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Published on Web 11/23/2004

Nucleic Acid-Triggered Fluorescent Probe Activation by the Staudinger Reaction

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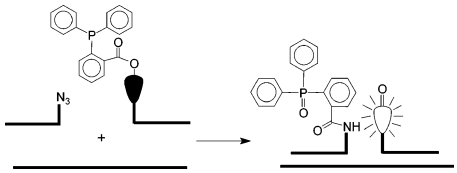
Biological and biomedical research could be greatly aided by probes that can detect specific RNA sequences in vivo with high sensitivity and selectivity and with low background signal.[1] Such reagents coupled with highly sensitive detection methods[2] could be used to monitor gene expression in cells or whole organisms and to detect and diagnose diseased or stressed cells. A number of approaches to developing such probes have been described and range from molecular beacons that fluoresce upon binding to a target,[3] to pairs of ODNs or analogues that fluoresce through a fluorescent energy transfer mechanism,[4] or through chemical release of a fluorescence molecule[5] or a quencher.[6] For in vivo use, reagents that are based on a chemical step require a biocompatible reaction that can proceed with a high rate and efficiency in water at 37° and pH 7. To this end, S_N2 reactions[6a−c] and carboxylic acid ester[5] and phosphodiester[6d] hydrolysis reactions have been explored. The ability of a nucleic acid sequence to trigger the release a molecule has also been proposed as a possible means of selectively releasing drugs as well as probes in a target cell containing a unique or uniquely overexpressed nucleic acid sequence (nucleic acid triggered prodrug or probe activation, or NATPA).[5,7] In this report, we show that the Staudinger reaction can also be used for NATPA to activate a fluorescent molecule in vitro.

The Staudinger reaction is the reaction between a phosphine and an azide, which produces a reactive aza-ylide,[8] that hydrolyzes almost spontaneously to give a primary amine and the corresponding phosphine. Bertozzi and co-workers have described a clever and useful modification of the Staudinger reaction in which an ester group on the phosphine captures the aza-ylide intermediate by intramolecular cyclization, producing an amide efficiently and selectively under physiological conditions.[9] This Staudinger ligation reaction is biocompatible and orthogonal to most biological functionalities, and it has found extensive application to peptide ligation and to both in vitro and in vivo bioconjugation.[10] Bertozzi and co-workers have also shown that a fluorescent dye can be activated by the Staudinger reaction as a direct result of the change in the oxidation state of phosphorus.[11]

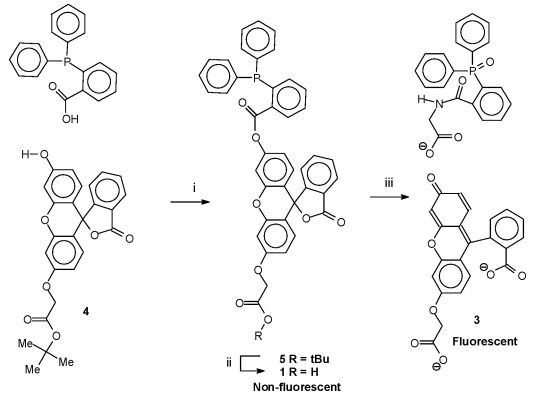
We realized that the amide bond formation in the Staudinger ligation reaction could also be used to activate a probe if one replaces the methyl ester used in most applications with an ester of a probe molecule (Scheme 1). To investigate this possibility we examined the reaction between the monoalkylated fluorescein ester of 2-carboxytriphenylphosphine **1** with α-azido acetic acid **2** (Scheme 2). Esters of monoalkylated fluoresceins are not fluorescent, but become fluorescent when hydrolyzed.[12]

Ester **1** was prepared from the *tert*-butyl ester of mono-*O*-carboxymethylfluorescein **4** by coupling with 2-carboxytriphenylphosphine in the presence of DCC to form **5**, followed by deprotection with TFA/CH_2Cl_2 (Scheme 2). Azidoacetic acid was synthesized by a published procedure.[13] No significant increase in absorbance at 454 nm indicative of **3** was observed for ester **1** when incubated in aqueous acetonitrile at neutral pH, but when azidoacetic acid was added, a time and concentration dependent increase in absorbance was observed (Figure 1). The apparent second-order rate constant for this reaction was calculated to be 0.017 M^{-1} s^{-1} (1.0×10^{-6} μM^{-1} min^{-1}), which is quite close to that reported for related reactions.[11,14]

Scheme 1



Scheme 2[a]



[a] Reaction conditions: (i) DCC, DMAP, CH_2Cl_2, 12h, room temperature, 91%; (ii) 1:1 CF_3COOH/CH_2Cl_2, 2h, 0 °C then room temperature, 93%; (iii) $N_3CH_2CO_2H$, 2:5 CH_3CN/H_2O, 10 mM phosphate buffer, pH = 7.0, room temperature, 2 h.

To test the suitability of the Staudinger ligation reaction for NATPA we designed the three component system shown in Scheme 3 in which ester **1** was tethered to the amino terminus of one peptide nucleic acid (PNA), and azidoacetic acid **2** was tethered to the carboxy terminus of another PNA through the ϵ-amino group of lysine. A Staudinger ligation reaction between **6** and **7** would be expected to produce PNA **9** and fluorescent PNA **10**. PNA was chosen because of its higher affinity toward complementary DNA and RNA than DNA,[15] its ability to invade regions of secondary structure,[16] its inability to cause degradation of the target RNA by RNaseH,[17] and its superior stability in serum and in cells.[18] Additionally, we have found herein that PNA coupling and final deprotection conditions are compatible with the ester being used.

