Bioconjugate Chemistry

Article

pubs.acs.org/bc

# Reductive Alkylation and Sequential Reductive Alkylation-Click Chemistry for On-Solid-Support Modification of Pyrrolidinyl Peptide Nucleic Acid

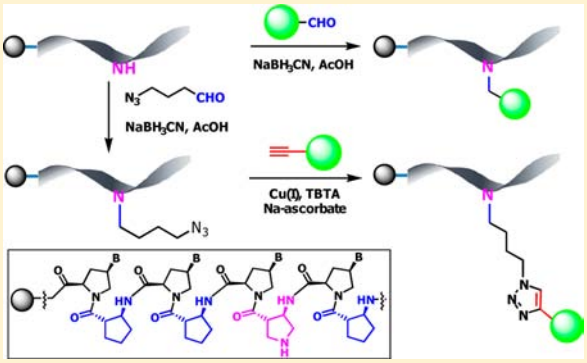
Boonsong Ditmangklo,[‡] Chalothorn Boonlua,[§] Chaturong Suparpprom,[‡] and Tirayut Vilaivan*[,§]

[‡]Department of Chemistry and Center of Excellence for Innovation in Chemistry, Faculty of Science, Naresuan University, Phitsanulok 65000, Thailand

[§]Organic Synthesis Research Unit, Department of Chemistry, Faculty of Science, Chulalongkorn University, Phayathai Road, Patumwan, Bangkok 10330, Thailand

Supporting Information

**ABSTRACT:** A methodology for the site-specific attachment of fluorophores to the backbone of pyrrolidinyl peptide nucleic acids (PNAs) with an $\alpha/\beta$-backbone derived from D-prolyl-(1*S*,2*S*)-2-aminocyclopentanecarboxylic acid (acpcPNA) has been developed. The strategy involves a postsynthetic reductive alkylation of the aldehyde-containing labels onto the acpcPNA that was previously modified with (3*R*,4*S*)-3-aminopyrrolidine-4-carboxylic acid on the solid support. The reductive alkylation reaction is remarkably efficient and compatible with a range of reactive functional groups including Fmoc-protected amino, azide, and alkynes. This allows further attachment of readily accessible carboxyl-, alkyne-, or azide-containing labels via amide bond formation or Cu-catalyzed azide–alkyne cycloaddition (CuAAC, also known as click chemistry). The label attached in this way does not negatively affect the affinity and specificity of the pairing of the acpcPNA to its DNA target. Applications of this methodology in creating self-reporting pyrene- and thiazole orange-labeled acpcPNA probes that can yield a change in fluorescence in response to the presence of the correct DNA target have also been explored. A strong fluorescence enhancement was observed with thiazole orange-labeled acpcPNA in the presence of DNA. The specificity could be further improved by enzymatic digestion with S1 nuclease, providing a 9- to 60-fold fluorescence enhancement with fully complementary DNA and a less than 3.5-fold enhancement with mismatched DNA targets.




## INTRODUCTION

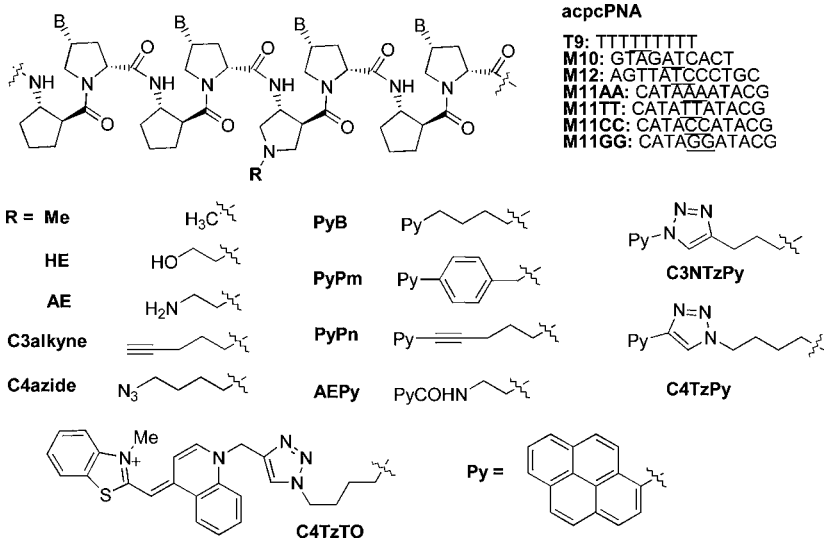
Fluorescence labeled oligonucleotides and analogues that are self-reporting, i.e., can exhibit a fluorescence change in response to the presence or absence of the correct DNA target, are useful probes for DNA sequence determination in homogeneous solution. Examples of such probes are molecular beacons which consist of two interacting fluorophores attached to different ends of the probes.[1,2] These dual-labeled probes are, however, expensive to make and, since both ends are occupied, further functionalization is difficult. In recent years, there has been considerable interest in singly labeled probes that can behave like molecular beacons.[3] These "quencher-free" molecular beacons require a fluorophore that is sensitive to different local environments, such as base stacking, hydrogen bonding, or solvent polarity. A typical example of such a label is pyrene.[4] In order to maximize these differences, the labels are often placed at internal positions of the probe. This can be achieved by incorporating a monomer carrying the fluorescence label or by postsynthetic labeling of the DNA that is prefunctionalized with a reactive group, such as an amino group for acylation or an alkyne/azide group for click chemistry.[5−8]

Peptide nucleic acid (PNA) is one of the most interesting analogues of DNA for diagnostic applications due to its many desirable characteristics including strong affinity, high sequence specificity, and excellent stability against nucleases and proteases.[9] The uncharged backbone of PNA allows the design of molecular beacons that do not require a stem-loop structure, in contrast to DNA beacons.[10−13] Despite numerous developments in the area of DNA probes,[3,4,14−19] only a few singly labeled self-reporting PNA probes have been described in the literature.[20−25] Realizing the potential of the recently introduced pyrrolidinyl PNA with $\alpha/\beta$-peptide backbones, such as 2-aminocyclopentanecarboxylic acid PNAs (acpcPNAs)[26−28] and their analogues,[29−31] in the area of molecular diagnostics, we have become interested in development of singly labeled fluorescence pyrrolidinyl PNAs as self-reporting probes. For example, we have reported synthetic strategies for labeling both the nucleobase[32,33] and backbone[34] of acpcPNA

**Received:** November 8, 2012
**Revised:** February 11, 2013
**Published:** March 22, 2013

   dx.doi.org/10.1021/bc3005914 | *Bioconjugate Chem.* 2013, 24, 614−625

**Scheme 1. Sequences of the acpcPNAs and Structures of the Labels Used in This Study, Labeling Sites are Indicated by Underscore**




with pyrene moieties and demonstrated the fluorescence enhancement of these pyrene-labeled acpcPNAs upon pairing with the complementary DNA targets. The backbone labeling strategy[34] is highly attractive because it does not alter the pairing behavior of the nucleobases, and because it can be easily carried out in a postsynthetic fashion by amide bond formation with the pyrrolidine nitrogen atom of the (3*R*,4*S*)-3-amino-pyrrolidine-4-carboxylic acid (APC)-modified acpcPNA. The latter point is very important because there is then no need to synthesize prelabeled monomers. However, the originally used amide chemistry created at least three problems. First, the introduction of the label resulted in a substantial decrease in the duplex stability ($\Delta T_m$ −10 to −30 °C) compared to the corresponding unmodified or APC-modified acpcPNA. This may possibly involve steric hindrance of the label and of the amide bond itself. Second, the possibilities of tertiary amide bonds to exist in two slowly interconvertable rotamers may influence the conformation and hybridization properties of the modified PNA. Finally, overacylation by reactions with unprotected C residues has occasionally been observed.

To overcome the problems associated with the amide bond chemistry described above, a new strategy for the postsynthetic backbone-labeling of acpcPNA based on reductive alkylation and sequential reductive alkylation-click chemistry is developed here (Scheme 1). The applicability of this strategy for the labeling of acpcPNA with an environmentally sensitive label, including pyrene and thiazole orange, and that these labeled acpcPNAs can behave as self-reporting fluorescence hybridization probes, is demonstrated. Although performed in a limited context, this study should reveal the potential of reductive alkylation and its combination with other more established chemistries for the postsynthetic modification of other classes of biomolecules.

