fluorogenic coumarin derivatives **4** and **13**, by the reaction of their corresponding diamino derivatives with maleic anhydride.[16] Diamino derivative **3** was prepared according to a previously published synthetic route,[17] which also formed the basis of the route used to prepare the novel diamine **12**. For most of the steps, the synthesis was straightforward; however, the key step involving the synthesis of the maleimide ring was problematic. As has been reported, the weak nucleophilicity of the aromatic amines and the limited solubility of the synthetic precursors lower the yield of this transformation that introduces two maleimide groups, each requiring two reaction steps.[18]

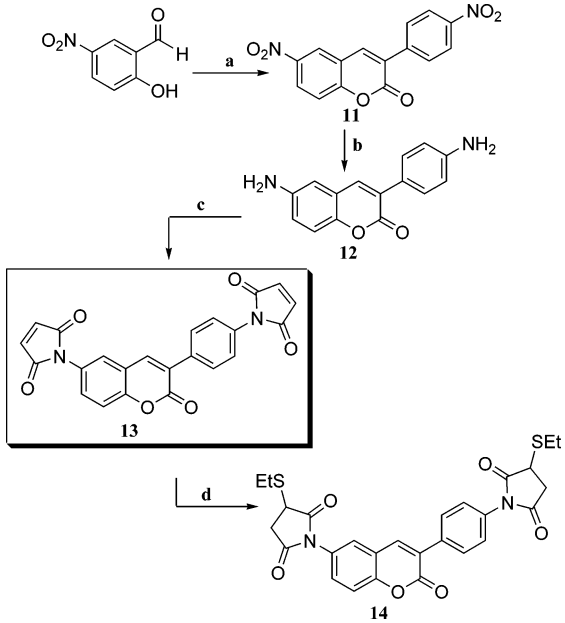
In Schemes 3 and 4 are shown the syntheses of fluorogens **15** and **20**, dimaleimide derivatives of naphthalene. The synthesis of **15** required only the facile addition of the maleimide group to the commercially available diaminonaphthalene, whereas the synthesis of **20** was more circuitous.[19] Nitration of

Scheme 1 [a]



[a] (a) *N*-Acetylglycine, NaH, Ac_2O, rt, 61%; (b) Pd/C (10% H_2O), $NaBH_4$, MeOH, 57%; (c) EtOH, HCl, reflux, 65%; (d) (i) maleic anhydride, $CHCl_3$, reflux, (ii) Ac_2O, AcONa, 100 °C, 20% (two steps); (e) EtSH, $CHCl_3$ or DMSO, rt, 83−85%; (f) BMMB, NEt_3, $CHCl_3$, reflux, 78%.

Scheme 2 [a]



[a] (a) 4-Nitrophenylacetic acid, Ac_2O, NaH, rt, 95%; (b) Pd/C (10% H_2O), $NaBH_4$, MeOH, 63%; (c) (i) maleic anhydride, $CHCl_3$, reflux, (ii) Ac_2O, AcONa, 100°C, 20% (two steps); (d) EtSH, DMSO, rt, 89%.

commercially available 1,8-naphthalic anhydride yielded 3,6-dinitro derivative **17** and a small amount of the 3,5- and 4,5-dinitro derivatives, whose formation was limited to <20% at reaction temperatures between 10 and 20 °C.[19] These derivatives were easily separable by chromatography, giving compound **17** in good yield. From this anhydride, the imide of L-aspartic acid

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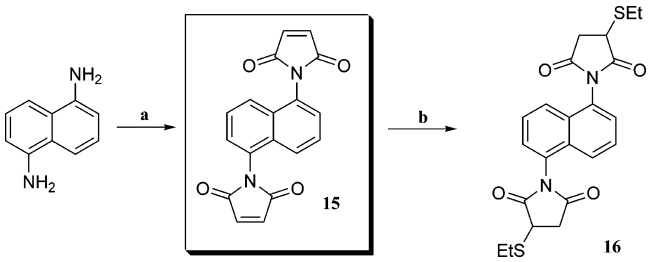
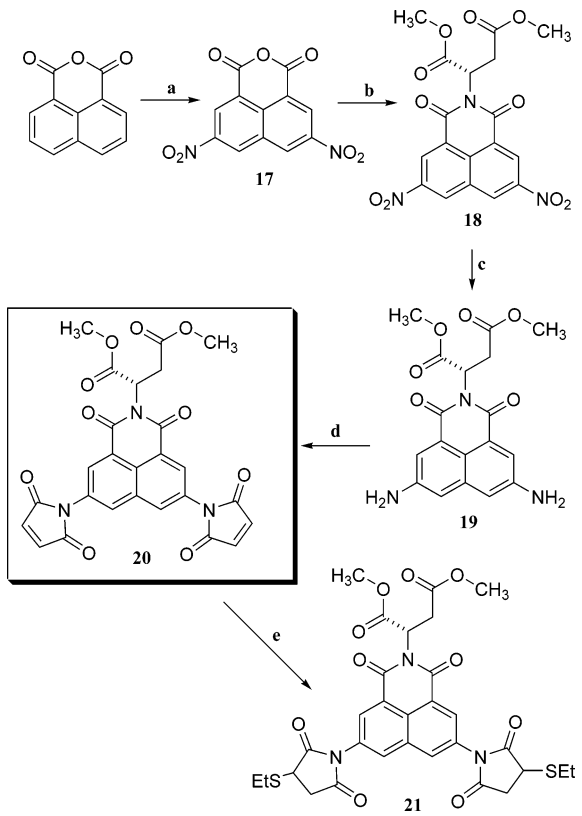
Scheme 3 [a]



[a] (a) (i) Maleic anhydride, $CHCl_3$, reflux, (ii) Ac_2O, AcONa, 100 °C, 75% (two steps); (b) EtSH, DMSO, rt, 87%.

