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On the necessity of nucleobase protection for 2-thiouracil for Fmoc-based pseudo-complementary PNA oligomers synthesis

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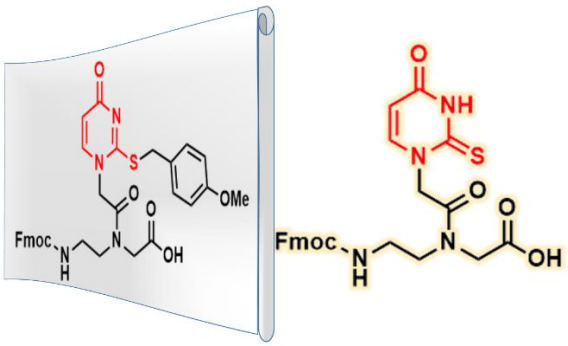


Abstract

A selection of benzyl-based protecting groups for thiouracil (^{S}U) for the synthesis of pseudo-complementary peptide nucleic acid (PNA) have been evaluated. The 4-methoxybenzyl protecting group that has found use for ^{S}U during Boc-based oligomerization is also suitable for Fmoc-based oligomerization. Furthermore, it is demonstrated that ^{S}U protection is unnecessary for the successful synthesis of thiouracil-containing PNA. The new 2-thiothymine (^{S}T) PNA monomer has also been prepared and incorporated into an oligomer and it's binding to complementary PNA evaluated.

Introduction

Peptide nucleic acid is an oligonucleotide analog that has attracted constant interest since it was first described.[1] Many modifications have been made around the original design in order to tune its biophysical and chemical properties.[2,3,4] The modifications can be broadly characterized as involving the repeating structure of the backbone, the nature of the inter-residue linkage, the connection to the nucleobase or the nucleobase itself, Figure 1a. We, and others, have been interested in nucleobase-modified PNAs for some time.[5,6] Recently, the synthesis of 2-thiouracil PNA monomer, Figure 1b, by Kittaka and coworkers[7] has prompted us to report our findings.

One of the attractive features of PNA is its hybridization properties. The neutral polyamide backbone of PNA is usually credited with producing high affinity and highly selective binding that has a reduced dependence on salt concentration.[8,9] PNA oligomers have the ability to form complementary duplexes with DNA or RNA, but may form triplexes with appropriate sequences. In fact, the first report of PNA binding was the unusual strand invasion triplex formation by a single-stranded polypyrimidine PNA on a double stranded target.[1] However, unmodified PNA oligomers are not efficient at targeting mixed-base sequences within a double-stranded complex, which is a serious limitation for PNAs applications involving gene targeting.[10,11] In order to form a strand-displaced complex within a double-helical target, PNA oligomers complementary to each strand of the DNA duplex are required. This leads to the use of PNAs that are complementary to each other that unavoidably partake in strong hybridization to each other rather than with the DNA target. In order to favor hybridization with DNA in preference to PNA, the PNA-PNA duplex must be destabilized relative to PNA-DNA duplex stability. The selective destabilization of PNA-PNA duplexes can be achieved using pseudo-complementary nucleobases, first reported by Gamper and coworkers in DNA.[12] In 2002, Nielsen *et al.* developed the use of pseudo-complementary nucleobases in PNA (pcPNAs) compatible with a graded acidolysis (Merrifield-type) peptide synthesis.[13,14] They reported pcPNAs possessing 2,6-diaminopurine (D) and 2-thiouracil (^{S}U) in place of natural adenine (A) and thymine (T), respectively. Steric hindrance between the 2-amino group of D and the 2-thiocarbonyl group of ^{S}U resulted in destabilization PNA-PNA duplexes, Figure 1c.

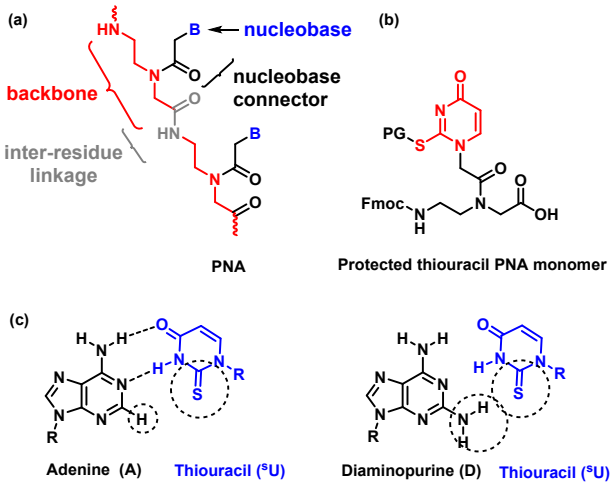


Figure 1. (a) Chemical structure of the first generation of PNA with structural features labeled; (b) Structure of a thiouracil PNA monomer with a generic nucleobase protecting group (PG); (c) Illustration of selective binding of thiouracil to adenine versus diaminopurine based on steric fit.

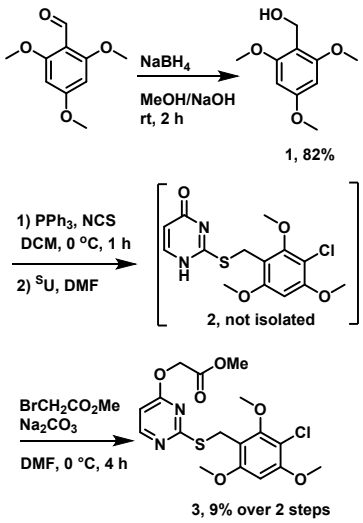
The first synthesis of pcPNA by Nielsen and coworkers conformed to the Boc-based oliogomerization strategy and the nucleobase protecting groups that were employed were the 4-methoxybenzyl moiety for ^{S}U and *N6*-benzyloxycarbonyl group for D.[15] Although the Fmoc-based monomer for D was reported in 2012,[16] it was not until 2017 that the ^{S}U Fmoc-based monomer was reported,[7] thus permitting Fmoc-based synthesis of pcPNAs. The ^{S}U protecting group used in this instance was 2-methyl-4-methoxybenzyl, which was somewhat surprising given that a modest change would engender compatibility with global deprotection conditions for Fmoc-based synthesis, i.e. generally ~95% trifluoroacetic acid (TFA) versus the TFA/trifluoromethanesulfonic acid (TFMSA) conditions usually used Boc-based synthesis.

In our work, we presumed the nucleobase protection of thiouracil (4-methoxybenzyl group) would persist during the oligomerization and be removed by high acid conditions usual for Boc-based synthesis. Thus, we began to investigate thiouracil protecting groups with potentially high acid lability. Taking inspiration from polypeptide synthesis, we attempted to install a trityl group under basic (using trityl chloride) or acidic (using tritanol) conditions to no avail. Due to the lack of reaction under standard tritylation conditions we optimistically attempted direct alkylation of the *N1* of thiouracil. Although a method has been reported to achieve *N1* allylation without thicarbonyl protection,[17] similar conditions failed to give *N*-alkylation using *tert*-butyl bromoacetate. Our attention then turned to electron-rich benzyl derivatives such as the Tmob

(trimethoxybenzyl) which has been used as a cysteine side-chain protecting group for Fmoc-based peptide synthesis,[18] and the related 2,4-dimethoxybenzyl group. We have also investigated Kittaka's 2-methyl-4-methoxybenzyl protecting group relative to 2-methoxybenzyl and 4-methoxybenzyl for thiouracil. We also report herein, the synthesis of 4-methoxybenzylthiothymine PNA monomer (^{S}T). Finally, we demonstrate that *S*-protection of thiouracil is not required for successful oligomerization of PNA.

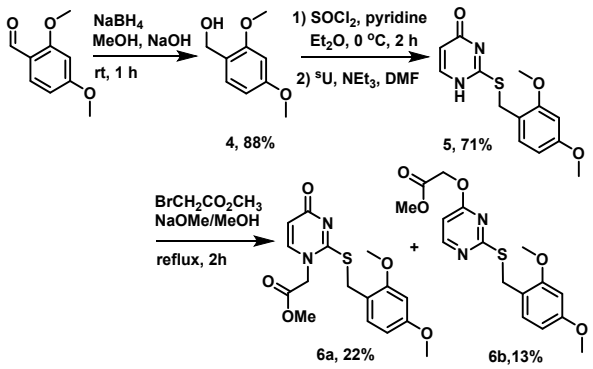
Results and Discussion

With the utility of the Tmob group,[18] trialkoxybenzyl linkers to resins[19] and peptide backbones,[20] for Fmoc-based synthesis already established, we sought to prepare the *S*-2,4,6-trimethoxybenzyl derivative of thiouracil according to Scheme 1. Commercially available 2,4,6-trimethoxybenzaldehyde was reduced to 2,4,6-trimethoxybenzyl alcohol (**1**)[21] and then was used for the sequential *in-situ* NCS-mediated conversion to the benzyl chloride (**2**, not isolated) and alkylation of 2-thiouracil to give the undesired *O4*-derivative **3**, as identified by the relative downfield shift of the acetate methylene protons relative to *N1*-alkylation.[22,7] Not only was the undesired regioisomer obtained in the alkylation reaction, but the electron rich trimethoxybenzyl group had been chlorinated under the reaction conditions. While the unintended chlorination was not cause enough to abandon this approach, the poor overall yields and inability to achieve *N1*-alkylation lead us to consider other potential protecting groups.