## EXPERIMENTAL PROTOCOL

**General.** 1-Ethynylpyrene (**aPy**) was obtained from Alfa Aesar (USA). All other chemicals were purchased from Fluka, Merck, or Aldrich Chemical Co., Ltd., and were used as received without further purification. Commercial grade solvents for column chromatography were distilled before use. Solvents for reactions and crystallization were reagent grade and used without purification. Methanol for HPLC use was HPLC grade, obtained from BDH, and filtered through a 13 mm diameter 0.45 μm membrane filter (Nylon Lida) before use. Anhydrous *N*,*N*-dimethylformamide ($H_2O \leq 0.01\%$) for solid phase peptide synthesis was obtained from RCI LabScan. Milli-Q water was obtained from a Millipore (USA) ultrapure water system with a 0.22 μm Millipak 40 filter unit. All oligonucleotides were purchased from the Bioservice Unit, National Science and Technology Development Agency (Thailand).

$^1$H and $^{13}$C NMR spectra were recorded in deuterated solvents using a Bruker Avance 400 NMR instrument operating at 400 MHz ($^1$H) and 100 MHz ($^{13}$C), respectively. Chemical shifts ($\delta$) are reported in parts per million (ppm) relative to tetramethylsilane (TMS) or using the residual protonated solvent signal as a reference ($CDCl_3$ $\delta$ 7.27, DMSO-$d_6$ $\delta$ 2.50 ppm). Coupling constants ($J$) are for proton−proton coupling unless otherwise noted and are reported in hertz (Hz). Multiplicities were abbreviated as follows: singlet (s), doublet (d), triplet (t), quartet (q). Splitting patterns that could not be interpreted or easily visualized are designated as multiplet (m) or broad (br).

**Synthesis of Alkyne-Modified Thiazole Orange (aTO).** *4-Methyl-1-(prop-2-ynyl)quinolinium Bromide (**aLP**).* A solution of lepidine (429.6 mg, 3.0 mmol) and propargyl bromide (80% (w/w) in toluene, 420 μL) (3.75 mmol, 1.25 equiv) in MeCN (5 mL) was stirred for 3 days at room temperature. The resulting precipitate was collected by filtration and dried under vacuum to afford the desired product as a light-gray solid (507.1 mg, 64.5%). $^1$H NMR (400 MHz, DMSO-$d_6$) $\delta$ (ppm): 3.04 (3H, s, $CH_3$), 4.00 (1H, s, CH), 6.05 (2H, s, $CH_2$), 8.09 (1H, t, $J$ = 7.7 Hz, ArH), 8.15 (2H, d, $J$ = 6.1 Hz, ArH), 8.34 (1H, d, $J$ = 9.8 Hz, ArH), 8.57 (2H, d, $J$ = 9.8 Hz, ArH), 9.60 (1H, d, $J$ = 6.1 Hz, CH); $^{13}$C NMR (100 MHz, DMSO-$d_6$) $\delta$ (ppm): 160.1, 148.1, 136.4, 135.3, 129.9, 128.9, 127.3, 122.9, 119.4, 81.2, 75.7, 46.8, 19.9; IR (ATR) $\nu_{max}$($cm^{-1}$): 3153, 2916, 2114, 1615, 1526; HRMS (ESI+): $m/z$ calcd for $C_{13}H_{12}N^+$: 182.0970 $[M]^+$ found: 182.1004.

*Alkyne-Modified Thiazole Orange (**aTO**).* Triethylamine (0.24 mL, 1.65 mmol) was added to a suspension of 3-methyl-2-(methylthio)-benzothiazolium *p*-toluenesulfonate[35] (242.6


dx.doi.org/10.1021/bc3005914 | *Bioconjugate Chem.* 2013, 24, 614−625

mg, 0.66 mmol) and 4-methyl-1-(prop-2-ynyl)quinolinium bromide (172.9 mg, 0.66 mmol) in $CH_2Cl_2$ (4 mL). The red mixture was stirred under exclusion of light at room temperature for 16 h. All volatiles were removed under reduced pressure. The residue was then suspended in 5 mL of MeOH and the solution was stored at 4 °C for 24 h. The resulting precipitate was collected by filtration and dried under reduced pressure to afford the desired product as a red solid (154.7 mg, 57.3%). $^1$H NMR (400 MHz, DMSO-$d_6$) $\delta$ (ppm): 3.75 (1H, s, C≡CH), 4.05 (3H, s, $CH_3$), 5.50 (2H, s, $CH_2$), 6.95 (1H, s, CH), 7.35 (1H, d, $J$ = 7.2 Hz, ArH), 7.45 (1H, t, $J$ = 7.6 Hz, ArH), 7.60 (1H, t, $J$ = 7.8 Hz, ArH), 7.75 (1H, t, $J$ = 7.6 Hz, ArH), 7.85 (1H, d, $J$ = 8.4 Hz, ArH), 8.00 (1H, t, $J$ = 7.8 Hz, ArH), 8.10 (2H, d, $J$ = 9.2 Hz, ArH), 8.60 (1H, d, $J$ = 7.2 Hz, ArH), 8.80 (1H, d, $J$ = 8.4 Hz, ArH; $^{13}$C NMR (100 MHz, DMSO-$d_6$) $\delta$ (ppm): 161.0, 149.4, 148.6, 143.5, 136.7, 133.2, 128.3, 126.8, 125.7, 124.8, 124.2, 124.0, 122.9, 118.1, 113.3, 107.6, 89.0, 78.9, 77.1, 43.6, 34.1; IR (ATR) $\nu_{max}$ (cm$^{-1}$): 3120, 2922, 2111, 1616, 1506; HRMS (ESI+): $m/z$ calcd for $C_{21}H_{17}N_2S^+$: 329.1112 $[M]^+$ found: 329.1138.

**Synthesis of APC-Modified acpcPNA.** The APC-modified acpcPNA was synthesized manually on Tentagel S-RAM resin from the respective Fmoc-protected monomers at a 1.5 $\mu$mol scale according to the previously published protocol.[26,34] Lysine was always included at the *C*-termini to improve water solubility. After completion of the synthesis, the *N*-terminal Fmoc group was removed and the free amino group was capped by acetylation. The acpcPNA on the solid support was split into 0.5 $\mu$mol portions for further labeling experiments.

**Modification of acpcPNA by Reductive Alkylation on Solid Support.** The trifluoroacetyl (Tfa) protecting group on the APC residue, as well as the nucleobase side chain protecting groups (Bz, Ibu), was first removed by treatment of the resin-supported fully protected APC-modified acpcPNA with 1:1 aqueous ammonia-dioxane at 60 °C overnight. Each deprotected APC-modified acpcPNA (0.5 $\mu$mol) was treated with the appropriate aldehyde (15 $\mu$mol, 30 equiv) in the presence of $NaBH_3CN$ (30 $\mu$mol, 60 equiv) and HOAc (30 $\mu$mol, 60 equiv) in MeOH (100 $\mu$L) at room temperature overnight. The progress of the reaction was monitored by MALDI-TOF mass spectrometry (MS) after cleavage of a small portion of the acpcPNA from the solid support.

**Modification of acpcPNA by Sequential Reductive Alkylation-Click Chemistry on Solid Support.** The resin-supported, fully side-chain deprotected APC-modified acpcPNAs (0.5 $\mu$mol) obtained as above was treated with 4-azidobutanal[36] (15 $\mu$mol, 30 equiv) in the presence of $NaBH_3CN$ (30 $\mu$mol, 60 equiv) and HOAc (30 $\mu$mol, 60 equiv) in MeOH (100 $\mu$L) at room temperature overnight. This azide-modified acpcPNA, still on the solid support, was reacted with 1-ethynylpyrene (**aPy**) or *N*-propargylated thiazole orange (**aTO**) (7.5 $\mu$mol, 15 equiv) in the presence of tris[(1-benzyl-1*H*-1,2,3-triazol-4-yl)methyl]amine[37] (TBTA, 30 $\mu$mol, 60 equiv), tetrakis(acetonitrile) copper(I) hexafluorophosphate (15 $\mu$mol, 30 equiv) in 3:1 (v/v) DMSO:$^t$BuOH and (+)-sodium-L-ascorbate (60 $\mu$mol, 120 equiv) in $H_2O$ at room temperature overnight.[8] The progress of the reaction was monitored by MALDI-TOF MS after cleavage of a small portion of the acpcPNA from the solid support.