Scheme 4 [a]



[a] (a) H_2SO_4, HNO_3, 10−20 °C, 72%; (b) CH_3CN, TEA, L-aspartic acid dimethyl ester·HCl, 90 °C, 45%; (c) H_2/Pd/C, 150 psi, THF, 49%; (d) (i) maleic anhydride, $CHCl_3$, reflux, (ii) Ac_2O, AcONa, 100 °C, 19% (two steps); (e) EtSH, DMSO, rt, 89%.

dimethyl ester (**18**) was formed in moderate yield over the two steps. Palladium-catalyzed reduction yielded the corresponding diamine **19** in modest (unoptimized) yield, with complete consumption of starting material, suggestive of the formation of side products or degradation. The subsequent reaction with maleic anhydride[16] gave the desired fluorogen **20** in 19% yield over these two steps.

Although the yields of the individual steps of these four synthetic schemes have not been optimized, we were easily able to scale-up these reactions to provide enough of each fluorogen for the subsequent characterization of their reactivity and fluorescence.

Thiol Addition Reaction. The addition of 3 mM ethanethiol to 1 mM solutions of fluorogen **4**, **13**, or **20**, prepared in DMSO or 9:1 H_2O/DMSO, leads to a marked increase in fluorescence. The fluorescence spectra obtained upon excitation at 351 nm

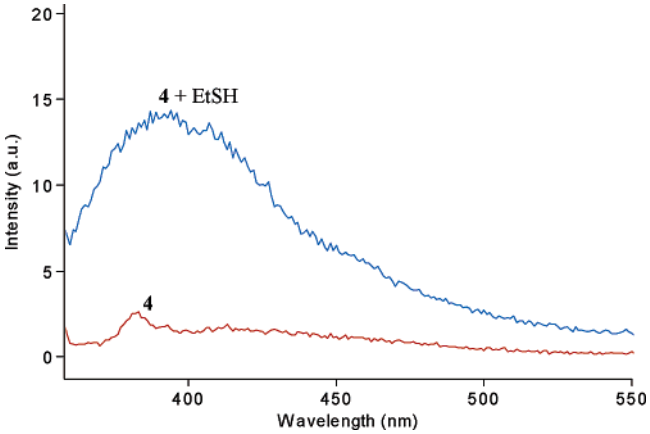


Figure 1. Marked increase in fluorescence (λ_{exc} = 351 nm, λ_{em} = 405 nm) upon the addition reaction of 3 mM ethanethiol with the two maleimide groups of fluorogen **4** (1 mM in DMSO).

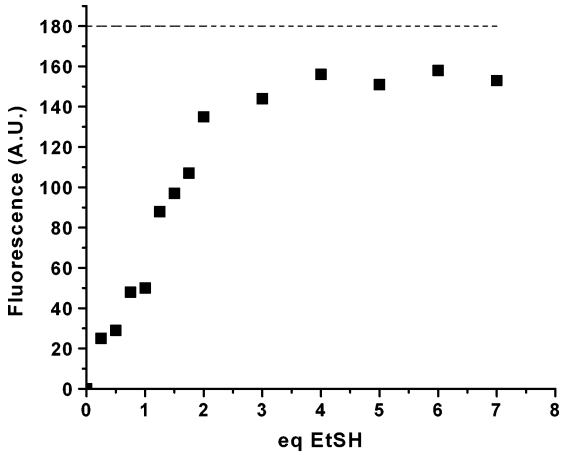


Figure 2. Increase in fluorescence (λ_{exc} = 367 nm, peak height at λ_{em} = 450 nm) upon addition of up to 2 equiv of ethanethiol to fluorogen **4** (1 mM in DMSO). Dotted line shows fluorescence of the same concentration of authentic thiol adduct product **7**, synthesized and purified independently (see Scheme 1).

before and after the reaction of ethanethiol with compound **4** in DMSO are shown in Figure 1.

The increase in fluorescence intensity was studied for compounds **4** and **13** and found to be directly proportional to the number of equivalents of thiol added, increasing with added thiol until a plateau was reached at ∼2.2 equiv of thiol, consistent with the formation of a fluorescent dithiolated adduct (i.e., formation of **7** (Scheme 1), shown in Figure 2 for fluorogen **4**). On the basis of this observation, we reasoned that compounds **4** and **13** should also react efficiently with 1 equiv of a dithiol bearing two sulfhydryl groups separated by an appropriate distance. As can be seen in Figure 3, the reaction of **4** (1 mM in DMSO) with 4,4′-bis(mercaptomethyl)benzophenone (BMMB, synthesized according to established literature procedures)[20,21] leads to an increase in fluorescence proportional to the number of equivalents of dithiol added until a plateau is reached at 1 equiv of dithiol. That this plateau was reached at ∼1.0 equiv of dithiol demonstrates the superior ability of an appropriately designed dithiol (BMMB) to react with both maleimide groups of an appropriately designed fluorogen (**4**) to form a single

