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Improved Nucleic Acid Triggered Probe Activation through the Use of a 5-Thiomethyluracil Peptide Nucleic Acid Building Block

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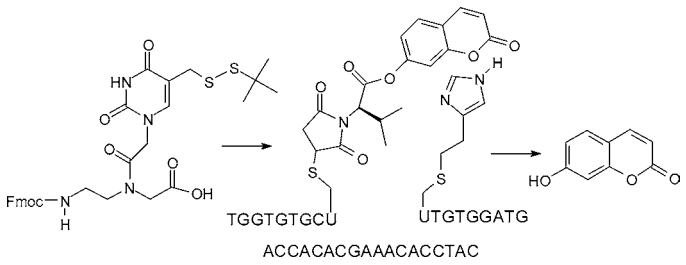
ABSTRACT



To improve the efficiency of a nucleic acid triggered probe activation (NATPA) system a 5-thiomethyluracil peptide nucleic acid (PNA) building block has been synthesized. Attachment of imidazole and a coumarin ester to uracils at the ends of two PNAs resulted in a 550 000-fold acceleration of DNA-triggered coumarin release relative to imidazole and a 6-fold increase in k_{cat} relative to a system which had these groups attached to the amino and carboxy ends of PNAs.

Recently, we described a new idea for the design of chemotherapeutic agents and diagnostic probes that makes use of a unique or overexpressed nucleic acid sequence specific to the disease state to trigger the activation of a cytotoxic drug or reporter molecule.[1] In one recent formulation of this idea, a disease-specific mRNA is used to template the association of a PNA that is linked to a prodrug or probe via an ester (the prodrug or probe component), with another PNA bearing an imidazole group (the catalytic component), which then catalyzes the release of the drug.[2] Because of the base lability of the ester, the prodrug or probe was linked to an amino terminal cysteine on the PNA following automated solid-phase synthesis via addition of the thiol group to a maleimide substituent at neutral pH and ambient temperature. The imidazole was introduced into the catalytic component via a carboxy terminal histidine. While we were able to show that both DNA and *E. coli* 5S rRNA could trigger the release of hydroxycoumarin from a pair of complementary PNAs, we found that the rate of coumarin release was low, presumably due in part to the lack of sufficient preorganization of the catalyst and the ester due to attachment to the ends of the PNAs by long flexible linkers (Figure 1). We reasoned that greater preorganization, and

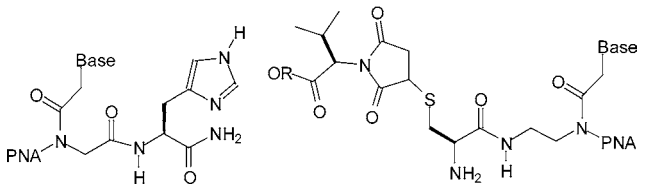


Figure 1. Previous PNA-based NATPA system.

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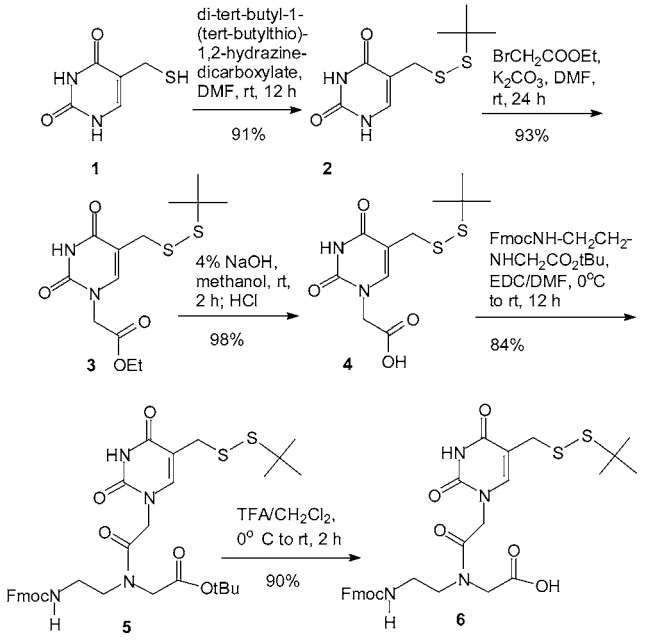
therefore a greater rate of release, could be achieved by linking the catalyst and ester to the bases via short tethers and that 5-thiomethyluracil would be an ideal choice for coupling to a maleimido ester. Herein we describe an eight-step synthesis of a 5-thiomethyluracil PNA building block **6** (Scheme 1) for Fmoc synthesis from commercially

Scheme 1. Synthesis of the PNA Building Block



available 5-hydroxymethyluracil and show how it can be used to synthesize both the catalytic and probe components of a DNA-triggered probe-releasing system. We also show that this C5-linked system is more efficient in releasing hydroxy coumarin than our previous system.