**Cleavage and Purification.** The acpcPNA was cleaved from the solid support by treatment with trifluoroacetic acid (TFA) (500 $\mu$L, 30 min). The procedure was repeated three times. The combined TFA washing was evaporated under a stream of nitrogen and the acpcPNA was precipitated by the addition of diethyl ether. After washing with more diethyl ether and air-dried, the crude acpcPNA was dissolved in 120 $\mu$L water, filtered through a 0.45 $\mu$m membrane and purified by reverse-phase HPLC. Reverse phase HPLC experiments were performed on Water Delta 600 system equipped with gradient pump and Water 996 photodiode array detector. An ACE $C_{18}$ HPLC column 4.6 × 150 mm, 5 $\mu$m particle size was used for both analytical and semipreparative purposes. Peak monitoring and data processing were performed using the Waters Empower software. Fractions from HPLC were collected manually, with selection of peaks being assisted by real-time HPLC chromatogram monitoring. The fractions containing the desired acpcPNA were combined and freeze-dried to obtain the purified acpcPNA, which was characterized by MALDI-TOF MS on a Microflex MALDI-TOF mass spectrometer (Bruker Daltonics) using $\alpha$-cyano-4-hydroxycinnamic acid (CCA) as the matrix.

**Fluorescence Experiments.** Evaluation of the probe-DNA binding fluorescence, including monitoring of the S1 nuclease digestion to determine if the probe bound specifically or not (vide infra), were performed on a Cary Eclipse Fluorescence Spectrophotometer (Agilent Technologies) using 5 nm excitation and emission slits, auto setting for the excitation and emission filters, and smoothing set to moving average.

**S1 Nuclease Digestion.** The sample for digestion by *Aspergillus oryzae* S1 nuclease (Sigma-Aldrich) was prepared by mixing the calculated amounts of stock oligonucleotide and acpcPNA solution together in 30 mM sodium acetate buffer (pH 4.6) containing 1.0 mM zinc acetate, 5% (v/v) glycerol to a final volume of 500 $\mu$L in a 10 mm quartz cell. The enzyme stock (1000 units (U)/mL, 5 $\mu$L) was added to give the final concentration of 5 U/mL. The progress of the enzymatic digestion was monitored by fluorescence spectrophotometry (kinetic mode).

## RESULTS AND DISCUSSION

**Functionalization of acpcPNA by Reductive Alkylation.** Initial attempts to directly alkylate the APC-modified acpcPNA on the solid support by propargyl bromide as a model alkyl halide in the presence of various bases/solvents were not successful. In all cases MALDI-TOF MS analyses of the products revealed the presence of both unreacted acpcPNA and the bis-propargylated product in addition to the desired monopropargylated acpcPNA. On the other hand, less reactive alkyl halides, such as homopropargyl tosylate, did not react at all. Accordingly, we turned our attention to a reductive alkylation approach, which has been used with considerable success on the solid phase,[38−40] including for the synthesis of aegPNA.[41] It was, however, unknown from the literature whether the reductive alkylation will be compatible with the unprotected amino groups of the nucleobases C, A, and G. A model reductive alkylation reaction was, therefore, performed on a short APC-modified acpcPNA tetramer (GCTA) in the presence of butyraldehyde (30 equiv), $NaBH_3CN$ (60 equiv), and HOAc (60 equiv) in methanol. Subsequent MALDI-TOF MS analyses revealed that the reductive alkylation proceeded cleanly, as shown by the disappearance of the mass signal of the starting acpcPNA (calcd. $m/z$ 1537.7; found 1536.8), accompanied by a new peak corresponding to the product (calcd. $m/z$ 1594.7; found 1593.4) after 12 h. No peaks corresponding to side-products derived from quaternization or


dx.doi.org/10.1021/bc3005914 | *Bioconjugate Chem.* 2013, 24, 614−625

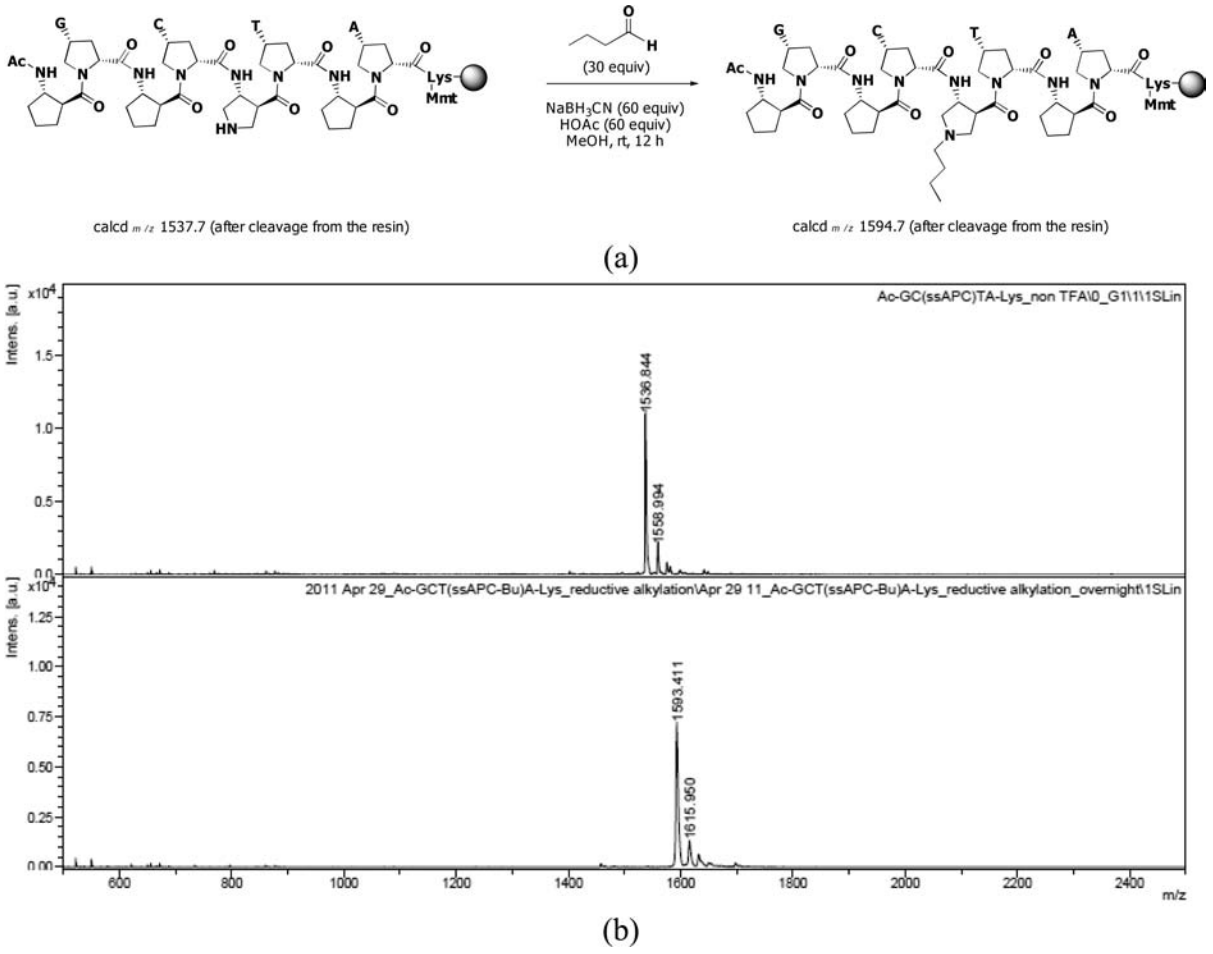



**Figure 1.** Reductive alkylation reaction of an acpcPNA tetramer: (a) Reaction scheme and (b) MALDI-TOF MS results of the acpcPNA before (top) and after (bottom) the reductive alkylation.