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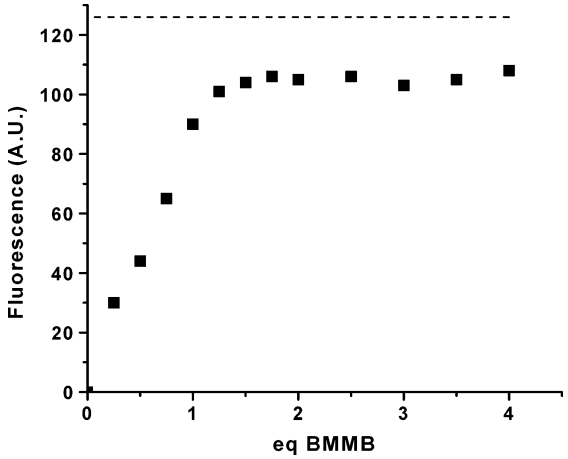
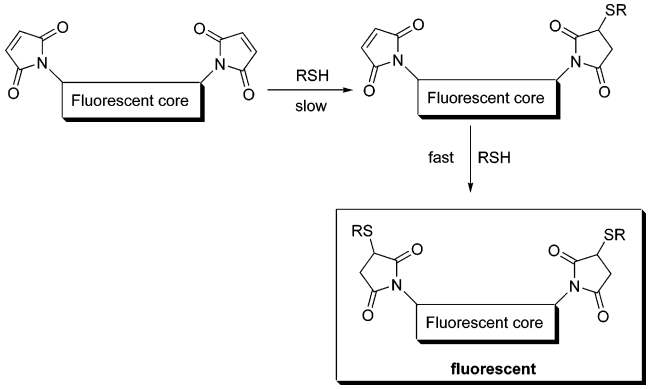


Figure 3. Increase in fluorescence (λ_{exc} = 367 nm, peak height at λ_{em} = 450 nm) upon addition of up to 1 equiv of dithiol BMMB to fluorogen **4** (1 mM in DMSO). Dotted line shows fluorescence of the same concentration of authentic dithiol adduct product **10**, synthesized and purified independently (see Scheme 1).

Scheme 5



dithiol adduct (**10**), as opposed to undergoing two separate intermolecular reactions. That is, after fluorogen **4** undergoes an initial addition reaction with BMMB, the second addition reaction with the pendant sulfhydryl group of BMMB is intramolecular and presumably much faster. In the case of simple thiols, the second thiol addition reaction would also be intermolecular and not subject to relative acceleration from any entropic advantage, so it remained to be seen if the first or second addition reaction were rate-limiting. If the first intermolecular addition reaction is indeed significantly slower than the addition of a second equivalent of simple thiol to the monothiolated adduct (Scheme 5), then the rate law for the formation of fluorescent addition product should be first order in thiol concentration, based on a rate-limiting first addition reaction (eq 1).

$$\frac{d[P]}{dt} = k_1[RSH][\mathbf{4}] \qquad (1)$$

Conversely, if the second step of Scheme 5 were slow, the rate law would be second order in simple thiol, reflecting the presence of 2 equiv of thiol in the activated complex at the transition state of the rate-limiting step (eq 2).

$$\frac{d[P]}{dt} = k_2[RSH][\mathbf{4}][RSH] = k_2[RSH]^2[\mathbf{4}] \qquad (2)$$

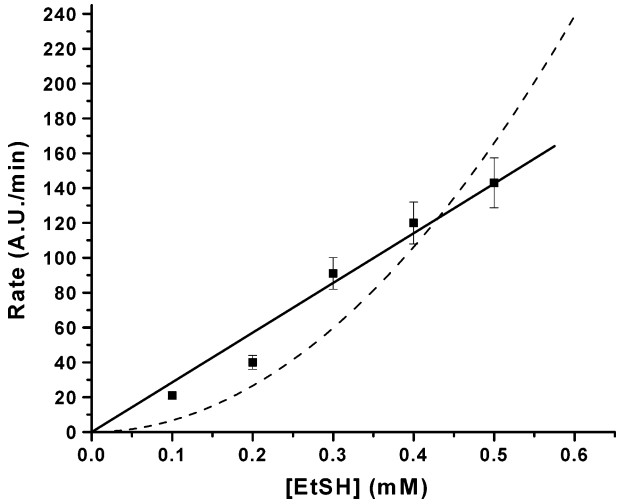


Figure 4. Linear dependence of the rate of thiol addition reaction of **4** (0.1 mM in DMF) on concentration of added ethanethiol (solid line). Reaction rate was measured as a time-dependent increase of fluorescence (λ_{exc} = 332 nm, intensity at λ_{em} = 419 nm). Shown as a dotted line is the poor fit of the rate data to a rate law second order in thiol.

In fact, when the concentration of added thiol was varied, the rate of the fluorogenic reaction was found to increase linearly, and not exponentially, as shown in Figure 4. This first-order rate law shows that even for simple thiols, the second addition reaction is *at least as rapid* as the first. The initially formed monothiolated adduct may or may not be activated toward the second thiol addition reaction; the rate law experiment indicates only that the second addition reaction is *not significantly slower* than the first. In the case of dithiol addition, this second addition reaction with a sulfhydryl group would be intramolecular and presumably even faster.