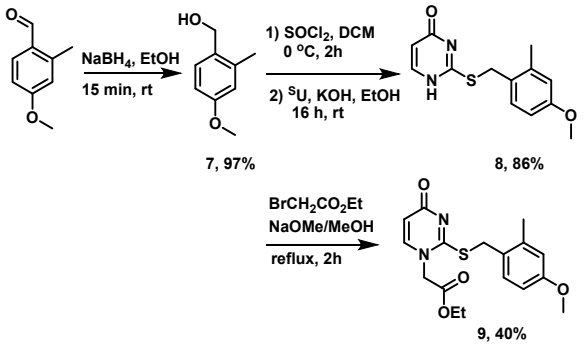
Scheme 1. Attempted synthesis of *N1*-alkylated 2,4,6-trimethoxybenzyl-protected thiouracil.

Next, we set out to examine the 2,4-dimethoxybenzyl group for 2-thiocarbonyl protection, as illustrated in Scheme 2, which outlines the synthesis of the nucleobase acetic acid submonomer. Once again, starting with commercial 2,4-dimethoxybenzylaldehyde, smooth reduction to the alcohol followed by in-situ conversion to the benzyl chloride and alkylation with thiouracil provided the protected nucleobase **5**. The *N1*-methyl acetate moiety was installed in low (**6a**, 22%), but serviceable yield, partially due to formation of the *O4*-alkylation product (**6b**, 13%).



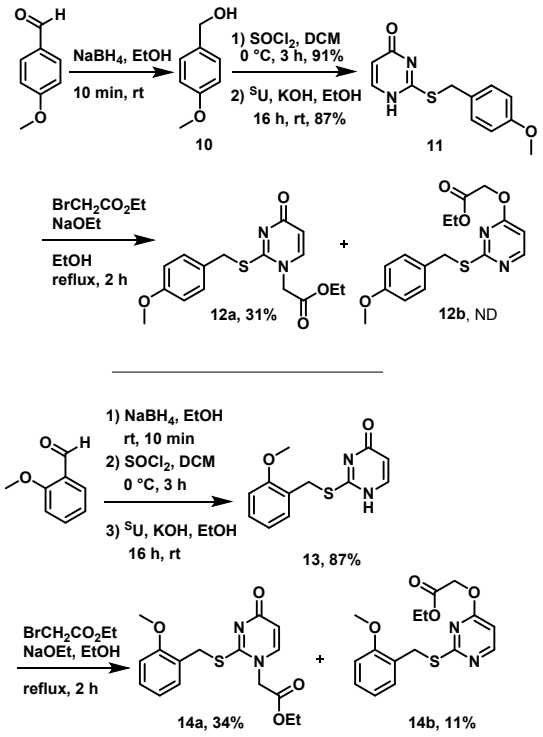
Scheme 2. Synthesis of methyl 2-(2-(2,4-dimethoxybenzyl)thiouracil-1-yl)acetate (**6**). ^{S}U = 2-thiouracil.

For comparison of benzyl-based protecting group lability toward acidolysis, we have prepared Kittaka's 2-methyl-4-methoxybenzyl derivative (Scheme 3) as well as the 4-methoxybenzyl derivative known from Nielsen's work and the new 2-methoxybenzyl protected thiouracil. The 2-methyl-4-methoxybenzyl-protected nucleobase was approached from the aldehyde and reduction proceeded near quantitatively (97%) under the reported conditions. A variation on the conditions for conversion to the benzyl chloride was used, and subsequent *S*-alkylation proceeded smoothly furnishing **8** in 86% yield. The *N1*-alkylated product **9** was isolated in 40% yield after column chromatography, Scheme 3.



Scheme 3. Synthesis of ethyl 2-(2-(2-methyl-4-methoxybenzyl)thiouracil-1-yl)acetate (**9**).

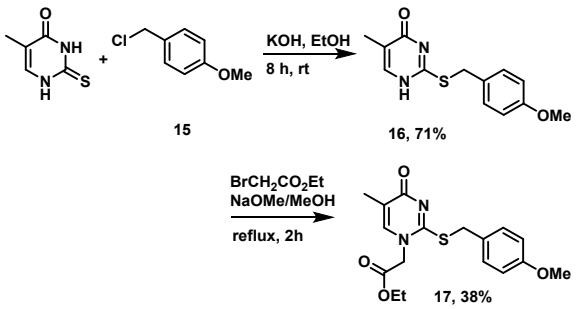
The 2-methoxybenzyl- and 4-methoxybenzyl-protected 2-thiouracil derivatives were prepared using conditions analogous to the preparation of the 2,4-dimethoxybenzyl derivative, both shown in Scheme 4. Compounds **6a**, **9**, **12a**, and **14a** were later used for comparing the rates of acidolysis under treatment with TFA.



Scheme 4. Synthesis of *N1*-(ethyloxycarboxymethyl)-*S*-(4-methoxybenzyl)-2-thiouracil (**12a**) *N1*-(ethyloxycarboxymethyl)-*S*-(2-methoxybenzyl)-2-thiouracil (**14a**). ND = not determined.

To date, only the thiouracil nucleobase, and not thiothymine, has been studied as a pseudo-complementary PNA oligomers, so we have taken this opportunity to study the effect of

thiothymine on the hybridization stability when incorporated into pcPNA oligomers.[22] Thiothymine was prepared by literature methods from thymine via 1,3-dimethylthymine[23] followed by treatment with thiourea.[24] The nucleobase was then reacted with the 4-methoxybenzyl chloride (**15**), in a similar fashion as for the preparation of thiouracil **11**, to yield the protected thiothymine (**16**). The nucleobase (**16**) was then alkylated with ethyl bromoacetate using sodium ethoxide to yield the *N1*-alkylated **17** as the major product and the *O4*-alkylated isomer as the minor product which were separated by column chromatography, Scheme 5.



Scheme 5. Synthesis of thiothymine protected nucleobase submonomer **17**.

With a panel of compounds (**6a**, **9**, **12a**, **14a**, and **17**) now available, we were interested in ranking their lability toward TFA treatment to assess their suitability for Fmoc-based synthesis of pseudo-complementary PNAs. Initial studies on the 4-methoxybenzyl protected thiouracil **12a** found acidolysis of the thioether occurred rapidly under standard (95% TFA) global deprotection/resin cleavage conditions used in the Fmoc-based synthesis. ^{1}H NMR spectroscopy showed complete deprotection within 4 min after the addition of the TFA solution, *i.e.* the time needed to prepare the sample and introduce it into the NMR spectrometer. This result suggests that the 4-methoxybenzyl protecting group is most likely removed during Boc-based synthesis of the pcPNA oligomers which typically uses neat TFA for *N*-Boc removal during oligomerization. Since Boc-based synthesis of pcPNAs has been very successful, this further suggests that a 2-thiouracil protecting group is unnecessary for oligomerization, *vide infra*.

Due to the unexpectedly high lability of the 4-methoxybenzyl group, the acidolysis of the protecting groups was examined at much lower TFA concentration. The acidolysis proceeded at a rate convenient for monitoring using a solution of 2% TFA and 1% triethylsilane (TES) in deuterated chloroform. The progress of the acidolysis was followed by ^{1}H NMR; the relative integration of the benzylic protons to the methylene carbonyl protons was measured at 2 min

intervals over 2 hours or until complete deprotection had occurred for compounds **9**, **12a**, **14a**, and **17**, Figure S1-S4.

The change in integration of the benzylic protons was plotted against time to illustrate relative deprotection rates, Figure S5. Under the 2% TFA, the *S*-(4-methoxybenzyl)thiouracil **12a** and the *S*-(4-methoxybenzyl)thiothymine **17** deprotected at similar rates, but with thiothymine being marginally slower than thiouracil. The higher stability of the protonated thiothymine, due to the C5-methyl group, compared to the thiouracil nucleobase results in a slightly slower acidolysis of the thioether. In the case of both **12a** and **17**, the majority of the nucleobase was deprotected after 2 hours (2% TFA, 1% TES). Acidolysis of the *S*-(2-methyl-4-methoxybenzyl)thiouracil **9** was significantly faster than the acidolysis of the *S*-(4-methoxybenzy)thiouracil **17**, with a half-life of approximately 13 min, Table 1. The 2,4-dimethoxybenzylthiouracil **6a** was the most acid labile of the protecting groups studied, with complete deprotection occurring within 4 min of treatment with a solution of 2% TFA implying a half-life of reaction of < 1 min. The 2-methoxybenzyl group was removed anomalously slowly under these conditions, but could be removed completely in 50 min with 95% TFA.

The change in the benzylic integration over time was treated as a first order reaction in order to determine reaction half-life, Figure S6. Plots of the natural logarithm of integration versus time showed a linear relationship consistent with first order kinetics of the acidolysis reaction which yielded reaction rates and half-life data shown in Table 1.