**Table 1. Percentage Yields, Mass (MALDI-TOF MS), and $T_m$ Data of 10mer acpcPNA (M10) with Various Labels Obtained via the Reductive Alkylation Process Described Herein**

| entry | PNA[a] | aldehyde | yield[b] | $m/z$ (calcd) | $m/z$ (found) | $T_m$ (°C)[c] |
|---|---|---|---|---|---|---|
| 1 | **M10-Me** | HCHO | 19.3 | 3574.8 | 3575.4 | 58.2[d] |
| 2 | **M10-HE** | $HOCH_2CHO$ dimer | 1.6 | 3604.8 | 3604.0 | 58.7[d] |
| 3 | **M10-AE** | $FmocNHCH_2CHO$[e] | 14.6 | 3604.7 | 3603.7 | 65.4[d] |
| 4 | **M10-C4azide** | $N_3(CH_2)_3CHO$ | 9.7 | 3657.8 | 3656.0 | 57.7[d] |
| 5 | **M10-C3alkyne** | 4-pentyn-1-al | 17.9 | 3626.8 | 3626.3 | 59.7[d] |
| 6 | **M10-PyB** | pyrene-1-ylbutyraldehyde | 2.9 | 3816.1 | 3816.9 | 49.4[f] |
| 7 | **M10-PyPm** | 4-(pyrene-yl)-benzaldehyde | 7.2 | 3850.1 | 3852.5 | 46.4[f] |
| 8 | **M10-PyPn** | 5-(pyrene-1-yl)pentynal | 2.7 | 3828.2 | 3831.6 | 51.4[f] |

[a]For the PNA sequences and definition of the labels see Scheme 1. [b]Isolated yield after HPLC purification based on the scale of the synthesis. [c]With complementary DNA (5′-AGTGATCTAC-3′). [d]Condition: 10 mM sodium phosphate buffer pH 7.0, [PNA] = 1.0 $\mu$M and [DNA] = 1.2 $\mu$M. [e]The Fmoc group was removed by a brief treatment with 20% (w/v) piperidine in DMF prior to cleavage of the acpcPNA from the solid support. [f]Conditions: 10 mM sodium phosphate buffer pH 7.0, [PNA] = 2.5 $\mu$M and [DNA] = 3.0 $\mu$M.

alkylation of the exocyclic amino groups of nucleobases could be observed (Figure 1).

Encouraged by the above results and to demonstrate the applicability of this reductive alkylation strategy, a series of backbone-modified acpcPNAs was prepared starting from the same mix-base 10mer acpcPNA (**M10**) and various aldehydes, as shown in Table 1. The reaction proceeded well with most aliphatic and aromatic aldehydes tested, including functionalized aldehydes, such as glycoaldehyde, Fmoc-aminoacetaldehyde, 4-azidobutanal, and 4-pentyn-1-al, as well as with various pyrene-substituted aldehydes. The low isolated yields obtained (ranging from 1.6% to 19%) were attributed to the inefficiency of the acpcPNA synthesis and HPLC purification process rather than the labeling procedure (vide infra), which were shown to be quantitative by MALDI-TOF MS and HPLC analyses in most cases. An exception was observed in the case of **M10-HE**, where the reaction required an elevated temperature and was accompanied by unknown side-reactions.

The modification of acpcPNA by the present reductive alkylation strategy resulted in a tertiary amino linkage, which


dx.doi.org/10.1021/bc3005914 | *Bioconjugate Chem.* 2013, 24, 614−625

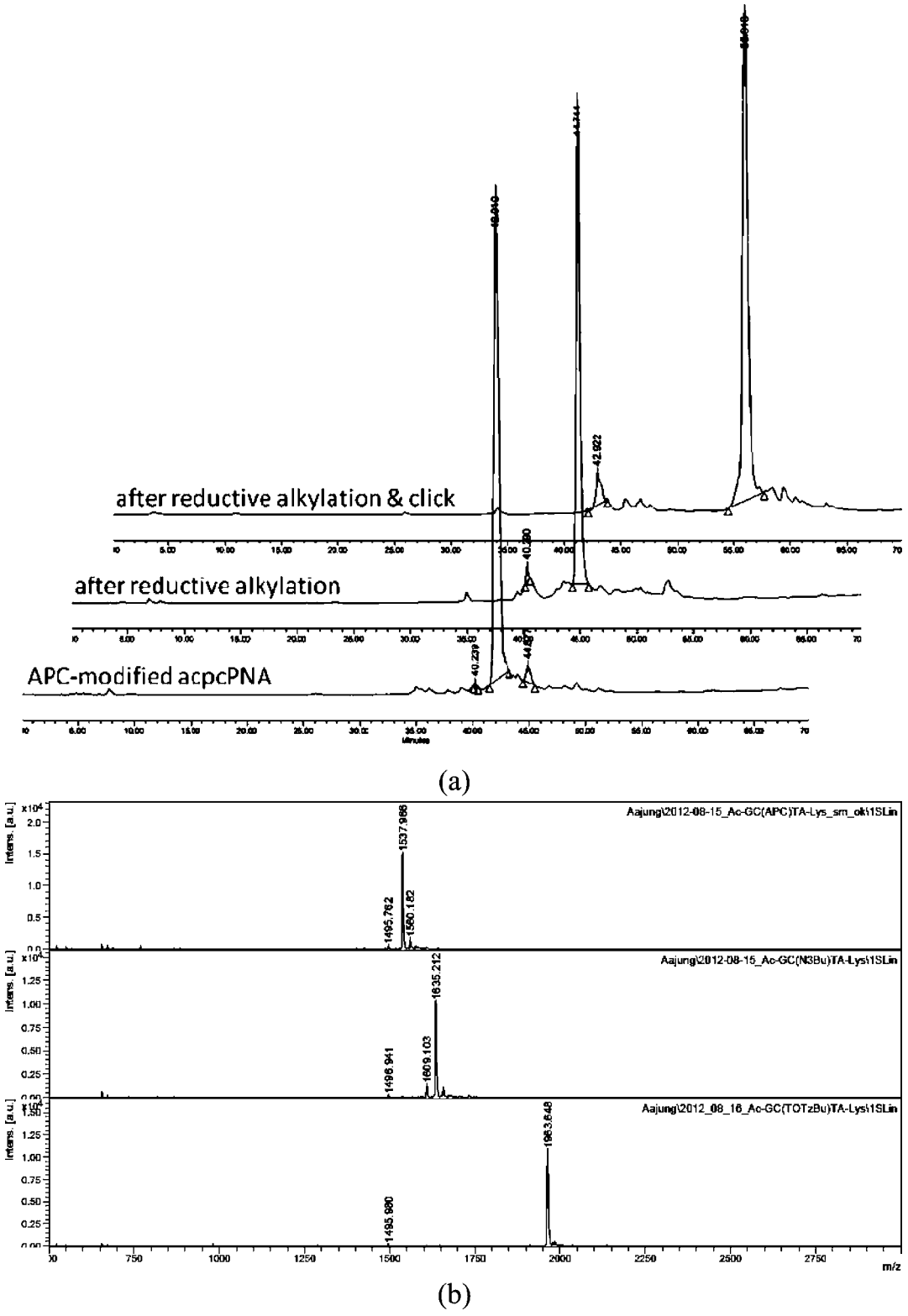



**Figure 2.** (a) Analytical HPLC chromatograms (254 nm) and (b) MALDI-TOF MS spectra of the reductive alkylation-click chemistry of the APC-modified acpcPNA tetramer before (top), after reductive alkylation with azidobutanal (middle), and then after click reaction with **aTO** (bottom). The peaks at 40.2 and 42.9 min in the HPLC chromatogram after the reductive alkylation and click reactions, respectively, were confirmed to not be the unreacted acpcPNA by MALDI-TOF MS analysis.