Product studies carried out for the reaction of **4** with simple thiols support the hypothesis that the second addition reaction is faster than the first. Initial experiments involved the addition of ethanethiol to **4** and attempts to separate and characterize the dithiolated and monothiolated adducts. However, no significant peak corresponding to the putative monothiolated adduct could be isolated by flash chromatography, so an experiment was designed to facilitate the separation of the monothiolated and dithiolated adducts by capillary electrophoresis (CE, see Experimental Section). By using 3-mercaptopropionic acid as a simple thiol, we ensured that at basic pH, any monothiolated adduct would have a single negative charge, due to ionization of the carboxylate group, whereas the dithiolated product would have two negative charges. The results of this analysis showed that during the reaction of **4** with up to 2 equiv of 3-mercaptopropionic acid, a trace quantity of monothiolated adduct was formed for reaction with <1.5 equiv of thiol. (For the reaction with **13**, no monothiolated product was detectable.[10]) Furthermore, the previously recorded fluorescence of the reaction mixture of **4** and 3-mercaptopropionic acid correlates linearly with the relative proportion of dithiolated adduct (data not shown).[10] Taken together, these experiments indicate that during the reaction of dimaleimido fluorogens with small thiols, very little monothiolated adduct is formed, and formation of dithiolated adduct predominates. Furthermore, the dithiolated adduct appears to be the sole contributor to the observed fluorescence; additional studies with bulkier protein thiols will be shown to

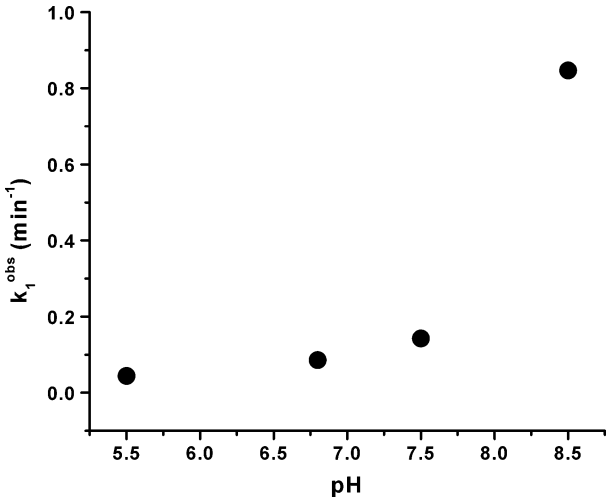


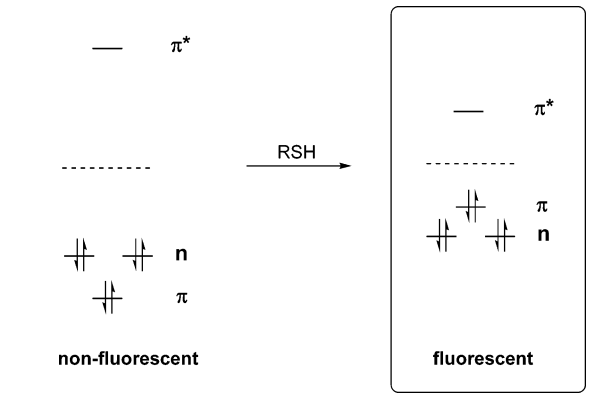
Figure 5. Graph of pH dependence of pseudo-first-order rate constants for the reaction of 2.0 mM EtSH with 0.1 mM **13**. Rate constants were measured for fluorescence increase ($\lambda_{exc} = 332$ nm, intensity at $\lambda_{em} = 419$ nm) in 9:1 H_2O/DMSO at room temperature.

corroborate the hypothesis that the monothiolated adduct does not fluoresce (vide infra).

Reactions involving nucleophilic addition of thiols typically proceed through nucleophilic attack by their thiolate anions, without the requirement for general base assistance. As such, reaction rates are observed to increase with pH until a plateau is reached above the pK_a of the nucleophilic thiol. The reaction of **13** with ethanethiol was studied for its pH dependence[11] from pH 5.5−9.0, under pseudo-first-order conditions (2.0 mM EtSH and 0.1 mM **13**) in buffered aqueous solutions with DMF added to a final concentration of 10% for solubility. Over this pH range, the thiol addition reaction was monoexponential, and the background reaction, potentially due to base-catalyzed maleimide hydration or hydrolysis,[12] was negligible, allowing the facile measurement[11] of the pseudo-first-order rate constants for the addition reaction as a function of pH (Figure 5). However, an apparent consecutive degradation reaction, resulting in a loss of fluorescence, became increasingly important at higher pH and prevented the measurement of first-order rate constants above pH 8.5. Due to this limitation, the kinetic pK_a expected to correspond to the pK_a of ethanethiol (10.6) could not be observed, but the pH dependence shown in Figure 5 is consistent with the attack of ethanethiol in its basic form. First-order rate constants for the reaction of 0.1 mM **13** with 2.0 mM ethanethiol in DMF were also measured as a function of temperature from 10 to 40 °C. Over this temperature range, the observed rate constants increased slightly with temperature, but varied by less than a factor of 2.

Fluorescence. To understand how the thiol addition reactions of fluorogens **4** and **13** result in the formation of more fluorescent products, it is instructive to consider how the maleimide groups quench fluorescence. It has been suggested[22,23] that their π-bonding orbitals are stabilized by the extended conjugation of a maleimide group. As such, in molecules bearing maleimide groups, the molecular orbital of highest energy (HOMO) corresponds to the lone pair electrons of the maleimide carbonyl group, and the lowest unoccupied orbital (LUMO) is its antibonding π^* orbital. Excitation leads to an excited state from which radiative relaxation is formally forbidden for the $\pi^* \rightarrow$ n transition; fluorescence is quenched, and relaxation takes place in a nonradiative fashion.[12,22,23] The thiol addition reaction of the maleimide group,[12,22,23] or its base-catalyzed hydrolysis at higher pH,[12] leads to a loss of conjugation, destabilization of the carbonyl group, and inversion of the energy levels of the π and n molecular orbitals. In the resulting thioalkyl succinimide group, radiative relaxation from the excited state can take place through the formally allowed $\pi^* \rightarrow \pi$ transition, restoring fluorescence of the aromatic core. In Scheme 6 is shown our quantitative representation of the orbital explanation proposed in the literature[12,22,23] for the dynamic quenching behavior observed with maleimides.