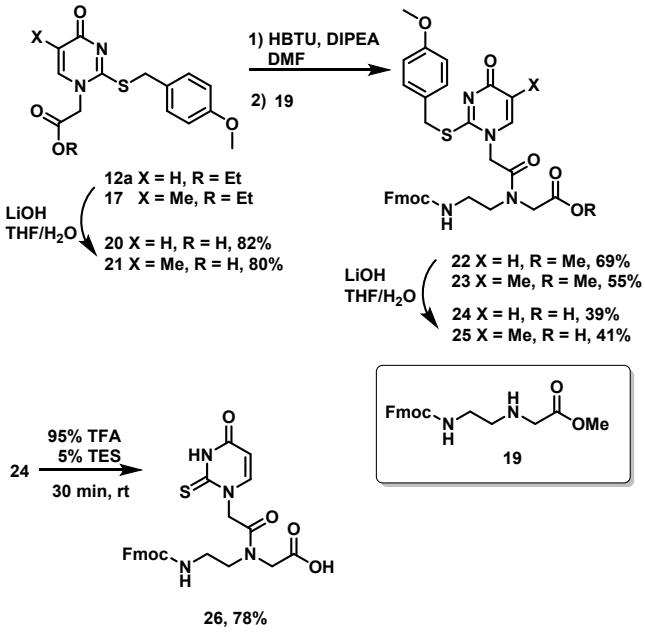
Table 1. Estimation of rates and half-life of acidolysis for different benzyl-type thio protection under dilute (2%) trifluoroacetic acid condition.

Compound	Protecting group	Nucleobase	Rate constant (k, min^{-1})	Half-life ($\boldsymbol{t_{1/2}}$, min)
12a	4-methoxybenzyl	^{S}U	0.0111	62
17	4-methoxybenzyl	^{S}T	0.0070	99
9	2-methyl-4-methoxybenzyl	^{S}U	0.0527	13
6a	2,4-dimethoxybenzyl	^{S}U	-	<1*
14a	2-methoxybenzyl	^{S}U	-	>>120†

* estimated using the observation that complete reaction (> four × $t_{1/2}$) required 4 minutes or less
† estimated based on the degree of changed observed within the 120 min experiment

In order to gain some insight on the resistance to acidolysis exhibited by **14a**, the possible structure for the protonated form was investigated by computational methods. In order to simplify the computation, a model compound with an ethyl acetate moiety replaced by a methyl group was used. Considering the likely mechanism for acidolysis (Figure S7), *i.e.* protonation of *N3* followed by heterolysis of the benzylic C-S bond, this intermediate was selected for study. The search for the lowest energy conformer at the Hartree-Foch 6-31G(D) level in vacuum, shown in Figure S7, revealed a short contact between protonated *N3* and the 2-methoxybenzyl group which is consistent with a moderate strength H-bond. It is posited that formation of the hydrogen bond with the proton on the *N3* position stabilizes the intermediate toward acidolysis because it reduces the ability of the methoxy group to stabilize the incipient benzylic carbocation. When the conformational search was repeated with the 3-methoxybenzyl- or 4-methoxybenzyl- protected thiouracil derivative no low energy conformers exhibited any interaction between the protecting group and the protonated *N3* position and no such interaction is available to the 2-methyl-4-methoxy derivative.

Proceeding to monomer synthesis and oligomerization, the previously prepared[25,26] Fmoc-based aminoethylglycine backbone hydrochloride **18** was used. The hydrochloride salt backbone was neutralized and extracted in chloroform to yield the neutral base as a clear and colourless oil (**19**) and used in the uronium-promoted condensation with the nucleobase acetic acid derivatives **20** and **21**, Scheme 6. Careful hydrolysis of the methyl ester protecting the C-terminus (**22**, **23**) using aqueous lithium hydroxide yielded the thiouracil and thiothymine PNA monomers **24** and **25**. The thiouracil PNA monomer lacking a nucleobase protecting group was prepared by the treatment of **24** with TFA to give monomer **26**. Monomer **26** was used to test the hypothesis a thiouracil PNA does not require a protecting group for successful oligomerization.



Scheme 6. Synthesis of protected 2-thiouracil and 2-thiothymine PNA monomers (**24**, **25**) and the unprotected thiouracil PNA monomer (**26**).

PNA sequences were prepared by automated peptide synthesis and it was found that the three PNA monomers performed well, with no significant difference noted for the lack of a nucleobase protecting group, Figure S6, Table S1.

The sequence prepared was the same as the one originally used by Nielsen.[14] Once the oligomers were in hand, thermal stability (T_m) analysis with complementary PNA was undertaken. The unmodified PNA control sequence showed excellent agreement with the reported literature value for T_m (68 °C). For this particular sequence, the thiouracil-containing oligomer showed a slight increase in T_m (~ ΔT_m = 0.3 °C per insert) with an unmodified PNA target. Interestingly, the thiothymidine showed a decrease in the thermal stability of the duplex from that of an unmodified PNA target (~ ΔT_m = -1.0 °C per insert), which may be useful in pcPNA applications although this phenomenon requires further study, Table 2.

Table 2. Thermal stabilities (T_m) of double-stranded PNA (dsPNA) complexes

PNA sequence* (N→C)	ds PNA† (°C)
H-Lys-GTAGATCACT-Lys-NH_2	67.5
H-Lys-G^{s}**T**AGA^{s}**T**CAC^{s}**T**-Lys-NH_2	64.5
H-Lys-G^{s}**U**AGA^{s}**U**CAC^{s}**U**-Lys-NH_2	68.5

* PNA oligomers are listed from the pseudo 5' terminus to the pseudo 3'-end. Lys = D-lysine, ^{s}**T** = 2-thiothymine ^{s}**U**= 2-thiouracil PNA residues. †The complementary PNA oligomer H-Lys-AGTGATCTAC-Lys-NH_2 was used.

In summary, this study has shown that the 4-methoxybenzyl protecting group used for Boc-based oligomerization of pcPNA synthesis containing thiouracil residues is also suitable for Fmoc-based oligomerization. Given that the 4-methoxybenzyl protecting group is removed rapidly under the same deprotection conditions as the *N*-Boc protecting group, the thiouracil protection most likely does not persist for Boc-based oligomer synthesis. This observation prompted us to examine the oligomerization of thiouracil PNA without nucleobase protection, which was successful. It is noteworthy that the solubility of the unprotected thiouracil PNA monomer is fine for oligomerization chemistry; thus, it appears that the only role the protecting groups serves is to allow regioselective alkylation of *N1* during monomer synthesis. A method to achieve regioselective *N1* alkylation in the absence of 2-thiocarbonyl protection would be a significant benefit to monomer preparation.

Although the 4-methoxybenzyl protecting group is suitable for Fmoc-based synthesis, milder cleavage conditions are achieved with Kittaka's 2-methyl-4-methoxybenzyl group. We have also demonstrated the preparation of a hyper labile protecting group, the 2,4-dimethoxybenzyl, which is removed within minutes with dilute TFA (2%) treatment. In comparison, the 2-methoxybenzyl protecting group was, in comparison, surprisingly robust and may find use in combination with highly acid labile resins (such as trityl-based) for the preparation of oligomers retaining the S-protecting group for further, post oligomerization chemistry. The origin of the resistance to acidolysis for the 2-methoxy group appears to be intramolecular stabilization of the protonated species and, if so, cannot be expected without this particular structural feature.

We have also prepared thiothymine-PNA and shown its incorporation into an oligomer. In the sequence context examined, the ^{S}T insert resulted in a mild destabilization (ΔT_m = -1.0/insert) relative to T, whereas ^{S}U had a slight stabilizing effect (ΔT_m = +0.3/insert). The sequence generality of this effect and its application to pseudo-complementary PNAs requires further investigation.

Experimental Section

General synthetic procedures

All chemicals were obtained from commercial sources and were of ACS reagent grade or higher and were used without further purification. Anhydrous and HPLC-grade solvents for PNA synthesis and chromatography were purchased from Caledon Laboratories. All other solvents were dried by passing through activated alumina columns. In all cases, sodium sulfate was used as the drying agent and solvent was removed by reduced pressure with Buchi Rotovapor. Thin layer chromatography was performed on Silicycle Silica Gel TLC F-254 plates. Unless otherwise specified, reported R_f values were measured using the same solvent system as for reaction monitoring. Flash chromatography was performed with Silicycle SiliaFlash® F60 230-400 mesh silica. Reactions performed at elevated temperature were done using a temperature-controlled oil bath.

All chemical shifts are reported in parts per million (δ), from tetramethylsilane (0 ppm), and are referenced to the residual proton in the respective solvent: $CDCl_3$ (7.26 ppm), DMSO-d_6 (2.49 ppm), methanol-d_6 (3.31 ppm) for ^{1}H NMR and $CDCl_3$ (77.0 ppm) and DMSO-*d6* (39.5 ppm) and methanol-d_6 (49.0 ppm) for ^{13}C NMR. Multiplicities are described as s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet) and br s (broad singlet). Coupling constants (*J*) are reported in Hertz (Hz). Spectra were obtained on Bruker-400 and INOVA-400 and INOVA-600 instruments. The ^{1}H NMR and ^{13}C NMR for PNA monomers performed in $CDCl_3$ show the presence of rotamers. High resolution mass spectra (HRMS) were obtained using electrospray ionization (ESI) with a time-of-flight (TOF) mass analyzer. Low resolution mass spectra (LRMS) and high resolution mass spectra (HRMS) with electron impact (EI) ionization were measured using a Thermo Scientific double focusing sector mass spectrometer, utilizing a reversed Nier Johnson geometry.