does not cause as much destabilization of the acpcPNA·DNA hybridization as observed in modification via tertiary amide linkage.[34] For example, modification of acpcPNA with small labels like methyl (**M10-Me**) and hydroxyethyl (**M10-HE**) resulted in only slightly lower $T_m$ values (**M10-Me**: 58.2 °C; **M10-HE**: 58.7 °C) compared to the unmodified (60.2 °C) or APC-modified (59.3 °C) acpcPNAs under the same conditions (at 1 $\mu$M acpcPNA, without salt). The hybrid of **M10-HE** and


dx.doi.org/10.1021/bc3005914 | *Bioconjugate Chem.* 2013, 24, 614−625

**Table 2. Percentage Yields and Mass (MALDI-TOF) Data of the Pyrene- and Thiazole Orange-Labeled acpcPNAs Obtained via the Sequential Reductive Alkylation-Click Reaction**

| entry | PNA[a] | modifiers | yield[b] | $m/z$ (calcd) | $m/z$ (found) |
|---|---|---|---|---|---|
| 1 | **T9-C4TzPy** | $N_3(CH_2)_3CHO$, **aPy** | 12.7 | 3504.1 | 3504.1 |
| 2 | **T9-C4TzTO** | $N_3(CH_2)_3CHO$, **aTO** | 8.3 | 3608.5 | 3605.5 |
| 3 | **M10-C4TzPy** | $N_3(CH_2)_3CHO$, **aPy** | 4.6 | 3884.1 | 3883.3 |
| 4 | **M10-C4TzTO** | $N_3(CH_2)_3CHO$, **aTO** | 5.2 | 3987.4 | 3984.4 |
| 5 | **M12-C4TzTO** | $N_3(CH_2)_3CHO$, **aTO** | 3.1 | 4613.1 | 4612.6 |
| 6 | **M10-C3NTzPy** | 4-pentyn-1-al, 1-azidopyrene | 3.0 | 3870.1 | 3870.0 |
| 7 | **M10-AEPy** | $FmocNHCH_2CHO$, pyrene-1-COOH | 9.9 | 3832.9 | 3831.7 |

[a]For the PNA sequences and definition of the labels see Scheme 1. [b]Isolated yield after HPLC purification based on the scale of the synthesis.

mismatched DNA 5′-AGTGTTCTAC-3′ gave a substantially lower $T_m$ (34.0 °C). The acpcPNA **M10-AE** with the aminoethyl substituent gave a $T_m$ of 65.4 °C which is 5.2 °C higher than the unmodified acpcPNA. This could be explained by the additional stabilizing electrostatic interaction between the protonated amino side-chain and the negatively charged phosphate groups on DNA. A rather high $T_m$ (41.5 °C) was also observed in the hybrid of **M10-AE** with the mismatched DNA sequence 5′-AGTGTTCTAC-3′, suggesting that the stabilization was nonspecific. This is fully consistent with the explanation above. Considerable destabilization was observed in the modified acpcPNAs with more bulky labels, such as pyrenebutyl (**M10-PyB**) that had a $T_m$ of 49.4 °C compared to 62.4 °C for the unmodified and 61.4 °C for the APC-modified 10mer acpcPNA (at 2.5 $\mu$M acpcPNA, without salt). Nevertheless, this $T_m$ value is still higher than the 45.7 °C for pyrenebutyryl-modified acpcPNA prepared via the acylation approach,[34] From these results, it can be concluded that reductive alkylation is an effective method for attaching various labels onto the acpcPNA backbone without adversely affecting the DNA hybridization properties. The compatibility of the reaction with functional groups, such as azide, alkyne, and protected amino groups, offers opportunities for further functionalization via click or acylation chemistries (vide infra).

**Modification of acpcPNA by Sequential Reductive Alkylation-Acylation and Reductive Alkylation-Click Chemistry.** In the reductive alkylation strategy presented here, the basic requirement is that the label must possess an aldehyde group. Although the commercial availability and shelf life of aldehyde-containing labels are limited, the ability to add reactive groups, such as amino or azide/alkyne groups, allows secondary modification via amide bond formation or click chemistry. Due to the popularity of these two labeling approaches, most labels are readily available in either or both carboxyl or clickable forms, and so the range of labels that can be added via this strategy is virtually unlimited. As an example, on additional modification by acylation chemistry, we demonstrated that the aminoethylated acpcPNA **M10-AE** could be further acylated with pyrene-1-carboxylic acid (activated as pentafluorophenyl ester) to give the expected pyrene-modified acpcPNA **M10-AEPy** ($m/z$ calcd. 3832.9, found 3831.7) in 9.9% isolated yield. It should be noted that the modification of the amino-functionalized acpcPNA in this way does not suffer from steric effects and conformational restrictions because the amide bond is not directly attached to the acpcPNA backbone. Accordingly, the $T_m$ of **M10-AEPy** (56.4 °C) was only 3.8 °C lower than that for the unmodified acpcPNA (60.2 °C) under similar conditions (1 $\mu$M acpcPNA, without salt).

The widespread use of click chemistry for the modification of biological molecules[5,6] has resulted in an extensive development of alkyne and azide-functionalized labeling groups, many of which are commercially available or easily synthesized. In order to demonstrate the applicability of this sequential reductive alkylation-click strategy, the APC-modified acpcPNA tetramer GCTA (the APC residue was placed between the two adjacent bases marked by the underscore) was first reductively alkylated with the readily available 4-azidobutanal.[36] The azide-functionalized acpcPNA was then clicked with an alkyne-modified thiazole orange (**aTO**) (15 equiv) in the presence of the Cu(I)-TBTA complex[37] to give the thiazole orange-modified acpcPNA tetramer. Both steps were conducted while the acpcPNA was still on the solid support. The progress of each reaction was monitored by MALDI-TOF MS and HPLC analyses, which confirmed the quantitative nature of both the reductive alkylation and the click steps (Figure 2). No traces of the starting materials (APC-modified acpcPNA and azide-modified acpcPNA) could be detected by both MALDI-TOF MS and HPLC analyses, and therefore both reactions were estimated to be >95% complete. Since both reactions proceeded essentially to completion overnight with a single coupling reaction for each step, no attempts were made to further optimize the reaction conditions. Following this sequential reductive alkylation-click chemistry on-solid-support, other thiazole orange- and pyrene-modified acpcPNAs were similarly synthesized (Table 2). Some representative MALDI-TOF analyses results of the crude reaction products from both the reductive alkylation and click steps are shown in Figures S22−S25 in the Supporting Information.

In order to explore the possibilities of the alternative click reaction via alkyne-functionalized acpcPNA, the APC-modified 10mer acpcPNA **M10** was first reductively alkylated with 4-pentyn-1-al. Then the subsequent on-solid-support click reaction with 1-azidopyrene gave the pyrene-modified acpcPNA **M10-C3NTzPy**, in which the pyrene moiety was linked to the triazole nitrogen atom, rather than the carbon as in **M10-C4TzPy**. For unknown reasons, this "inverse" click reaction (alkyne on the acpcPNA, azide on the label) could not be forced to completion. The mass peak corresponding to the starting **M10-C3alkyne** peak remained observable even after prolonged/repeated click reactions (Figure S25). Nevertheless, the desired pyrene-clicked acpcPNA could be isolated in an acceptable yield (3%) after reverse phase HPLC purification.

The seemingly relatively low isolated yields of the labeled acpcPNAs obtained in this work (3−13%) are, however, similar to previous reports on labeled PNA and DNA syntheses of comparable length and complexity.[8,32,35] Indeed, such low obtained yields are typical for solid phase synthesis and may be attributed to three factors: (i) the efficiency of the acpcPNA


dx.doi.org/10.1021/bc3005914 | Bioconjugate Chem. 2013, 24, 614−625

Table 3. Thermal Stability Data of Complementary and Single Mismatched DNA:Probe Hybrids for the Pyrene- and Thiazole Orange-Labeled acpcPNAs Obtained via the Sequential Reductive Alkylation-Click Reaction

| entry | PNA[a] | DNA[b] | $T_m$ (°C)[c] | $\Delta T_m$ (°C)[d] | note |
|---|---|---|---|---|---|
| 1 | **T9-C4TzPy** | **D9comp** | 67.6 | −12.4 | complementary |
| 2 | **M10-C4TzPy** | **D10comp** | 55.6 | −4.6 | complementary |
| 3 | **M10-C3NTzPy** | **D10comp** | 51.1 | −9.1 | complementary |
| 4 | **T9-C4TzTO** | **D9comp** | 75.8 | −4.2 | complementary |
| 5 | **T9-C4TzTO** | **D9smT** | 47.5 | −32.5 | single mismatched |
| 6 | **M10-C4TzTO** | **D10comp** | 60.3 | +0.1 | complementary |
| 7 | **M10-C4TzTO** | **D10smT** | 38.0 | −22.2 | single mismatched |
| 8 | **M10-C4TzTO** | **D10smC** | 37.2 | −23.0 | single mismatched |
| 9 | **M10-C4TzTO** | **D10smG** | 26.3 | −33.9 | single mismatched |
| 10 | **M10-C4TzTO** | **D10non1** | <20 | - | noncomplementary |
| 11 | **M10-C4TzTO** | **D10non2** | <20 | - | noncomplementary |
| 12 | **M10-C4TzTO** | **D11non** | <20 | - | noncomplementary |
| 13 | **M12-C4TzTO** | **D12comp** | 74.5 | −0.3 | complementary |
| 14 | **M12-C4TzTO** | **D12smA** | 58.6 | −16.2 | single mismatched |
| 15 | **M12-C4TzTO** | **D12smC_3′** | 59.8 | −15.0 | indirect single mismatched |
| 16 | **M12-C4TzTO** | **D12smC_5′** | 55.7 | −19.1 | indirect single mismatched |
| 17 | **M12-C4TzTO** | **D11non** | <20 | - | noncomplementary |