Scheme 6



For the application of this fluorogenic reaction to the detection of thiols and dithiols, two important criteria are the fluorescence intensity of the thiol addition product and the ratio of its fluorescence compared to that of its parent fluorogen. We studied the effect of electron-withdrawing and -donating substituents on these criteria by preparing the derivatives of fluorogen **4** shown in Scheme 1. Fluorogen **5**, bearing an electron-withdrawing ester group, was prepared according to a protocol similar to that used for fluorogen **4**, but using an ester-substituted maleic anhydride derived by oxidation of dimethyl L-tartrate according to a published procedure.[24] Fluorogen **6** bears an electron-donating methyl group and was obtained in similar yield by adaptation of the standard literature protocol,[16] using citraconic anhydride. The reaction of **5** and **6** with ethanethiol in DMSO led to the formation of thiol adducts **8** and **9**, respectively, whose fluorescence was characterized as shown in Table 1. Even from this small series, certain tendencies are apparent. Increasing the electron-donating capacity of the substituent appears to have a significant effect on the absorbance maximum corresponding to the wavelength of excitation, increasing by 37 nm from **8** to **9**. Although the emission wavelength increases very slightly over the same series, the net effect on the Stokes shift is a significant decrease with increasing electron-donating capacity of the substituent. A slight increase in the ratio of fluorescence intensity (before and after reaction with 2 equiv of ethanethiol) was also observed to follow increasing substituent electron-donating capacity, as expected.[25] The extinction coefficients of these derivatives differ by less

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Table 1. Effect of Substituents on the Spectral Properties of Derivatives of Fluorogen **4** and Their Corresponding Ethanethiol Adducts in DMSO

fluorogen	adduct	R	λ_{exc} (nm)	λ_{em} (fwhm)[a] (nm)	Stokes shift (nm)	I_F ratio[b]
4		H	360	450 (100)	90	7.2
	7		351		99	
5		CO_2Me	332	447 (100)	115	6.2
	8		336		121	
6		CH_3	373	455 (100)	82	8.9
	9		373		82	

[a] Wavelength corresponding to the center of the full width at half-maximum (fwhm) of the emission band, with the width in parentheses. [b] Calculated by the ratio of the areas under the fluorescence emission bands of the fluorogens and their respective thiol adduct products.

than 10%, indicating that the observed differences in fluorescence intensity are due to variation of the quantum yields of the fluorophores. In light of this trend, the methoxy derivative of **4** was also prepared,[26] but the thiol addition reaction was apparently suppressed upon introduction of such a strong electron-donating group on the maleimide group; thus, no thiol adduct was formed. While these substituent effects on fluorescence may provide data for detailed spectroscopic discussion outside the objectives of this work, it is clear that a simple change of substituents does not provide sufficient improvement, with respect to the criteria mentioned above for the development of a sensitive labeling method.

An alternative strategy for significantly altering the desired fluorescent properties involves replacement of the fluorophore core. For example, the addition of a phenyl group to the fluorescent core of **4** provided fluorogen **13**, a compound whose thiol adduct (**14**) fluoresces 52 times more intensely in DMSO, compared to the 7-fold increase observed upon reaction of **4** (Table 2). Similarly, fluorophore **15** reacts with ethanethiol in DMSO to form a dithiolated adduct (**16**) that is more fluorescent than **15** by a factor of 20 (Table 2). However, **15** proved to be insoluble in water, even upon the addition of up to 10% DMSO, rendering its practical application to the labeling of proteins problematic. Fluorophore **20** was thus prepared as a more polar naphthalene derivative; its ethanethiol adduct (**21**) was found to fluoresce 20 times more intensely in DMSO and was easily detected at micromolar concentrations. Furthermore, this naphthalene fluorophore was also found to be soluble in aqueous solutions containing as little as 5% DMSO. The change of solvent from DMSO to water−DMSO was also found to have a substantial effect on the spectral properties of the four different parent fluorogens and their corresponding thiol adducts. Their excitation and emission wavelengths measured in 9:1 H_2O/DMSO, as well as their quantum yields, measured[27] using appropriate fluorescence standards,[25,28,29] are also reported in Table 2. It is important to note than in all cases, for either solvent system, the ratio of the fluorescence intensity of a given fluorogen compared to that of its thiol adduct corresponds very closely to the ratio of their quantum yields. That is, the increase in fluorescence intensity is due to an increase in fluorescence efficiency; this is consistent with the observation that the extinction coefficients of the fluorogens do not vary significantly upon reaction with thiols. Finally, the large effect of solvent upon the measured spectral properties (Table 2) illustrates the importance of using the more relevant aqueous data in evaluating which fluorogens would be appropriate for application to protein labeling. From the data obtained in 9:1 H_2O/DMSO, it is apparent that while fluorogen **4** gives the greatest increase in quantum yield after reaction with thiols, the thiol adducts of fluorogens **13** and **20** fluoresce most intensely. For the sake of sensitivity in the envisaged protein-labeling method, compounds **13** and **20** were selected for their greater fluorescent efficiency for further studies with test proteins.