We previously chose peptide nucleic acid (PNA) to recognize the triggering mRNA sequence because of a number of features that make it ideal for in vivo use. PNA is a oligonucleotide analogue in which the nucleobases are linked to an *N*-(2-aminoethyl)glycine backbone instead of a sugar−phosphate backbone.[3] PNA has higher affinity for complementary RNA sequences than natural ODNs and can invade duplex regions, presumably because of its lack of charge.[4] PNA also has much higher biological stability than ODNs in serum and in cells.[5] For these reasons, PNA has shown great promise for the design of nucleic acid-targeted therapeutic agents, probes, and tools, either by itself or when modified by other molecules, some of which are chemically sensitive.[3,6]

Because of the basic and/or acidic conditions used in various steps in solid-phase synthesis and final deprotection, modification of nucleic acids and analogues with sensitive functional groups, such as aryl esters, is best accomplished following automated synthesis and deprotection, with chemoselective reactions that can proceed under near neutral conditions at ambient temperature. Thiol groups have been particularly useful in this regard and have been used to link oligonucleotides to such molecules as biotin and fluorescent probes[7] as well as to peptides, proteins, and gold particles.[8]

C5-(Thioalkyl)uridines are particularly useful for both internal and terminal modifications of DNA. Substituents at the C5 position of pyrimidine nucleosides do not interfere with Watson−Crick base pairing, and project out into the major groove of duplex DNA thus allowing for more precise conformational control compared to substituents linked to the ends of DNA. Another advantage of linking to the bases is that a modification can be introduced anywhere in a sequence since the number and position of thiol-bearing building blocks can be controlled by oligonucleotide synthesis. While the synthesis of a series of 5-(thioalkyl)uridines and 5-(thioalkyl)-2′-deoxyuridines has been described,[9] the preparation of 5-(thioalkyl)uracil PNA building blocks has not yet been reported. A *p*-methoxybenzyl-protected 5-thiopropynyl PNA monomer has been reported, but its incorporation into PNA has not.[10] To reduce the conformational degrees of freedom of the probe and catalyst in the nucleic acid triggered probe activating system, we decided to synthesize the 5-thiomethyluracil PNA building block **6** (Scheme 1).

Thiomethyluracil **1** was obtained in three steps from hydroxymethyluracil according to a known procedure.[11] Treatment of **1** with di-*tert*-butyl-1-(*tert*-butylthio)-1,2-hydrazine dicarboxylate[12] in DMF at room temperature for 12 h gave 5-(*tert*-butyldithio)methyluracil **2** in 91% yield and in 51% overall yield from 5-hydroxymethyluracil. Compound **2** has also been synthesized in 58% overall yield in three steps from 5′-hydroxymethyluracil via the in situ preparation of 5-thiomethyluracil.[9b] Compound **2** was alkylated with ethyl bromoacetate in the presence of potassium carbonate in DMF to give ester **3** in 93% yield. Ester **3** was hydrolyzed with 4% NaOH in methanol followed by acidification to give acid **4** in 98% yield. EDC-mediated

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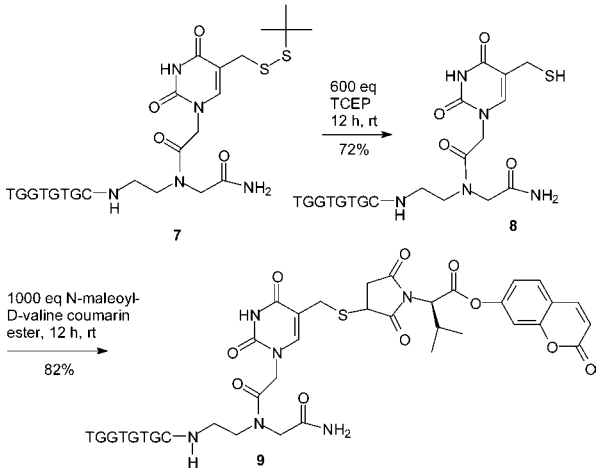
coupling of **4** to *tert*-butyl-*N*-Fmoc-aminoethylglycinate[13] afforded **5** in 84% yield. Finally, deprotection of the *tert*-butyl ester with TFA/DCM gave the desired PNA monomer **6** in 90% yield and in 35% overall yield from 5-hydroxymethyluracil.