[a]For the PNA sequences and definition of the labels see Scheme 1. [b]DNA sequence (5′→3′): **D9comp** = dAAAAAAAAA; **D9smT** = dAAAATAAAA; **D10comp** = dAGTGATCTAC; **D10smT** = dAGTGTTCTAC; **D10smC** = dAGTGCTCTAC; **D10smG** = dAGTGGTCTAC; **D10non1** = dTATCATGTAT; **D10non2** = dTCTGAATTTA; **D11non** = TCTGCATTTAG ; **D12comp** = dGCAGGGATAACT; **D12smA** = dGCAGGAATAACT; **D12smC_3′** = dGCAGGGCTAACT, **D12smC_5′** = dGCAGCGATAACT. [c]Conditions: 10 mM sodium phosphate buffer pH 7.0, [PNA] = 1.0 $\mu$M and [DNA] = 1.2 $\mu$M. [d]Relative to unmodified acpcPNA under similar conditions. $T_m$ of hybrids with complementary DNA; Ac-TTTTTTTTT-Lys (80.0 °C), Ac-GTAGATCACT-Lys (60.2 °C), Ac-AGTTATCCCTGC-Lys (74.8 °C).

synthesis, (ii) the efficiency of the labeling and cleavage from the solid support, and (iii) loss during the HPLC purification. In an attempt to delineate these three effects, the synthesis of the TO-labeled acpcPNA tetramer Ac-GCTA-Lys$NH_2$ was repeated and compared to the unmodified acpcPNA (Table S1). The efficiency of the acpcPNA synthesis, as measured from the Fmoc deprotection, was between 78% and 80%, which translates to an ∼97% coupling efficiency per coupling step, which is consistent with our previous work.[26] The synthesis efficiency figure will obviously decrease with the length of the acpcPNA (calculated values for 10-, 11-, and 12-mers were 54%, 51%, and 48%, respectively). From the overall isolated yields of 55% and 35% for the unlabeled and TO-labeled acpcPNA, respectively, the combined efficiency of the labeling and cleavage and the HPLC purification was calculated to be 69% and 45.5%, respectively. Assuming quantitative TO-labeling (as suggested by HPLC and MALDI-TOF analyses of the crude products) and similar cleavage yields, these figures can be taken to represent the HPLC recovery yields. The lower HPLC recovery yield of the TO-labeled acpcPNA is then attributed to the tendency of hydrophobic labels to be retained on the HPLC column.

As an alternative to the on-solid-support click reaction, the intermediate azide- and alkyne-modified acpcPNAs, such as **M10-C4azide** or **M10-C3alkyne**, could be cleaved from the solid support and purified by HPLC (see Table 1) for further clicking in the solution phase. This may be useful in situations where the click reaction could not be performed on the solid support, for example, with macromolecular or acid-sensitive clicking partners.

**Thermal Stability and Fluorescence Properties of the Reductively Alkylated-Clicked acpcPNA.** The thermal stabilities of DNA hybrids of the reductively alkylated-clicked acpcPNAs were investigated by UV spectrophotometry, monitoring at 260 nm, and the results are summarized in Table 3. The $T_m$ values for the complementary DNA hybrids of the pyrene-modified acpcPNAs were lowered by 4.6 to 12.4 °C compared to the respective unmodified acpcPNAs. On the other hand, the TO-modified acpcPNAs gave comparable or only slightly lower $T_m$ values relative to the unmodified acpcPNAs ($\Delta T_m$ −4.2 to +0.1 °C). This suggests that the TO label contributes to additional stabilization of the acpcPNA·DNA hybrid, possibly by intercalation or minor groove binding analogous to the binding of TO to double-stranded DNA.[42−44] This stabilization is sufficient to compensate for the destabilization due to steric bulkiness of the dye. All single mismatched acpcPNA·DNA hybrids gave a substantially lower $T_m$ than the complementary hybrids ($\Delta T_m$ −14.7 to −34.0 °C) indicating that the base pairing specificity in the labeled acpcPNA system was still maintained.

In order to investigate the potential of these acpcPNAs as hybridization-responsive fluorescent DNA probes, the fluorescence experiments of the representative 10mer acpcPNAs modified with pyrene and thiazole orange (**M10-PyB**, **M10-PyPm**, **M10-PyPn**, **M10-C4TzPy**, **M10-C3NTzPy**, and **M10-C4TzTO**) were performed. The fluorescence spectra of the labeled acpcPNAs upon excitation at 345 nm (pyrene labels) or 508 nm (TO labels) measured before and after addition of the complementary DNA are shown in Figure 3. In all cases, the fluorescence spectra of the acpcPNA in the single-stranded and duplex forms were quite different. For the pyrene-labeled acpcPNAs, moderate fluorescence increases were observed upon hybridization with DNA when the pyrene was linked through a relatively flexible linker (**M10-PyB** and **M10-PyPn**) at 5.5-fold and 3-fold higher than that in the single-stranded forms, respectively (Figure 3a,c). Other pyrene-labeled acpcPNAs gave a less desirable fluorescence quenching upon hybridization with DNA. However, the largest fluorescence increase was observed with the TO-labeled **M10-C4TzTO** (Figure 3f) in which the duplex state showed a very strong