PNA **7** was prepared by standard Fmoc-PNA synthesis with a carboxy terminal Mtt-protected lysine, which was selectively deprotected with 2% dichloroacetic acid and then coupled to azidoacetic acid in the presence of HATU/2,6-lutidine/DIEA prior to cleavage from the resin and complete deprotection with TFA/

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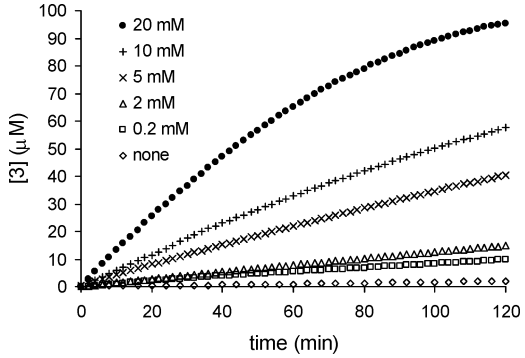


Figure 1. Rate of formation of **3** from 100 μM **1** as a function of the concentration of **2** in 2:5 CH_3CN/H_2O, 10 mM phosphate, pH 7, at room temperature. The amount of **3** was monitored by its absorbance at 454 nm.

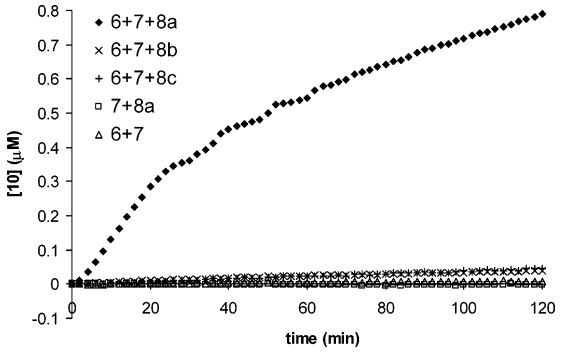
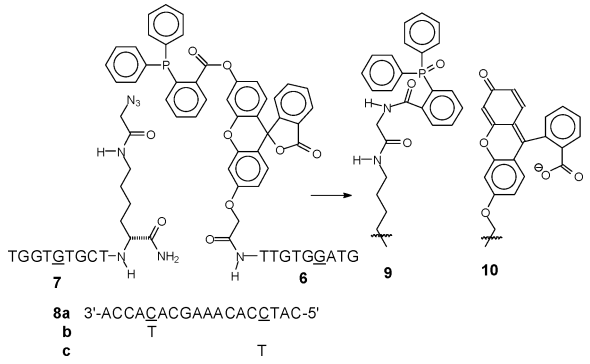


Figure 2. Rate of formation of **10** from 2 μM **6** in 10 mM phosphate, pH 7, at room temperature in the presence or absence of 5 μM **7** and 5 μM **8a**, **b**, or **c**. The amount of **10** was monitored by its fluorescence at 514 nm (454 nm excitation). See SI for expanded plots of the data.

Scheme 3



m-cresol. PNA **6** was prepared by coupling the ester **1** to the amino terminal amine prior to cleavage and deprotection.

The ester linkage of PNA **6** is very stable in aqueous solution at pH 7, as indicated by the lack of increase in fluorescence of a 2 μM solution of **6** over a period of 2 h (Figure 2). When 2 μM **6** and 5 μM **7** were incubated together under the same conditions, **10** formed with an initial rate of 8×10^{-5} $\mu M \cdot s^{-1}$, which is 8 times that calculated for the reaction of 2 μM **1** and 5 μM **2**, and may be due to association of the PNA molecules. When 2 μM **6** and 5 μM **7** were incubated in the presence of 5 μM of the fully complementary strand **8a**, **10** formed with an initial rate of 0.015 $\mu M \cdot s^{-1}$, which corresponds to a 188-fold increase in rate over the reaction in the absence of DNA template. The reaction appeared to approach only 50% completion, presumably due to the presence of some oxidized phosphine resulting from exposure to air during purification and handling. The reaction also showed good mismatch discrimination, dropping in initial rate by 37- and 31-fold opposite templates with the mismatch opposite the azido (**8b**) and fluorogenic (**8c**) components, respectively (Figure 2).

Further work will be needed to see how efficient the activation of fluorescence can be made by adjusting the length and conformational properties of the linkers to the azide and ester. For in vivo use, additional substitution of the fluorogenic component may be necessary to increase the stability of the phosphine to oxidation and to minimize fluorescence activation by enzymatic hydrolysis.[5b] The PNAs will also have to be rendered membrane-permeable and may also have to be lengthened to bind efficiently to the low concentration of mRNAs present inside human cells.[19]

Acknowledgment. Dedicated to Peter Dervan on the occasion of his 60th birthday. Supported by NIH CA92477 with assistance of the Washington University Mass Spectrometry Resource with support from Grant No. P41RR0954.

Supporting Information Available: Experimental procedures and analytical data for the synthesis of compounds **1**, **2**, **4**−**7**, and analysis of the kinetics. This material is available free of charge via the Internet at http://pubs.acs.org.

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JA0452626