2,4,6-Trimethoxybenzyl alcohol (1). To a solution of 2,4,6-trimethoxybenzaldehyde (1.00 g, 5.10 mmol) and 0.5 M sodium hydroxide in methanol (30 mL) was added sodium borohydride (290 mg, 7.65 mmol) portionwise. The resultant solution was stirred for 2 h, then the reaction was quenched with 30 mL water. The solution was concentrated under reduced pressure to approximately 30 mL, then the product was extracted into ether (3 x 20 mL). The organic layers were combined, washed with brine, dried over sodium sulfate, then the solvent evaporated to yield a clear, colourless oil which crystallized overnight (830 mg, 4.19 mmol, 82%). Spectroscopic analysis conformed to previous reports.[27] ^{1}H NMR (400 MHz, DMSO-*d6*) δ 6.17 (s, 2H), 4.35 (d, *J* = 5.5 Hz, 2H), 4.13 (t, *J* = 5.5 Hz, 1H), 3.75 (s, 6H), 3.73 (s, 3H). ^{13}C{^{1}H} NMR (100 MHz, DMSO-*d6*) 160.9, 159.5, 110.5, 91.0, 56.0, 55.61, 51.7.

2-(2,4,6-Trimethoxy-3-chlorobenzyl)thiouracil (2). To a solution of **1** (2.00 g, 10.1 mmol) and triphenyl phosphine (1.94 g, 15.1 mmol) in 100 mL dichloromethane at 0 °C, was added *N*-chlorosuccinimide (1.62mg, 12.1 mmol). The solution was stirred for 1 h, then added to a suspension of 2-thiouracil (1.29 g, 10.1 mmol) and sodium carbonate (2.79 g, 20.2 mmol) in 100 mL dimethylformamide. The mixture was stirred for 90 min., then neutralized with 40 mL saturated citric acid. The solution was diluted with 150 mL water, then extracted in ethyl acetate (3 x 150 mL). The organic layers were combined, washed with water (4 x 150 mL), and brine (150 mL), dried over sodium sulfate, then the solvent was evaporated under reduced pressure to yielded crude compound **2** combined with triphenylphosphine oxide as an off white solid (2.55 g). The crude product was used for further synthesis.

Methyl 2-(2-(2,4,6-trimethoxy-3-chlorobenzyl)thiouracil-*O*4-yl)acetate (3). To a solution of crude **2** (2.55 g) and sodium carbonate (2.03 g, 11.1 mmol) in 120 mL dimethylformamide at 0 °C was added methyl bromoacetate (1.05 mL, 11.1 mmol), then the reaction was stirred at 0 °C for 15 min, then allowed to warmed to room temperature. The reaction was stirred for 4 h, then diluted with water (200 mL) and extracted with ethyl acetate (3 x 120 mL). The organic layers were combined, washed with water (4 x 100 mL) and brine (100 mL), dried with sodium sulfate, filtered, then the solvent was evaporated under reduced pressure, yielding a crude orange oil (3.84 g). The crude product was purified by column chromatography on silica gel (1:9 to 1:1 ethyl acetate: hexanes) to yield the pure product as light-yellow crystals (234 mg, 0.56 mmol, 9.0% from **1**). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.24 (d, *J* = 5.7 Hz, 1H), 6.48

(d, J = 5.7 Hz, 1H), 6.27 (s, 1H), 4.89 (s, 2H), 4.37 (s, 2H), 3.85 (s, 3H), 3.82 (s, 3H), 3.79 (s, 3H), 3.71 (s, 3H). $^{13}C\{^1H\}$ NMR (100 MHz, $CDCl_3$) δ 172.2, 168.7, 167.5, 157.9, 157.7, 156.3, 156.0, 111.5, 108.6, 103.4, 92.6, 62.5, 61.8, 56.4, 56.1, 52.3, 24.7. LRMS (EI) calcd for $C_{17}H_{19}ClN_2O_6S$ $[M]^+$ 414.07; found 414.09.

2,4-Dimethoxybenzyl alcohol (4). To a solution of 2,4-dimethoxybenzaldehyde (5.00 g, 30.09 mmol) and 0.5 M sodium hydroxide in 200 mL methanol was added sodium borohydride (1.37 g, 36.11 mmol) portionwise. The solution was stirred for 1 h, then the reaction was quenched with water. The solution was concentrated under reduced pressure, then the product was extracted into ether. The organic layers were combined, washed with brine, dried over sodium sulfate, filtered, then the solvent was evaporated under reduced pressure to yield **13** as a clear, colourless oil which crystalized overnight (4.45 g, 26.46 mmol, 88%). Spectra matched those reported by Integrated Spectral Database System of Organic Compounds with data that were obtained from the National Institute of Advanced Industrial Science and Technology (Japan). ^1H NMR (400 MHz, $CDCl_3$) δ 7.17 (d, J = 8.0 Hz, 1H), 6.50-6.42 (m, 2H), 4.61 (s, 2H), 3.84 (s, 3H), 3.81 (d, 3H), 2.27 (br s, 1H). $^{13}C\{^1H\}$ NMR (100 MHz, $CDCl_3$) δ 160.7, 158.6, 129.7, 121.8, 103.9, 98.6, 61.7, 55.4, 55.3.

2-(2,4-Dimethoxybenzyl)thiouracil (5). A solution of 2,4-dimethoxybenzyl alcohol (**4**) (4.12 g, 24.5 mmol) and pyridine (3.95 mL, 49.0 mmol) in 50 mL dry ether was cooled to 0 °C, then thionyl chloride (4.89 mL, 67.4 mmol) was added dropwise to solution over 15 min. The solution was stirred for 90 min at 0 °C, then quenched with 50 mL ice cold water. The ethereal layer was collected, and the aqueous layer was extracted with ether (3 x 25 mL). The organic layers were combined, washed with water (50 mL), and 5:1 saturated sodium chloride: saturated sodium bicarbonate (3 x 60 mL), and then dried over sodium sulfate. Dimethylformamide (10 mL) was added to solution, then the ether was evaporated under reduced pressure. The resulting dimethylformamide solution was added dropwise to a solution of 2-thiouracil (1.57 g, 12.26 mmol) and triethylamine (2.05 mL, 14.71 mmol) in 50 mL dimethylformamide at 0 °C. The resultant solution was stirred for 4 h at 0 °C, then diluted with water and extracted with dichloromethane (3 x 40 mL). The dichloromethane layers were combined, washed with water (4 x 50 mL), and brine (50 mL), dried over sodium sulfate then the solvent was evaporated under reduced pressure. The resulting white power was triturated with cold ether, yielding **5** as a pure white solid (2.41 g, 8.66,

71%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.89 (d, *J* = 6.6 Hz, 1H), 7.31 (d, *J* = 8.2 Hz, 1H), 6.49-6.40 (m, 2H), 6.20 (d, *J* = 6.6 Hz, 1H), 4.42 (s, 2H), 3.86 (s, 3H), 3.81 (s, 3H). ^{13}C{^{1}H} NMR (100 MHz, $CDCl_3$) δ 164.4, 162.6, 160.9, 158.4, 154.8, 131.4, 116.6, 110.8, 104.3, 98.5, 55.5, 55.4, 29.7. HRMS (EI) calcd for $C_{13}H_{14}N_2O_3S$ $[M]^+$ 278.0725; found 278.0720.

Methyl 2-(2-(2,4-dimethoxybenzyl)thiouracil-1-yl)acetate (6a). To a solution of sodium (165 mg, 7.18 mmol) in 15 mL methanol was added **5** (1.00 g, 3.59 mmol), then the solution was stirred and heated to reflux. Methyl bromoacetate (0.79 mL, 7.18 mmol) was added to the reaction, the solution was stirred for 2 h at reflux, then cooled to room temperature and the solvent was evaporated under reduced pressure. The resulting residue was dissolved in 25 mL water then extracted with a solution of 3:1 dichloromethane: methanol (2 x 80 mL). The organic layers were combined, dried over sodium sulfate, filtered, then the solvent was evaporated under reduced pressure to yield a white residue. The residue was purified by column chromatography on silica gel with gradient elution of 5% to 10% methanol in dichloromethane to yield **6a** as a pure white solid (289 mg, 0.79 mmol, 22%) and **6b** as a yellow solid, independently prepared below, in 13 % yield. (. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.34 (d, *J* = 8.9 Hz, 1H), 7.07 (d, *J* = 7.6 Hz, 1H), 6.41-6.34 (m, 2H), 6.02 (d, *J* = 7.6 Hz, 1H), 4.47 (s, 2H), 4.44 (s, 2H), 3.77 (s, 3H), 3.74 (s, 3H), 3.73 (s, 3H). ^{13}C{^{1}H} NMR (400 MHz, $CDCl_3$) δ 167.8, 166.4, 163.8, 160.9, 158.6, 143.6, 131.8, 115.9, 110.0, 104.1, 98.5, 55.3, 53.1, 52.6, 41.9, 31.3. HRMS (EI) calcd for $C_{16}H_{18}N_2O_5S$ $[M]^+$ 350.0936; found 350.0949.