dx.doi.org/10.1021/bc3005914 | Bioconjugate Chem. 2013, 24, 614−625

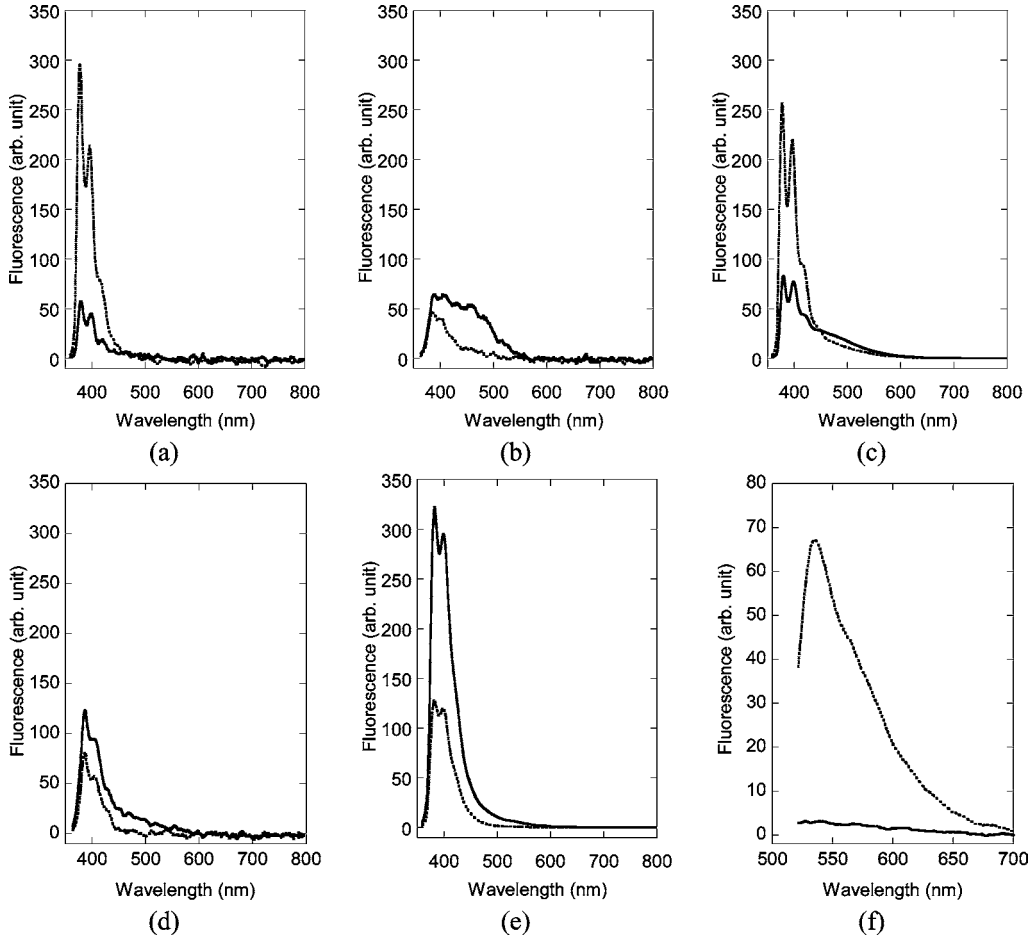



**Figure 3.** Fluorescence spectra of (a) **M10-PyB**, (b) **M10-PyPm**, (c) **M10-PyPn**, (d) **M10-C4TzPy**, (e) **M10-C3NTzPy**, and (f) **M10-C4TzTO** in the absence (——) or presence (---) of complementary DNA (**D10comp**). Conditions: 2.5 $\mu$M acpcPNA, 3.0 $\mu$M DNA, 10 mM sodium phosphate buffer pH 7.0, excitation wavelength = 345 nm (**M10-PyB**, **M10-PyPm**, **M10-PyPn**, **M10mer-C4TzPy**, and **M10-C3NTzPy**) or 508 nm (**M10-C4TzTO**).

increase in fluorescence at 535 nm (ca. 22-fold) upon hybridization with complementary DNA. The increased fluorescence is likely to be due to the restriction of internal rotation of the thiazole orange upon intercalating/groove binding to the acpcPNA·DNA duplex.[43,44]

**Fluorescence Enhancement of TO-Modified acpcPNA Probes: Specific vs Nonspecific DNA Binding.** Since the TO-labeled acpcPNA **M10-C4TzTO** showed the largest fluorescence increase upon hybridization with DNA, it was chosen for further investigation of the binding specificity. Although the $T_m$ measurement suggests that the binding of TO-labeled acpcPNAs to DNA is highly specific, as shown by the large decrease in $T_m$ (−22.2 to −33.9 °C) relative to the complementary pair, the fluorescence spectra of various mismatched hybrids also showed a disappointingly large fluorescence increase. In one case (hybrid with **D10smT**), the fluorescence increase even exceeded that of the complementary hybrid (Figure S26a and Table S2). Non-complementary DNA gave a lower, but not negligible, fluorescence increase despite the absence of duplex formation according to UV melting analysis results (Table 3, entries 10 and 11). These facts, together with the known ability of free TO to bind to single-stranded DNA that results in a sequence-dependent increase in its fluorescence,[43] suggest that the enhanced fluorescence in the presence of noncomplementary DNA targets originated from the nonspecific binding of thiazole orange (positively charged) to single-stranded DNA (negatively charged) without the involvement of the acpcPNA part. This proposal is also supported by the significant red-shift and hypochromism of the TO absorption peaks in the presence of both complementary and noncomplementary DNA, where the $\lambda_{max}$ of **M10-C4TzTO** without DNA was 510 nm compared to 512−518 nm with DNA (Figure S26b), and also by the fact that the **M10-PyB** with an uncharged pyrene label showed excellent discrimination between complementary and mismatched DNA targets (Table S2). Attempts to increase the hybridization stringency by increasing the temperature, changing the salt concentration, and using different buffers could not completely eliminate the nonspecific fluorescence increase. Whether the observed increase in the fluorescence upon **M10-C4TzTO** binding to the complementary DNA target was due to the same nonspecific binding mechanism or more specific interactions that require the presence of a duplex, such as intercalation or groove binding, was then evaluated by S1 nuclease digestion.

**S1 Nuclease Digestion Improves the Specificity of TO-Modified acpcPNA Probes.** S1 nuclease specifically hydrolyzes single-stranded DNA, and fully complementary aegPNA·DNA duplexes are much more stable toward degradation by S1 nuclease than aegPNA·DNA duplexes containing mismatched bases.[45−47] Therefore, S1 nuclease digestion should be able to differentiate whether the enhanced


dx.doi.org/10.1021/bc3005914 | *Bioconjugate Chem.* 2013, 24, 614−625

**Table 4. Fluorescence Ratio of TO-Modified acpcPNA in the Presence and Absence of DNA ($F/F_0$) before and after S1 Nuclease Digestion[b,c]**

| PNA[a] | DNA[b] | $F/F_0$[c] before digestion | $F/F_0$[c,d] after digestion | $\frac{(F/F_0)_{comp}}{(F/F_0)_{mm}}$ before digestion | $\frac{(F/F_0)_{comp}}{(F/F_0)_{mm}}$ after digestion |
|---|---|---|---|---|---|
| **T9-C4TzTO** | **D9comp** | 25.7 | 20.3 | - | - |
| | **D9smT** | 34.6 | 1.6 | 0.8 | 12.7 |
| | **D9smC** | 45.5 | 2.9 | 0.6 | 7.0 |
| | **D9smG** | 30.7 | 1.5 | 0.8 | 13.5 |
| **M10-C4TzTO** | **D10comp** | 43.6 | 10.9 | - | - |
| | **D10smT** | 24.7 | 1.6 | 1.8 | 6.8 |
| | **D10smC** | 17.4 | 1.4 | 2.5 | 7.8 |
| | **D10smG** | 11.6 | 1.3 | 3.8 | 8.4 |
| | **D11non** | 17.9 | 1.0 | 2.4 | 10.9 |
| **M12-C4TzTO** | **D12comp** | 83.6 | 49.0 | - | - |
| | **D12smA** | 53.6 | 3.4 | 1.6 | 14.4 |
| | **D12smC_3′** | 41.5 | 1.9 | 2.0 | 25.8 |
| | **D12smC_5′** | 83.8 | 2.0 | 1.0 | 24.5 |
| | **D11non** | 16.7 | 0.7 | 5.0 | 70.0 |

[a]For the PNA sequences and definition of the labels see Scheme 1. [b]DNA sequence (5′→3′), mismatched base specified by underlining: **D9comp** = dAAAAAAAAA; **D9smT** = dAAAATAAAA; **D9smC** = dAAAACAAAA; **D9smG** = dAAAAGAAAA; **D10comp** = dAGTGATCTAC; **D10smT** = dAGTGTTCTAC; **D10smC** = dAGTGCTCTAC; **D10smG** = dAGTGGTCTAC; **D11non** = dTCTGCATTTAG; **D12comp** = dGCAGGGATAACT; **D12smA** = dGCAGGAATAACT; **D12smC_3′** = dGCAGGGCTAACT; **D12smC_5′** = dGCAGCGATAACT. [c]Fluorescence spectra were measured in 30 mM sodium acetate buffer pH 4.6, 1 mM zinc acetate, 5% (v/v) glycerol, 5 U/mL S1 nuclease, [PNA] = 1.0 $\mu$M and [DNA] = 1.0 $\mu$M, excitation wavelength = 510 nm, PMT gain = high. [d]Detection wavelengths and digestion times used were: **T9-C4TzTO**, 532 nm and 15 min; **M10-C4TzTO**, 535 nm and 10 min; **M12-C4TzTO**, 528 nm and 60 min.

fluorescence of TO-modified acpcPNA is due to binding with specific or nonspecific DNA targets. To confirm that bound acpcPNA is effective in protecting DNA from S1 nuclease digestion, preliminary experiments were performed by monitoring the fluorescence of **T9-C4TzTO** with complementary and single mismatched DNA as a function of time. After a 15 min digestion, the fluorescence of the mismatched hybrids had almost returned to the same level as that of the single-stranded acpcPNA ($F/F_0$ decreased from 30.7−45.5 to 1.5−2.9), while that of the complementary hybrid was only slightly decreased from the original value ($F/F_0$ decreased from 25.7 to 20.3) (Figure S27). The S1 nuclease digestion was then performed upon the other TO-modified acpcPNA-DNA hybrids, and the results are summarized in Table 4 and Figure 4.