For the synthesis of the probe component **9** (Scheme 2), monomer **6** was used to introduce 5-(*tert*-butyldithio)-

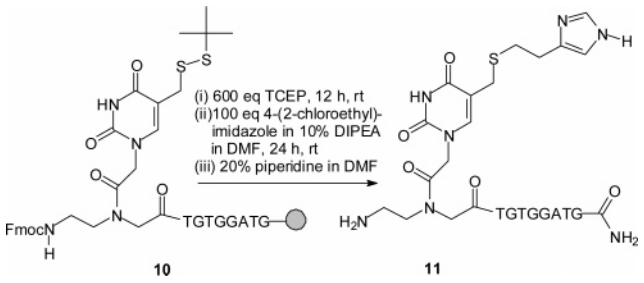
Scheme 2. Synthesis of the Probe Component



methyluracil into the carboxy terminus of the 9-mer PNA **7** by standard solid-phase Fmoc synthesis on an ABI 8909 PNA synthesis machine. Following synthesis, deprotection, and purification, the disulfide bond of the PNA was reduced with excess TCEP to give **8** and then incubated with excess *N*-maleoyl-D-valine coumarin ester[1] to give the PNA probe component **9** in 59% yield.

For the synthesis of the catalytic component **11** (Scheme 3), monomer **6** was used to introduce 5-(*tert*-butyldithio)-

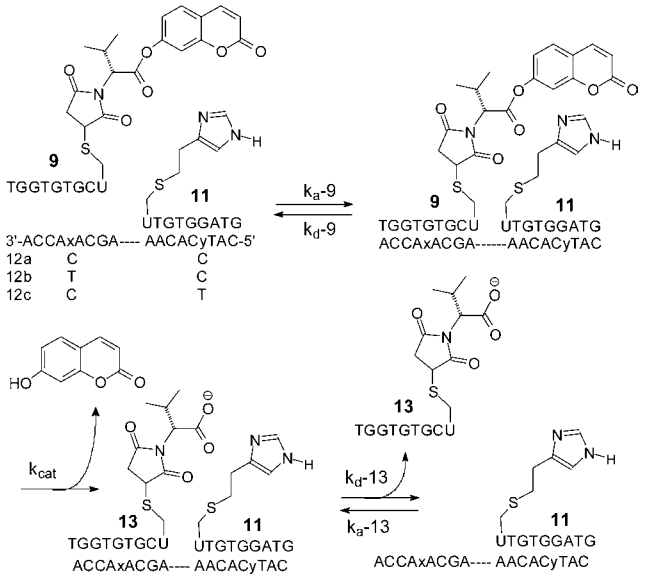
Scheme 3. Synthesis of the Catalytic Component



methyluracil into the amino terminus of the 9-mer PNA **10**. The disulfide bond was reduced with TCEP on the column and then treated with 4-(2′-chloroethyl)imidazole[14] in 10% DIPEA in DMF for 1 day. Final deprotection and cleavage from the resin, followed by reversed-phase HPLC purification, provided PNA **11**.

To determine the efficiency of fluorescent probe release, 4 μM PNA **9** was incubated with and without 4 μM PNA **11** and/or the fully complementary template **12a** in pH = 7.0 sodium phosphate buffer (Scheme 4). Efficient coumarin

Scheme 4. Kinetic Scheme for DNA-Triggered Coumarin Release



release only occurred when the catalytic and probe components were in the presence of the complementary DNA (Figure 2). The system was also found to be sensitive to mismatches, dropping 23- and 30-fold in rate with a

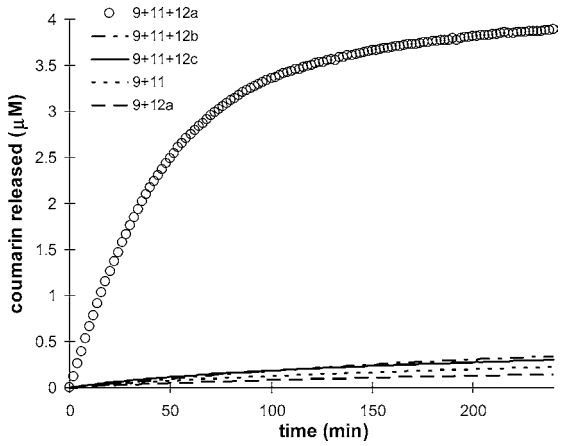


Figure 2. Kinetics of coumarin release with matched and mismatched DNA triggers. The prodrug component **9** (4 μM) was incubated in the presence or absence of the catalytic component **11** (4 μM), fully complementary DNA **12a** or mismatched DNAs **12b** and **12c** in 10 mM phosphate pH 7 buffer. Coumarin release was assayed by fluorescence (Ex = 350 nm, Em = 452 nm).