Finally, the effects of pH and temperature on fluorescence intensity were studied for compounds **4**, **13**, and **20** and their corresponding thiol adducts. Between pH 6.2 and 7.2, a negligible difference in fluorescence intensity was observed. From pH 7.2 to 8.2, a slight decrease of less than 20% was observed for some fluorogens. It is difficult to attribute this slight difference to a specific pH effect, as opposed to a typical general anionic effect.[25] In any case, at pH near neutrality, conditions under which the labeling reaction would normally be employed, the fluorescence is insensitive to slight variations in pH. A similar lack of sensitivity was observed with respect to changes in temperature. From 25 to 40 °C, the fluorescence was found to vary negligibly. From 40 to 80 °C, fluorescence was found to decrease by less than 10%, potentially due to partial decomposition of the thiol adduct product. These results also suggest that for the application of this labeling method under typical conditions, such as room temperature or 37 °C, slight variations in temperature would not have a significant effect on the observed fluorescence.

Protein Labeling. As mentioned earlier, many methods exist for the *non*-specific labeling of proteins, some of them taking advantage of the reactivity between maleimide groups and the sulfhydryl groups of exposed cysteine residues. The advantage of using fluorogens, such as **13** and **20**, containing two maleimide groups is their potential to show specificity for proteins presenting two cysteine residues separated by the corresponding distance between the maleimide groups. Our initial tests with proteins were thus designed to rule out the possibility that fluorogen **13** or **20** would simply react with 2 equiv of a protein bearing a single exposed cysteine residue, resulting in the formation of a (fluorescent) covalent dimer. For these experiments, the Cys35Ala mutant of thioredoxin (named C35A-TRX herein) was chosen as a simple test protein since it is small, monomeric, and contains only one cysteine residue, Cys32.[11] Furthermore, we utilized an expression vector encoding His-tagged thioredoxin, permitting facile purification of the protein.[30] The reaction of 45.8 μM **13** with 45.8 μM C35A-TRX for 1 h at 70 °C in 90% 25 mM MOPS (pH 7)/10% DMSO did *not* lead to an increase in fluorescence, under conditions in which at least one maleimide group of **13** would be expected to react. On the basis of our observations of the reaction of **13** with small molecule thiols, we hypothesized that **13** reacts with 1 equiv of C35A-TRX to form a monothiolated addition product that does not fluoresce since it still bears one intact maleimide group. This was confirmed by performing a second incubation, where excess ethanethiol was added to the reaction mixture of

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Table 2. Effect of Different Fluorophore Cores and Solvent on the Spectral Properties of Fluorogens **4**, **13**, **15**, and **20** and Their Corresponding Ethanethiol Adducts

		Neat DMSO				9:1 H_2O/DMSO			
fluorogen	adduct	λ_{exc} (nm)	λ_{em} (fwhm)[c] (nm)	I_F ratio[d]	Φ^e ($\times 10^{-3}$)	λ_{exc} (nm)	λ_{em} (fwhm)[c] (nm)	I_F ratio[d]	Φ^e ($\times 10^{-3}$)
4	**7**	351[a]	405 (82)	7.0	1.22[f] 10.4	381	531 (200)	50	0.050[g] 2.45
13	**14**	384	460 (92)	52	0.740[g] 42.0	376	436 (82)	27	0.471[g] 14.7
15	**16**	328	398 (82)	20	0.604[f] 14.5	*i*			
20	**21**	509[b]	578 (80)	19	3.69[h] 79.7	346	503 (181)	8.0	0.614[f] 4.30

[a] Compounds **4** and **7** were both excited at 351 nm for calculation of their quantum yields. [b] Excitation spectrum of **21** shows another peak at 544 nm, but 509 nm was used for the reported spectral data. [c] Wavelength corresponding to the center of the full width at half-maximum (fwhm) of the emission band, with the width in parentheses. [d] Calculated by the ratio of the areas under the fluorescence emission bands of the fluorogens and their respective thiol adduct products. [e] Calculated by the rapid approximation method published in ref 27. [f] Quinine sulfate in 0.1 M H_2SO_4 used as a standard (see page 53 of ref 25). [g] 7-Diethylamino-4-methylcoumarin in methanol used as a standard (see ref 28). [h] Rhodamine 110 in ethanol used as a standard (see page 144 of ref 29). [i] Compound **15** was insoluble under these conditions.

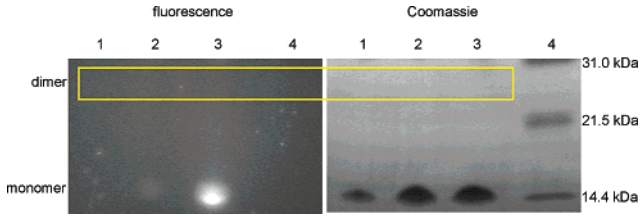

Figure 6. SDS−PAGE analysis of fluorescent labeling of C35A-TRX, a protein containing one Cys residue, with fluorogen **13** (45.8 μM **13** with 45.8 μM C35A-TRX for 1 h at 70 °C in 90% 25 mM MOPS (pH 7)/10% DMSO). Lane 1: C35A-TRX. Lane 2: C35A-TRX with **13**. Lane 3: C35A-TRX with **13** and then excess EtSH for 4 h. Lane 4: molecular weight markers. Note the presence of the fluorescent band corresponding to the 1:1 adduct and the absence of any dimeric protein adduct.