Methyl 2-(2-(2,4-dimethoxybenzyl)thiouracil-*O*4-yl)acetate (6b). To a solution of **5** (1.00 g, 3.59 mmol) and sodium carbonate (1.24 g, 8.98 mmol) in 60 mL dimethylformamide was added methyl bromoacetate (0.52 mL, 5.39 mmol) then the solution was heated to 80 °C. The reaction was stirred for 2 h at 80 °C, then diluted with water (70 mL) and extracted with dichloromethane (4 x 50 mL). The dichloromethane layers were combined, washed with water (4 x 60 mL) and brine (60 mL), dried over sodium sulfate, filtered, then the solvent was evaporated under reduced pressure, yielding a yellow solid (1.23 g, 3.37 mmol, 94%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.31 (d, *J* = 5.7 Hz, 1H), 7.31 (d, *J* = 8.2 Hz, 1H), 6.55 (d, *J* = 5.7 Hz, 1H), 6.50-6.40 (m, 2H), 4.93 (s, 2H), 4.34 (s, 2H), 3.86 (s, 3H), 3.81 (s, 3H), 3.78 (s, 3H). ^{13}C{^{1}H} NMR (400 MHz, $CDCl_3$) δ 172.0, 168.6, 167.5, 160.4, 158.6, 157.8, 130.8, 117.6, 104.1, 103.5, 98.6, 62.4, 55.5, 55.4, 52.2, 29.7.

2-Methyl-4-methoxybenzyl alcohol (7). To a solution of 2-methyl-4-methoxybenzaldehyde (2.03 g, 13.5 mmol) in 32 mL ethanol was added sodium borohydride (519 mg, 13.7 mmol). The resultant solution was stirred for 15 min., then the reaction was quenched by the addition of 240 mL water. The solution was acidified with 4 M hydrochloric acid, then the product was extracted in diethyl ether (3 x 80 mL). The ethereal layers were combined, washed with water (60 mL), and brine (60 mL), and then dried over sodium sulfate. The solvent was evaporated under reduced pressure to yield the pure product as a clear, colourless oil (2.00 g, 13.1 mmol, 97%), which matched literature characterization: ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.19 (d, 1H, J = 8.3 Hz), 6.71 (d, 1H, J = 2.7 Hz), 6.68 (dd, 1H, J = 2.7, 8.3 Hz), 4.59 (s, 2H), 3.76 (s, 3H), 2.33 (s, 3H), 1.59 (s, 1H).

2-(2-Methyl-4-methoxybenzyl)thiouracil (8). To a solution of 2-methyl-4-methoxybenzyl alcohol **7** (2.00 g, 13.1 mmol) in 80 mL dry dichloromethane at 0 °C was added thionyl chloride (1.05 mL, 14.4 mmol) dropwise, then the solution was stirred for 2 h at 0 °C. The reaction was added to a solution of ice cold saturated sodium bicarbonate (250 mL), the dichloromethane layer was separated, and then the aqueous layer was extracted with diethyl ether (2 x 60 mL). The organic layers were combined, washed with water (80 mL), and brine (80 mL), and subsequently dried over sodium sulfate. The solvent was evaporated under reduced pressure to yield a clear colourless oil. The resulting oil was dissolved 8 mL ethanol then added to a solution of 2-thiouracil (833 mg, 6.58 mmol) in 8 mL ethanol and 8 mL aqueous potassium hydroxide (474 mg, 8.45 mmol KOH in H_2O). The resultant reaction was stirred overnight at room temperature, then the solvent was evaporated under reduced pressure. A solution of saturated sodium bicarbonate (20 mL) was added to the resultant residue then the precipitate was collected by filtration, washed with water, ethanol, ethyl acetate, and diethyl ether to yield the product as pure white crystals (1.49 g, 5.60 mmol, 86%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.29 (d, 1H, J = 8.3 Hz), 6.79 (d, 1H, J = 2.1 Hz), 6.72 (dd, 1H, J = 2.1 Hz, 8.3 Hz), 4.35 (s, 2H), 3.72 (s, 3H), 2.32 (s, 3H). The ^{1}H NMR spectrum was consistent with previous report.[7]

Ethyl 2-(2-(2-methyl-4-methoxybenzyl)thiouracil-1-yl)acetate (9). To a solution of sodium (273 mg, 11.4 mmol) dissolved in ethanol (13 mL) was added 2-(2-methyl-4-methoxybenzyl)thiouracil (1.49 g, 5.60 mmol). The resultant suspension was stirred and heated to reflux until the solids dissolved, then ethyl bromoacetate (1.26 mL, 11.4 mmol) was added dropwise, then the solution was stirred at reflux for 2 h. The mixture was cooled to room

temperature, then the ethanol was evaporated under reduced pressure. Water (15 mL) was added to the resulting residue, and the product was extracted in a solution of 3:1 dichloromethane: methanol (2 x 60 mL). The organic layers were combined, dried over sodium sulfate, and then evaporated under reduced pressure. The resultant residue was purified by column chromatography on silica gel (ethyl acetate to 9:1 ethyl acetate: methanol) to yield the product as a pure white solid (781 mg, 2.2 mmol, 40%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.25 (d, 1H, J = 8.2 Hz), 7.08 (d, 1H, J = 7.6 Hz), 6.69 (d, 1H, J = 2.6 Hz), 6.66 (dd, 1H, J = 2.6, 8.2 Hz), 6.08 (d, 1H, J = 7.6 Hz), 4.45 (s, 2H), 4.44 (s, 2H), 4.20 (q, 2H, J = 7.1 Hz), 3.74 (s, 3H), 2.30 (s, 3H), 1.23 (t, 3H, J = 7.1 Hz). This corresponded closely to the ^{1}H NMR spectrum previously reported in the literature.[7]

4-Methoxybenzyl alcohol (10). To a solution of 4-methoxybenzaldehyde (10.0 g, 95% purity, 73 mmol) in EtOH (40 mL) was added $NaBH_4$ (3.0 g, 80 mmol) portion wise and the mixture was stirred at room temperature for 10 min. After the addition of H_2O (300 mL), the reaction mixture was acidified until pH = 4 with 4 M HCl and extracted with Et_2O (80 mL ×3). The combined organic layers were washed with H_2O, brine, dried over anhydrous sodium sulfate, and solvent evaporated *in vacuo* after filtration to give alcohol **2** (9.2 g, 66.6 mmol, 91%) as a clear colorless oil. The ^{1}H NMR spectrum is consistent with that previously reported:[14] ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.27 (d, J = 8.4 Hz, 2H), 6.86 (d, J = 8.4 Hz, 2H), 4.58 (s, 2H), 3.77 (s, 3H), 1.61 (br s, 1H).

2-(4-Methoxybenzyl)thiouracil (11). To a solution of **10** (9.2 g, 66.6 mmol) in CH_2Cl_2 (200 mL) was added $SOCl_2$ (5.4 mL, 74.2 mmol) dropwisely at 0 °C. After stirring at the same temperature for 20 min, the reaction mixture was poured into saturated aqueous $NaHCO_3$ (250 mL) with crushed ice. The resulting mixture was extracted with Et_2O (100 mL ×3). The combined organic layers were washed with H_2O, brine, dried over anhydrous sodium sulfate, and solvent evaporated *in vacuo* after filtration to yield the crude 4-methoxybenzyl chloride **15** (9.7 g, 8.46 mL, 62 mmol) which was subjected to the next reaction without further purification. To a suspension of 2-thiouracil (5 g, 39 mmol) in EtOH (50 mL) was added aqueous KOH solution (2.8 g KOH in 20 mL H_2O, 50.7 mmol) and the mixture was warmed to 45 °C to completely dissolve 2-thiouracil. After cooling to room temperature, crude chloride **15** (62 mmol) in EtOH (10 mL) was added to the solution and the reaction mixture was stirred at room temperature overnight. Mixture was evaporated to dryness and the crude product was suspended in saturated aqueous

$NaHCO_3$ (50 mL). The resulting precipitate was collected by filtration and washed with H_2O, EtOH, EtOAc, and Et_2O to give **11** (8.4 g, 33.9 mmol, 87%) as a white solid. The ^1H NMR and ^{13}C NMR spectra of the product is matched the literature spectra reported by Nielsen et. al.[28] ^1H NMR (400 MHz, DMSO-d_6) δ 12.43 (br s, 1H), 7.41 (d, J = 7.6 Hz, 1H), 7.33 (d, J = 8.6 Hz, 2H), 6.88 (d, J = 8.7 Hz, 2H), 4.35 (s, 2H), 3.73 (s, 3H); $^{13}C\{^1H\}$ NMR (100 MHz, DMSO-d_6) δ 176.6, 161.5, 159.0, 142.6, 130.7, 129.2, 114.4, 105.7, 55.5, 33.7.