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mismatch in either the catalytic (**12b**) or probe (**12c**) binding sites of the triggering DNA.

As in previous studies,[1,2] the initial rate of DNA triggered coumarin release exhibited saturation kinetics (Figure 3)

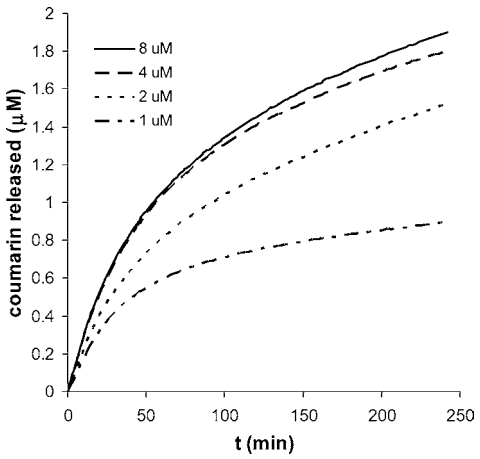

Figure 3. Kinetics of coumarin release as a function of probe component concentration. The catalytic component **11** (2 μM) and DNA trigger **12a** (1 μM) were incubated with probe component **9** in 10 mM phosphate pH 7 buffer. Coumarin release was assayed by fluorescence (E_x = 350 nm, E_m = 452 nm).

indicative of reversible binding of substrate **9** to the DNA trigger sequence (Scheme 4). A plot of the initial rates as a function of the concentration of **9** could be fit to a Michaelis-Menten equation with a k_{cat} of 5.0 ± 0.2 × 10^{-4} s^{-1} and a K_m of 0.8 ± 0.1 μM (see the Supporting Information). The k_{cat} value is 6 times that of 8.4 × 10^{-5} s^{-1} for the DNA-triggered PNA-based system in which the ester and imidazole were tethered to the amino and carboxy ends of the PNA (Figure 1)[2] and 11 times that of 4.4 × 10^{-5} s^{-1} observed for coumarin release from a DNA-triggered DNA-based system.[1b] Based on (k_{cat}/K_m)/k_{Im}, the C5-linked DNA-triggered probe activating system shows the greatest acceleration of coumarin release (550 000-fold based on k_{Im} = 1.13 × 10^{-3} M^{-1} s^{-1} for imidazole-catalyzed hydrolysis of *N*-malemido-D-valine coumarin ester).[1b]

The reaction also exhibited catalysis, as evidenced by the release of more moles of coumarin than the number of moles of the catalytic complex **11·12a** in the presence of excess substrate **9** (Figure 2). The reaction showed biphasic or burst kinetics,[15] appearing to be fast and substrate concentration dependent during the first turnover (pre-steady-state regime), but slow and concentration independent during the second turnover (steady-state regime). This type of kinetics is indicative of a rate-limiting step following coumarin release. Indeed, the rate of coumarin release in the presence of saturating substrate (8 μM **9**) could be fit to the burst equation [coumarin] = [**11·12a**]$_o$(($1 - e^{-k_{cat}t}$) + k_2t) from which the steady-state rate constant (k_2) was determined to be (6.46 ± 0.02) × 10^{-5} s^{-1} (see the Supporting Information). The value of k_2 is remarkably similar to that of 6.2 × 10^{-5} s^{-1} calculated for the dissociation rate constant (k_d) of an 8-mer PNA·DNA duplex from published data.[16] This similarity suggests that dissociation of the hydrolyzed 9-mer PNA product **13** from the catalytic complex **11·12a** (k_d−13, Scheme 4) is limiting the turnover rate of the reaction. If so, it should be possible to increase the turnover rate by decreasing the length of PNA **9** and hence the dissociation rate of its hydrolysis product, PNA **13**. Future studies will be aimed at increasing the overall catalytic efficiency of both DNA- and RNA-triggered coumarin release by optimizing the length of the substrate PNA, and the lengths of the linkers to the ester and imidazole subunits.

In conclusion, we have developed an efficient route for the synthesis of a 5-thiomethyluracil PNA monomer for solid-phase Fmoc synthesis that allows for postsynthetic modification of PNA in the major groove. Most importantly, we have shown that attachment of imidazole and a coumarin ester to the C5 of uracil substantially increases the efficiency of DNA-triggered prodrug activation over those systems in which these groups are attached to the chain termini,[2] thereby paving the way to better RNA-triggered systems.

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Supporting Information Available: Abbreviations used, experimental procedures and analytical data for compounds **2**−**9** and **11**, and analyses of the kinetic data. This material is available free of charge via the Internet at http://pubs.acs.org.

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