C35A-TRX, previously incubated with **13** as described above. This second incubation with ethanethiol over 4 h would have the effect of converting all of the unreacted maleimide groups to their thiol adducts, removing their ability to quench the latent fluorescence of the initially formed 1:1 protein−fluorogen derivative. Subsequent analysis by SDS−PAGE (Figure 6) shows clearly that under these conditions, **13** reacts with 1 equiv of C35A-TRX but does not react further *and displays negligible fluorescence*. Apparently, the steric bulk of even the small C35A-TRX protein, being much more significant than that of the small molecule thiols studied previously, hinders the reaction of a second equivalent of protein. This suggests that it may be possible to specifically label target proteins bearing dicysteine motifs capable of recognizing their corresponding dimaleimide fluorogens. Any background reaction with proteins bearing only one cysteine residue would not lead to increased fluorescence (a false positive result) since the monothiolated fluorogen does not fluoresce.

This hypothesis was then tested further using proteins designed to react with our fluorogenic dimaleimides. To react efficiently with their complementary dimaleimides, these proteins must contain two cysteine residues whose pendant thiol groups would be solvent exposed, sterically unhindered, and separated by an appropriate distance, namely, that between the corresponding maleimide groups, as determined by molecular modeling. We chose to work with α-helical proteins since this secondary structural motif is of sufficiently limited conformational flexibility so as to allow the precise positioning of cysteine residues. Specifically, the C99S mutant of a fragment of Fos[31] was chosen as a starting point for the proof of principle experiment since this protein is known to be α-helical[32] and had previously been expressed in this form.[33] Site-directed mutagenesis was carried out to introduce a cysteine residue (L56C), separated by two turns of the α-helical motif from the native Cys49, positioning the thiol groups approximately 10 Å apart.[34] The plasmids encoding for both the monocysteine and dicysteine mutants of Fos were subcloned into an expression vector that added an N-terminal His-tag, facilitating the subsequent purification of these test proteins (named mCys-Fos and diCys-Fos) by IMAC after overexpression in XL-1 Blue *Escherichia coli* cells (see Experimental Section).

Purified mCys-Fos and diCys-Fos were then tested in vitro for their ability to react with fluorogens **13** and **20**. After incubation of 0.22 mM protein with 0.5 mM **20** or 0.05 mM **13** at 25 °C overnight, glycerol was added, and the reaction mixtures were analyzed by SDS−PAGE using UV illumination and then Coomassie blue staining. From the resulting gel shown in Figure 7, it is clear that even under these extended reaction conditions, diCys-Fos is efficiently labeled by either fluorogen, whereas mCys-Fos is not. Moreover, the fluorogens tested do not react with 2 equiv of mCys-Fos, as evidenced by the absence of a band corresponding to the molecular weight of the expected covalent dimeric protein−fluorogen adduct. This selectivity for reaction with 1 equiv of dithiol protein, rather than 2 equiv of monothiol protein, is consistent with the behavior observed previously with small organic thiols and dithiols (vide supra), presumably based on a kinetic advantage of the entropically favored system.[7,10,11] Finally, the difference in fluorescent intensity of the respective bands may be due to an apparent selectivity of diCys-Fos for **20** over **13**. Presumably the reaction of diCys-Fos, whose Cys residues are separated by ∼10 Å, would be faster with **20** (∼10 Å between maleimides) than with **13** (∼15 Å between maleimides). Furthermore, at higher concentrations of **13**, even less fluorescence was observed (results not shown), possibly due to self-quenching or an

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(33) Furthermore, this plasmid was made available through the generous donation of Dr. T. Curran of St. Jude Children's Research Hospital, corresponding author of ref 31.
(34) On the basis of coordinates from Protein DataBank file 1FOS. Although at higher concentrations, Fos is known to form a homodimer (see ref 32), and the distance between the intermolecular cysteine residues in the putative homodimer would render impossible the reaction of one Cys residue from each helical protein with a single fluorogen molecule.

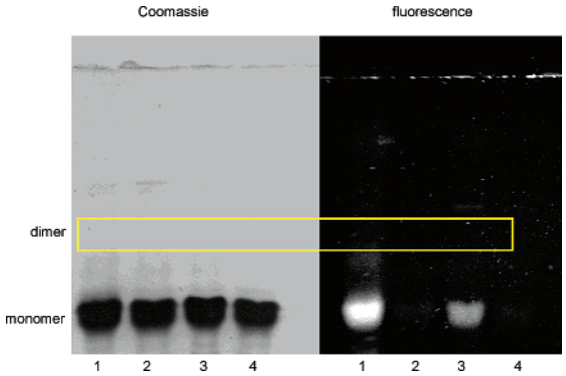

Figure 7. SDS−PAGE analysis of fluorescent labeling of diCys-Fos, a protein containing two Cys residues, by fluorogen **13** or **20**, with no labeling of mCys-Fos, a protein containing one Cys residue (0.22 mM protein, labeled overnight at 25 °C in 90% 25 mM MES buffer (pH 6.0)/10% DMSO). Lane 1: diCys-Fos with **20** (0.5 mM). Lane 2: mCys-Fos with **20** (0.5 mM). Lane 3: diCys-Fos with **13** (0.05 mM). Lane 4: mCys-Fos with **13** (0.05 mM).

increased propensity of *2 equiv* of the larger fluorogen to react with 1 equiv of diCys-Fos, leading to the formation of a non-fluorescent adduct. We are currently investigating this phenomenon to determine if it may be exploited as a means of spatial selectivity between pairs of target proteins and fluorogens.