***N*1-(Ethyloxycarboxymethyl)-*S*-(4-methoxybenzyl)-2-thiouracil (12a)**. Under a nitrogen atmosphere, sodium (620 mg, 27 mmol) was dissolved in refluxing absolute EtOH (40 ml), and was added to a suspension of **11** (6.4 g, 26 mmol) in EtOH (30 mL) and heated to reflux. To the reaction mixture was added ethyl bromoacetate (1.26 mL, 11.4 mmol) and reflux was continued for 2 h. Ethyl bromoacetate (4.7 g, 3.1 mL, 27 mmol) was added and reflux was continued for 3 h. The resulting mixture was cooled to room temperature and EtOH was removed under reduced pressure. Water (15 mL) was added, and the mixture was extracted with CH_2Cl_2 – MeOH (3:1, 60mL) twice. The organic phases was evaporated to dryness, taken up in ethyl acetate/hexane, and again evaporated. The residue was triturated in cold ethyl acetate (10 ml), whereby the product precipitated. It was filtered off, and washed with cold ethyl acetate to afford **7a** as a white solid. Yield: 2.7 g (2.7 g, 8 mmol, 31%). The ^1H NMR and ^{13}C NMR spectra of the product matched the literature spectra reported by Nielsen et. al.[14] ^1H NMR (600 MHz, DMSO-d_6) δ 7.64 (d, J = 7.6 Hz, 1H), 7.31 (d, J = 8.7 Hz, 2H), 6.85 (d, J = 8.7 Hz, 2H), 5.91 (d, J = 7.5 Hz, 1H), 4.76 (s, 2H), 4.33 (s, 2H), 4.13 (q, J = 7.1 Hz, 2H), 3.70 (s, 3H), 1.15 (t, J = 7.1 Hz, 3H); $^{13}C\{^1H\}$ NMR (100 MHz, DMSO-d_6) δ, 167.0, 166.4, 162.4, 158.8, 145.7, 130.5, 127.8, 113.7, 108.7, 61.7, 55.1, 52.5, 34.3, 13.9. HRMS (EI) calcd for $C_{16}H_{18}N_2O_4S$ 334.0987 [M^+]; found 334.0990.

2-(2-Methoxybenzyl)thiouracil (13). To a solution of 2-methoxybenzaldehyde (5 g, 95% purity, 36.5 mmol) in EtOH (20 mL) was added $NaBH_4$ (1.5 g, 40 mmol) portion wise and the mixture was stirred at room temperature for 10 min. After the addition of H_2O (150 mL), the reaction mixture was acidified until pH=4 with 4 M HCl and extracted with Et_2O (40 mL × 3). The combined organic layers were washed with H_2O, brine, dried over anhydrous sodium sulfate, and solvent evaporated *in vacuo* after filtration to give 2-methoxybenzyl alcohol (4 g, 28.9 mmol, 79%) as a clear yellow oil. Then, to this alcohol (4 g, 28.9 mmol) in CH_2Cl_2 (100 mL) was added $SOCl_2$ (5.4 mL, 32.0 mmol) dropwisely at 0 °C. After stirring at the same temperature for 20 min, the

reaction mixture was poured into saturated aqueous $NaHCO_3$ (150 mL) with crushed ice. The resulting mixture was extracted with Et_2O (100 mL × 3). The combined organic layers were washed with H_2O, brine, dried over anhydrous sodium sulfate, and solvent evaporated *in vacuo* after filtration. The crude 2-methoxybenzyl chloride (4.7 g, 4.1 mL, 30 mmol) was subjected to the next reaction without further purification. To a suspension of 2-thiouracil (2.4 g, 19 mmol) in EtOH (25 mL) was added aqueous KOH solution (1.4 g KOH in 10 mL H_2O, 25.3 mmol) and the mixture was warmed to 45 °C to completely dissolve 2-thiouracil. After cooling to room temperature, the crude, freshly prepared 2-methoxybenzyl chloride (30 mmol) in EtOH (5 mL) was added to the solution and the reaction mixture was stirred at room temperature overnight. The mixture was evaporated to dryness and the crude product was suspended in saturated aqueous $NaHCO_3$ (50 mL). The resulting precipitate was collected by filtration and washed with H_2O, EtOH, EtOAc, and Et_2O to give **13** (5.4 g, 21.9 mmol, 87%) as a white solid. ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.90 (d, J = 6.5 Hz, 1H), 7.37 (dd, J = 7.4, 1.8 Hz, 1H), 7.28 (ddd, J = 8.2, 7.4, 1.8 Hz, 1H), 7.02 (dd, J = 8.3, 1.1 Hz, 1H), 6.89 (td, J = 7.4, 1.1 Hz, 1H), 6.09 (d, J = 6.5 Hz, 1H), 4.34 (s, 2H), 3.83 (s, 3H); ^{13}C{^{1}H} NMR (100 MHz, DMSO-d_6) δ 174.6,157.7, 130.8, 129.6, 124.9, 120.8, 111.4, 56.0, 29.4. HRMS (ESI-TOF) calcd for $C_{12}H_{12}N_2O_2S$ (**13**) $[M+H]^+$ 248.06195; found 248.06235.

***N*1-(Ethyloxycarboxymethyl)-*S*-(2-methoxybenzyl)-2-thiouracil (14a).** Under a nitrogen atmosphere, sodium (528 mg, 23 mmol) was dissolved in refluxing absolute EtOH (40 ml), and was added to a suspension of **13** (5.4 g, 21.9 mmol) in EtOH (25 mL) and heated to reflux. To the reaction mixture was added ethyl bromoacetate (1.21 mL, 11.4 mmol) and reflux was continued for 2 h. Ethyl bromoacetate (4.0 g, 2.6 mL, 23 mmol) was added and reflux was continued for 4 h. The resulting mixture was cooled to room temperature and EtOH was removed under reduced pressure. Water (15 mL) was added, and the mixture was extracted with CH_2Cl_2 – MeOH (3:1, 60 mL) twice. The combined organic layers were dried over anhydrous sodium sulfate and concentrated *in vacuo* after filtration. The residue was purified by column chromatography on silica gel (for **14b** hexane-EtOAc = 5:1 to 1:1; for **14a,** EtOAc to EtOAc-MeOH = 9:1) to afford **14a** (2.5 g, 7.4 mmol, 34%) as a white solid, **14b** (810 mg, 2.42 mmol, 11%) as an oil. ^{1}H NMR (600 MHz, Chloroform-*d*) δ 7.43 (d, J = 7.6 Hz, 1H), 7.26 – 7.19 (m, 1H), 7.11 (d, J = 7.6 Hz, 1H), 6.89 – 6.80 (m, 2H), 6.03 (dd, J = 7.5, 1.7 Hz, 1H), 4.51 (d, J = 1.7 Hz, 2H), 4.48 (d, J = 1.7 Hz, 2H), 4.20 (q, J = 7.1 Hz, 2H), 3.81 (s, 3H), 1.22 (t, J = 7.1 Hz, 3H); ^{13}C{^{1}H} NMR (151 MHz,

Chloroform-*d*) δ 167.8, 165.9, 163.6, 157.6, 143.81, 131.2, 129.3, 123.7, 120.6, 110.4, 109.9, 62.5, 55.4, 52.7, 31.3, 13.9. HRMS (ESI-TOF) calcd for $C_{16}H_{18}N_2O_4S$ (**14a**) $[M+H]^+$ 334.0987; found 334.0990.

2-(4-Methoxybenzyl)thiothymine (16). To a solution of 25 mL ethanol was added thiothymine (1.00 g, 7.00 mmol) followed by aqueous potassium hydroxide (510 mg, 9.10 mmol) in 15 mL water. The solution was heated to 45 °C then 4-methoxybenzyl chloride **15** (1.42 mL, 10.5 mmol), prepared as previously described, was added to the reaction. The reaction was stirred for 8 h., then evaporated to dryness under reduced pressure. The resultant residue was suspended in a solution of 10% sodium bicarbonate (20 mL), then the precipitate was collected by filtration, and washed with water, methanol, and diethyl ether to yield a pure white solid (1.3 g, 4.96 mmol, 71%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.66 (br, 1H), 7.76 (s, 1H), 7.32 (d, J = 8.6 Hz, 2H), 6.87 (d, J = 8.6 Hz, 2H), 4.32 (s, 2H), 3.73 (s, 3H), 1.87 (s, 3H). HRMS (EI) calcd for $C_{13}H_{14}N_2O_2S$ (**16**) $[M]^+$ 262.0776; found 262.0770.

Ethyl 2-(2-(4-methoxybenzyl)thiothymin-1-yl)acetate (17). To a solution of sodium (210 mg, 9.15 mmol) in ethanol (15 mL) at reflux, was added 2-(4-methyoxybenzyl)thiothymine (1.2 g, 4.57 mmol) followed by ethyl bromoacetate (1.0 mL, 9.15 mmol). The reaction was stirred for 2 h. at reflux, then the solvent was evaporated under reduced pressure. The residue was dissolved in water (15 mL) and extracted with a solution of 3:1 dichloromethane: methanol (2 × 60 mL). The organic layers were combined, dried over sodium sulfate, and evaporated under reduced pressure. The crude oil was washed with a solution of 1:1 ethyl acetate: hexanes (3 × 10 mL), the washes were combined and evaporated under reduced pressure. The resultant solid was suspended in ice cold ethyl acetate and the product was collected by filtration, yielding a pure white solid **17** (612 mg, 3.45 mmol 38%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.62 (s, 1 H), 7.34 (d, J = 8.7 Hz, 2 H), 6.88 (d, J = 8.7 Hz, 2 H), 4.76 (s, 2 H), 4.37 (s, 2 H), 4.17 (q, J = 7.1, 2 H), 3.73 (s, 3 H), 1.82 (s, 3 H), 1.18 (t, J = 7.1, 3 H). ^{13}C{^{1}H} NMR (400 MHz, DMSO) δ 167.9, 167.5, 159.2, 142.1, 130.9, 128.6, 117.4, 114.4, 62.2, 55.5, 52.8, 34.9, 14.4, 13.8. HRMS (EI) calcd for $C_{17}H_{20}N_2O_4S$ (**17**) $[M]^+$ 348.1144; found; 348.1145.

Methyl (2-((((9H-fluoren-9-yl)methoxy)carbonyl)amino)ethyl)glycinate (19). To obtain the free base from the available hydrochloride salt (**19•**HCl, identified as **18**, Scheme 6**)**, it was dissolved in $CHCl_3$, washed with saturated aqueous $NaHCO_3$, dried (Na_2SO_4) and

concentrated in *vacuo* to give the free base of **19** as a colourless oil which was used promptly. ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.07 (s, 1H), 7.91 (d, J = 7.5 Hz, 2H), 7.69 (d, J = 7.5 Hz, 2H), 7.43 (t, J = 7.5 Hz, 2H), 7.35 (t, J = 7.4 Hz, 2H), 4.37 (d, J = 6.7 Hz, 2H), 4.24 (t, J = 6.7 Hz, 1H), 4.01 (s, 2H), 3.76 (s, 3H), 3.57 (s, 3H), 3.02 (t, J = 6.4 Hz, 2H). ^{13}C{^{1}H} NMR (100 MHz, DMSO-d_6) δ168.0, 159.4, 145.1, 142.6, 128.8, 128.1, 126.1, 120.9, 68.1, 53.5, 49.2, 48.2, 48.1, 38.1.

2-(2-(4-Methoxybenzyl)thiouracil-1-yl)acetic acid (20). To a solution of **12a** (1.7 g, 5.5 mmol) in a mixture of methanol (10 mL) and tetrahydrofuran (20 mL) was added 2 M lithium hydroxide (4.3 mL, 8.7 mmol) at room temperature. After stirring at the same temperature for 30 min, the solvent was removed *in vacuo*. The residue was dissolved in 5 mL water and solution was acidified with 2M hydrochloric acid. The resulting precipitate was filtered and washed with cold ethyl acetate and diethyl ether and then dried under a high vacuum to give **20** (1.25g, 4.1 mmol, 82%) as a white solid. The ^{1}H NMR and ^{13}C NMR spectra of the product is matched the literature spectra reported by Nielsen et. al.[14] ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.51 (d, J = 7.5 Hz, 1H), 7.34 (d, J = 8.6 Hz, 2H), 6.87 (d, J = 8.7 Hz, 2H), 5.79 (d, J = 7.4 Hz, 1H), 4.29 (s, 2H), 4.04 (s, 2H), 3.73 (s, 3H); ^{13}C{^{1}H} NMR (100 MHz, DMSO-d_6) δ167.6, 162.3, 159.0, 151.0, 146.8, 130.9, 128.9, 114.4, 108.1, 56.4, 55.6, 34.5.

2-(2-(4-Methoxybenzyl)thiothimin-1-yl)acetic acid (21). To a solution of **17** (250 mg, 0.72 mmol) dissolved in methanol (2.2 mL) and tetrahydrofuran (5 mL) was added 0.72 mL of 2 M lithium hydroxide, then the solution was stirred for 15 min. The solvent was evaporated under reduced pressure, then the residue was dissolved in 5 mL water and the solution was acidified with 2 M hydrochloric acid. The resultant precipitate was filtered and washed with cold ethyl acetate and diethyl ether which yielded a pure white solid (185 mg, 0.38 mmol, 80%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 13.53 (s, 1 H), 7.62 (s, 1 H), 7.34 (d, J = 8.4 Hz, 2 H), 6.88 (d, J = 8.4 Hz, 2 H), 4.64 (s, 2 H), 4.37 (s, 2 H), 3.73 (s, 2 H), 1.82 (s, 3 H). ^{13}C{^{1}H} NMR (400 MHz, DMSO) δ 168.9, 167.9, 161.6, 159.1, 142.3, 130.9, 128.7, 117.5, 114.4, 55.6, 52.9, 34.8, 13.8. HRMS (EI) calcd for $C_{15}H_{16}N_2O_4S$ $[M]^+$ 320.0831; found 320.0828.

Methyl *N*-(2-((((9H-fluoren-9-yl)methoxy)carbonyl)amino)ethyl)-*N*-(2-(2-(4-methoxybenzyl)thiouracil-1-yl)acetyl)glycinate (22). To a solution of **20** (700 mg, 2.28 mmol) in DMF (10 mL) were added HBTU (872 mg, 2.3 mmol) and DIPEA (674 mL, 3.96 mmol) at 0 °C. After stirring for 10 min, **19** (744.2 mg, 2.1 mmol) was added at the same temperature. The resulting

mixture was allowed to warm to room temperature and stirred for 4 h. The reaction mixture was diluted with EtOAc, poured into water, and extracted with EtOAc three times. The combined organic layers were washed with 5% sat. citric acid/H_2O (20 mL), saturated aqueous $NaHCO_3$, brine, dried over anhydrous sodium sulfate, and concentrated *in vacuo* after filtration. The residue was purified by column chromatography on silica gel (CH_2Cl_2-MeOH = 30:1 to 19:1) to afford **22** (912 mg, 1.41 mmol, 69%) as an amorphous solid. ^{1}H NMR (400 MHz, Chloroform-*d*) δ 7.83 – 7.33 (m, 5H), 7.22 – 6.98 (m, 1H), 6.94 – 6.73 (m, 2H), 6.14 – 6.00 (m, 1H), 5.72 (s, 1H), 5.32 (s, 2H), 4.82 – 4.22 (m, 6H), 4.26 – 4.00 (m, 3H), 3.86 – 3.31 (m, 7H); ^{13}C{^{1}H} NMR (100 MHz, $CDCl_3$) δ 169.2, 168.6, 168.3, 165.6, 163.5, 159.4, 156.6, 144.7, 143.8, 143.5, 141.3, 138.9, 134.8, 134.4, 131.8, 129.0, 128.8, 128.7, 128.6, 128.3, 127.8, 120.0, 119.9, 116.2, 111.3, 111.2, 109.5, 68.0, 67.5, 66.7, 66.6, 55.8, 55.1, 55.07, 52.2, 50.3, 49.2, 48.9, 48.6, 47.8, 47.2, 47.1, 39.1, 38.8, 38.6, 35.04, 34.96, 29.7, 19.5; HRMS (ESI-TOF) calcd for $C_{33}H_{32}N_4O_7SNa$ $[M+Na]^+$ 665.2046; found 665.2050.

Methyl *N*-(2-((((9H-fluoren-9-yl)methoxy)carbonyl)amino)ethyl)-*N*-(2-(2-(4-methoxybenzyl)thiothymin-1-yl)acetyl) glycinate (23). To a solution of **21** (280 mg, 0.87 mmol) in 7 mL dimethylformamide at 0 °C was added HBTU (338 mg, 0.89 mmol) and diisopropylethylamine (0.48 mL, 1.55 mmol) and stirred for 2 min., then **19** (375 mg, 0.96 mmol) was added to the reaction and the mixture was stirred for 2 h., then allowed to warm to room temperature. The reaction mixture was diluted with 15 mL ethyl acetate, poured into 40 mL water, then extracted with ethyl acetate (3 x 20 mL). The organic layers were combined, washed with 5% citric acid in water (20 mL), saturated sodium bicarbonate (20 mL), and brine (20 mL), then dried over sodium sulfate, filtered, after which the solvent was evaporated under reduced pressure. This yielded a crude orange oil which was purified by column chromatography on silica gel (1% methanol-dichloromethane to 5% methanol-dichloromethane) to yield compound **23** as a white solid (312 mg, 0.48 mmol, 55%) HRMS (ESI-TOF) calcd for $C_{35}H_{36}N_4O_7SNa$ $[M+Na]^+$ 679.2202; found 679.2205.