Conclusions

Novel fluorogens **4**, **13**, **15**, and **20** were synthesized, containing two maleimide groups attached directly to the fluorophore core. These fluorogens were characterized by their ability to undergo addition reactions with 2 equiv of simple thiols or 1 equiv of dithiol. With small molecule thiols, no monothiolated addition product was detected. Subsequent addition of a second equivalent of thiol is presumed to be more rapid and results in the nearly quantitative transformation into the dithiolated adduct. These adducts were found to fluoresce much more intensely than their parent fluorogens, and their fluorescence was only subtly affected by substituents on the maleimide rings. Preliminary tests with a recombinant protein, carried out in vitro, demonstrated that the monothiolated adduct formed through reaction with a protein bearing one cysteine residue does not fluoresce, limiting the likelihood of false positives in protein labeling. Finally, a recombinant α-helical protein designed to bear two cysteine residues in positions complementary to maleimide groups of our fluorogens was efficiently labeled in vitro, demonstrating the efficacy of the covalent fluorescent labeling reaction and the feasibility of the application of this method to the labeling of specific proteins. We are currently exploring this application in intact bacterial and mammalian cells.

Experimental Section

Materials. Fluorescence spectra were recorded using a Varian Cary Eclipse fluorimeter, and fluorescent analysis of electrophoresis gels was carried out on a SpectraPhoresis 100 apparatus.

Methods. Synthesis. The materials and methods used for the synthesis of all compounds shown in Schemes 1−4, along with the spectral data of their complete characterization, are reported in detail in the Supporting Information.

Capillary Electrophoresis. After reaction of 1 mM **4** with 0.25−3 mM 3-mercaptopropionic acid in 1:9 DMSO/H_2O overnight, the fluorescence of the reaction mixture was measured. To this mixture was then added 3 equiv of ethanethiol, converting all unreacted and monothiolated adducts to dithiolated adducts. Subsequent separation by CE permitted the distinction between the monocarboxylated dithiol adducts and the dicarboxylated dithiol adducts produced during the reaction in DMSO. Injection of ~20 nL of the reaction mixture diluted in 0.1 M CHES buffer at pH 9.0 and application of 15 kV potential led to the elution of the monothiolated adduct at 4.42 min and the dithiolated adduct at 8.88 min. Their relative concentrations were calculated by the areas under their peaks, with recorded UV detection at 370 nm. Note that the prior conversion to dithiol adducts was necessary since the maleimide group reacts slowly to form side products in the pH 9 buffer used for CE analysis.

Expression of Recombinant Cys35Ala Thioredoxin Mutant. A pQE32 expression plasmid (Invitrogen), into which the gene coding for the Cys35Ala mutant of thioredoxin had been cloned, was available from previous work in the laboratory.[30] Expression was carried out in transformed BL-21 cells, and the overexpressed, His-tagged protein was isolated and purified by immobilized nickel affinity chromatography as described previously.[11,30]

Expression of Recombinant Fos Mutants. The gene for mCys-Fos was amplified by PCR from the plasmid pDS56[31] using the primers 5′-(CACACAGGATCCACGGTCGTGCGCA) and 5′-(CACACAAAGCTTGTTAACCCAGGTC). The underlined base pairs were added to re-establish the correct open reading frame for insertion in a pQE32 expression vector. The product of the PCR was cleaved using BamHI and HindIII, purified by gel electrophoresis, and extracted using a QIAEXII kit (Qiagen). The same steps were taken with the vector pQE32 prior to ligation, resulting in a recombinant plasmid containing 3741 base pairs.

The gene coding for diCys-Fos was constructed using the megaprimer technique, where the primers 5′-(CACACAGGATCCACGGTCGTGCGCA) and 5′-(GGTTTCCGCCTGGAGGGTGTCGGTACATTCACGACGGCAGT) were used in order to create a megaprimer of 164 bp. After purification and extraction as described above, a second PCR was carried out using the megaprimer and the primer 5′-(CACACAAAGCTTGTTAACCCAGGTC). The product of this PCR was purified and treated with BamHI and HindIII when it was subcloned into pQE32 as described above.

The constructed plasmids, named pQE32-mCys-Fos and pQE32-diCys-Fos, and the coding for 107 aa proteins (12.4 kDa) were then used to transform XL-1 Blue *E. coli* cells. After propagation of these cells at 37 °C in 500 mL of LB (with 100 μg/mL of ampicillin) to an OD_{600} of 0.6, protein expression was induced by the addition of IPTG to a final concentration of 1 mM. After 3 h, the cell culture was centrifuged, and the cellular extract obtained by lysis in 0.1 M phosphate buffer (pH 8.0, with 5 mM imidazole) was mixed with 1 mL of Ni−NTA agarose. The slurry was placed in a 0.8 × 4 cm glass column and washed successively with 6 mL (pH 8.0) of phosphate buffer containing 5, 10, 20, and finally 250 mM imidazole at pH 7.5. The proteins were concentrated to 0.24 mM in 25 mM MES (pH 6.0) by centrifugation over a 10 kDa MWCO membrane and stored at 4 °C until use.

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Supporting Information Available: Spectral data and complete characterization of the compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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