***N*-(2-((((9H-Fluoren-9-yl)methoxy)carbonyl)amino)ethyl)-*N*-(2-(2-(4-methoxy-benzyl)thiouracil-1-yl)acetyl)glycine (24).** To a solution of **22** (570 mg, 0.87 mmol) in 15 mL tetrahydrofuran at 0 °C was added 10 mL 2M lithium hydroxide and solution was stirred for 30 min. The reaction was neutralized with 2 M hydrochloric acid then the solvent was evaporated under reduced pressure. The residue was suspended in 10 mL cold water with crushed ice and the

solid was collected by filtration. The solid was suspended in cold dichloromethane and produce was collected by filtration, yielding the PNA monomer (**24**) as a pure white solid (220 mg, 0.34 mmol, 39%). The ^{1}H NMR and ^{13}C NMR spectra of the product matched the literature spectra reported by Kittaka et al.[7] ^{1}H NMR (600 MHz, Methanol-d_4) δ 7.78 (t, J = 6.9 Hz, 2H), 7.62 (d, J = 7.4 Hz, 1H), 7.59 – 7.54 (m, 1H), 7.37 (t, J = 7.4 Hz, 2H), 7.29 (t, J = 7.4 Hz, 2H), 7.22 (d, J = 8.2 Hz, 1H), 7.13 (d, J = 8.3 Hz, 1H), 6.71 (d, J = 8.2 Hz, 1H), 6.57 (d, J = 8.2 Hz, 1H), 4.48 – 4.05 (m, 5H), 3.94 (d, J = 12.9 Hz, 2H), 3.66 (s, 2H), 3.56 – 3.51 (m, 2H), 3.47 (qd, J = 9.1, 8.1, 3.8 Hz, 4H), 3.36 – 3.32 (m, 2H). ^{13}C{^{1}H} NMR (100 MHz, DMSO-d_6) δ 170.6, 170.2, 166.6, 166.2, 165.8, 162.8, 162.7, 158.8, 158.7, 156.3, 156.1, 145.8, 143.8, 143.7, 140.7, 138.4, 131.4, 127.01, 127.06, 127.6, 125.1, 125.0, 120.1, 115.81, 115.77, 111.2, 111.1, 108.4, 79.2, 65.4, 55.0, 54.9, 52.3, 47.7, 46.8, 46.7, 37.8, 33.3, 19.0; HRMS (ESI-TOF) calcd for $C_{33}H_{32}N_4O_7SNa$ $[M+Na]^+$ 651.1889; found 651.1909.

***N*-(2-((((9H-Fluoren-9-yl)methoxy)carbonyl)amino)ethyl)-*N*-(2-(2-(4-methoxybenzyl)thiothymin-1-yl)acetyl) glycine (25).** To a solution of **23** (312 mg, 0.48 mmol) in 8 mL tetrahydrofuran at 0 ^{O}C was added 8 mL 2 M lithium hydroxide then the solution was stirred for 30 min. The reaction was neutralized with 2 M hydrochloric acid then the solvent was evaporated under reduced pressure. The residue was suspended in 10 mL ice cold water and the resultant precipitate was collected by filtration. The solid was suspended in cold dichloromethane, then the product was collected by filtration to yield the PNA monomer (**25**) as a white solid (126 mg, 20 mmol, 41%) ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.06 (m, 1H), 7.89 (d, J = , 2H), 7.67 (m, 2H), 7.43-7.27 (m, 7H), 6.80 (d, J = , 2H), 4.70 (s, 2H), 4.28 (s, 2H), 4.16 (s, 2H), 3.69 (s, 3H), 3.64 (s, 2H), 3.59 (s, 1H), 3.38 (m, 2H), 3.15 (m, 2H), 1.73 (s, 3H). ^{13}C{^{1}H} NMR (100 MHz, DMSO) δ 170.2, 168.2, 166.8, 162.0, 159.0, 156.5, 144.4, 142.5, 141.1, 130.8, 129.4, 128.8, 128.1, 127.8, 127.6, 125.9, 121.9, 120.5, 117.1, 114.3, 66.0, 55.5, 53.5, 53.0, 48.4, 47.2, 38.4, 34.9, 13.7. HRMS (ESI-TOF) calcd for $C_{34}H_{34}N_4O_7SNa$ $[M+Na]^+$ 665.2046; found 665.2030.

***N*-(2-((((9H-Fluoren-9-yl)methoxy)carbonyl)amino)ethyl)-*N*-(2-thiouracil-1-yl)acetylglycine (26).** A solution of **24** (120 mg, 0.18 mmol) in 15 mL trifluoroacetic acid and 0.75 mL triethylsilane was prepared and it was stirred for 30 min. The solvent was then evaporated under a nitrogen stream and the resulting residue was washed twice with toluene to remove the PMB protecting group from the nucleobase yielding the PNA monomer (**26**) as a pure white solid

(70 mg, 0.14 mmol, 78%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.1 (m, 1H), 7.89 (d, J = , 2H), 7.67 (m, 2H), 7.43-7.27 (m, 6H), 4.31 (s, 2H), 4.22 (s, 2H), 3.64 (s, 2H), 3.59 (s, 1H), 3.38 (m, 2H), 3.15 (m, 2H). ^{13}C{^{1}H} NMR (100 MHz, DMSO) δ 170.2, 168.2, 166.8, 162.0, 156.5, 144.4, 142.5, 141.1, 130.8, 129.4, 128.8, 128.1, 127.8, 127.6, 125.9, 121.9, 120.5, 117.1, 66.0, 53.4, 53.1, 47.2, 38.4, 35.1. HRMS (ESI-TOF) calcd for $C_{25}H_{24}N_4O_6SNa$ $[M+Na]^+$ 531.1317; found 531.1323.

General procedure for acidolysis studies of thioesters. To a solution of 0.02 mL trifluoroacetic acid, 0.02 mL triethylsilane and 0.96 mL deuterated chloroform was added to 3 mg of protected thiouracil or thiothymine (**6a**, **9**, **12a**, **14a**, and **17**) and the reaction was monitored by ^{1}H NMR at 2 min. intervals until the reaction was completed. The reaction was carried out at 25 °C and monitored for 2 h, or until reaction had gone to completion. Integration of benzylic protons was monitored to determine the rate of deprotection.

Oligomer synthesis. PNA oligomers were synthesized using the ABI 433A peptide synthesizer using manufacturer supplied "Fastmoc" cycles. Oligomerization was carried out using newly synthesized ^{S}U or ^{S}T monomers and commercially available Fmoc-A(Bhoc)-AEG-OH, Fmoc-G(Bhoc)-AEG-OH, and Fmoc-C(Bhoc)-AEG-OH, Fmoc-T(Bhoc)-AEG-OH (purchased from PolyOrg, Inc.), and *N*α-Fmoc-*N*ε-Boc-L-lysine (Chem-Impex International Inc.). Fmoc-RAM-PS was used as a solid support resin preloaded with L-lysine at 0.057 mmol/g. Synthesis was carried out on a 5.0 μmol scale. The synthesis cycle was only modified by using 20% 4-methylpiperidine in dimethylformamide for Fmoc deprotection.[29] Following automated synthesis, the resin was treated with a solution of 95% trifluoroacetic acid and 5% triethylsilane to cleave the oligomer from the resin and remove the protecting group from the nucleobases (Bhoc) and amino group (Boc). The solvent was then evaporated under a nitrogen stream, the resulting residue was washed twice with cold ether, dissolved in a solution of 0.05% trifluoroacetic acid in water then purified by reverse-phase HPLC. Reverse-phase HPLC was performed on an Agilent Microsorb-MV 100-5 C18 250 × 4.6 mm column heated to 50 °C. The purified PNA oligomer was eluted using a gradient (water/0.1% trifluoroacetic acid to acetonitrile/0.1% triflouroacetic acid) over 60 min.

Thermal stability analysis. Thermal stabilities (T_m) of complexes were measured in solutions of 100 mM NaCl, 10 mM sodium hydrogen phosphate, 0.1 mM EDTA, pH=7.0 with individual PNA strand concentrations of 2 μM. Absorbance at λ = 260 nm were measure at 0.5 °C intervals while the temperature was changed at a rate of 0.7 °C/min between 15-90 °C. T_m

values were measured in triplicate and determined by the first derivative method applied through the manufacturer supplied Varian WinUV Bio software.

Computations. Structures were constructed and minimized in Spartan '14 using a desktop computer at the Hartree Foch 6-31G* level. A computational search for low energy conformers was performed using the same basis set. A variety of trials were run wherein the initial dihedral angle of C2-S-CH_2-Ph bond was set to avoid starting with the 2-methoxy group interacting with the protonated N3. The model nucleobase protected with 3-methoxybenzyl- or 4-methoxybenzyl-protecting group were similarly modelled and subjected to equilibrium conformer search for comparison.

Associated Content

Supporting Information: ^{1}H NMR spectra for time course of acidolysis reactions of **9**, **12a**, **14a**, and **17**. Plots of first order reaction analysis of acidolysis of **9**, **12a**, **14a**, and **17**. HPLC chromatograms and HRMS characterization of PNA oligomers. Minimum energy structure for the model compound representing [**14a**-H]$^{+}$. Verbose output from the computational study. ^{1}H and ^{13}C{^{1}H} NMR spectra for compounds described in the experimental section.

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