In all cases, the fluorescence ratios ($F/F_0$) after hybridization to complementary DNA remained high while those of the hybrids with the single-mismatched and noncomplementary DNA returned to unity after the S1 nuclease digestion. The more stable mismatched hybrids of the 12mer acpcPNA **M12-C4TzTO** required a longer digestion time to effect complete digestion as compared to those of the 10mer acpcPNA **M10-C4TzTO**, regardless of the position of the mismatched base. The results from the S1 nuclease digestion confirmed that the fluorescence increase of the TO-labeled acpcPNA in the presence of complementary DNA is associated with the specific pairing between the two partners, and that S1 nuclease digestion can enhance the specificity of TO-modified acpcPNA in distinguishing between noncomplementary (including single-mismatched) DNA and complementary DNA. The discrimination factors, as defined by the ratio of $(F/F_0)_{comp}$ to $(F/F_0)_{mismatched}$, are in the range of 7.0−13.5, 6.8−10.9, and 14.4−70.0, for **T9-C4TzTO**, **M10-C4TzTO**, and **M12-C4TzTO**, respectively.

It is expected that this enzymatic digestion should also be applicable to TO-modified aegPNA probes. The advantage of using S1 nuclease to increase the specificity of the DNA

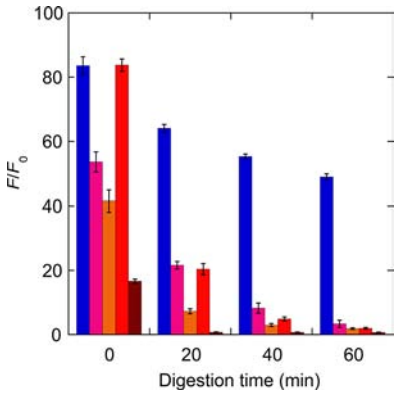



**Figure 4.** Kinetics of S1 nuclease digestion of complementary and single mismatched hybrids of **M12-C4TzTO**, as monitored from the fluorescence emission at 528 nm (**D12comp**: blue, **D12smA**: pink, **D12smC_3′**: orange, **D12smC_5′**: red, **D11non**: brown). The fluorescence ratios are shown as the mean ± 1 SD, derived from three independent experiments. Fluorescence spectra were measured in 30 mM sodium acetate buffer pH 4.6 containing 1 mM zinc acetate, 5% (v/v) glycerol, 5 U/mL S1 nuclease, [PNA] = 1.0 $\mu$M and [DNA] = 1.0 $\mu$M, with an excitation wavelength of 510 nm and PMT gain at high.

sequence detection by TO-labeled acpcPNA is that both the hybridization and enzymatic digestion can be carried out very conveniently under nonstringent conditions at ambient temperature without the need for temperature control, as is often employed in other TO-modified aegPNA probes.[20,23] This means that the fluorescence hybridization can be performed in a standard fluorescence spectrometer without temperature control accessories.

**Base Sequences Have No Effects on the Fluorescence of TO-Modified acpcPNA Probes.** The hybridization-responsive fluorescence probe design outlined here based on the internally TO-labeled acpcPNA offers some advantages


dx.doi.org/10.1021/bc3005914 | *Bioconjugate Chem.* 2013, 24, 614−625

**Table 5. Quantum Yield and Fluorescence Ratios between the Complementary Hybrids and Single-Stranded ($F/F_0$) TO-Labeled acpcPNAs before and after S1 Nuclease Digestion**

| entry | PNA[a] | $\Phi_F$(ss)[b] before digestion | $\Phi_F$(ds)/$\Phi_F$(ss)[b] before digestion | $\Phi_F$(ds)/$\Phi_F$(ss)[b,d] after digestion | $F/F_0$[c] before digestion | $F/F_0$[c,d] after digestion |
|---|---|---|---|---|---|---|
| 1 | **M10_C4TzTO** | 0.0022 | 41 | 8 | 40.2 | 9.0 |
| 2 | **M11AA_C4TzTO** | 0.0021 | 25 | 19 | 33.9 | 21.6 |
| 3 | **M11TT_C4TzTO** | 0.0015 | 36 | 29 | 50.0 | 39.3 |
| 4 | **M11CC_C4TzTO** | 0.0016 | 67 | 49 | 83.7 | 60.0 |
| 5 | **M11GG_C4TzTO** | 0.0023 | 86 | 36 | 120.2 | 46.9 |

[a]See Scheme 1 for PNA sequences and definition of labels. [b]Quantum yields were measured at 1.0 $\mu$M acpcPNA (ss) or 1.0 $\mu$M acpcPNA + 1.2 $\mu$M complementary DNA (ds) in S1 buffer (30 mM sodium acetate buffer pH 4.6, 1 mM zinc acetate, 5% (v/v) glycerol) at $\lambda_{ex}$ 480 nm using Rhodamine B as a standard ($\Phi_F$ = 0.46 in EtOH).[53] [c]Fluorescence ratios were measured under the same conditions as the quantum yield at $\lambda_{em}$ = 529 nm. [d]After S1 nuclease digestion for 20 min (**M10_C4TzTO**) or 45 min (**M11NN_C4TzTO**). By this time the $\Phi_F$(ds)/$\Phi_F$(ss) and $F/F_0$ values of noncomplementary hybrids were close to unity.

over other similar TO-labeled probes. TO-labeled DNA[48,49] generally gives a high background fluorescence due to the self-binding of the TO and the DNA, although it has recently been demonstrated that adjustment of the linker[50] or incorporation of an electron acceptor in the DNA strand[51] can partially solve the problem. PNA offers superior performance than DNA in this aspect because the interaction between the positively charged TO and the electrostatically neutral PNA should be less favorable. Nevertheless, the terminally TO-labeled aegPNA (so-called Light up probe) requires pyrimidine-rich sequences due to the high background fluorescence of purine-rich or mixed purine−pyrimidine probes.[21] The forced intercalation (FIT) probe carrying TO as a base surrogate in aegPNA tends to give only moderate fluorescence enhancement and usually requires temperature control for optimal results.[22,23] The reported large fluorescence enhancement of about 2 orders of magnitude when TO-backbone labeled $\gamma$-D-Lys aegPNA binds to DNA, was performed on a pyrimidine-rich sequence and did not address the issue of specificity.[52] In contrast, the TO-labeled acpcPNA probes in this study consistently showed a very low background fluorescence, regardless of the sequence and identity of the flanking bases around the position of the TO attachment (Table 5). The quantum yields were in the range of 0.0015 to 0.0023 which were comparable to the previously reported values for pyrimidine rich TO-labeled aegPNA.[20,21] Most notably, the single-stranded acpcPNA **M11GG_C4TzTO** carrying the TO label with two flanking G/G bases gave about the same quantum yield value compared to other flanking bases. This suggests that there are minimal interactions between the TO label and the single-stranded acpcPNA, which is in contrast a common problem for all TO-labeled aegPNA probes.[21] Upon hybridization to complementary DNA targets, large fluorescence increases were consistently observed in all cases irrespective of the flanking base sequences after S1 nucleases digestion. Accordingly, the TO-labeled acpcPNA probes introduced in this work can be used for DNA detection in a broad sequence context.

## CONCLUSION

In summary, we have reported an effective reductive alkylation strategy for the attachment of various labels at internal positions of the APC-modified acpcPNA. The acpcPNA modification can be performed on a solid support by either direct reductive alkylation with labels containing an aldehyde group, or by sequential reductive alkylation/acylation or reductive alkylation/click reactions employing amino-, azide-, or alkyne-functionalized aldehydes in the reductive alkylation step. The reductive alkylation, as well as the click reactions, took place quantitatively under mild conditions that are compatible with a range of functional groups. The applicability of the methodology was demonstrated by synthesizing a number of pyrene- and thiazole orange-labeled acpcPNAs and investigating the potential of these internally labeled acpcPNAs as hybridization-responsive fluorescent probes for DNA sequence determination under homogeneous conditions. The modified acpcPNAs retained a high stability and good specificity for binding to DNA, and yielded a different fluorescence in response to the presence or absence of the complementary DNA target. TO-labeled acpcPNAs gave a larger fluorescence increase upon hybridization with complementary DNA than the pyrene-labeled acpcPNAs, although the specificity was lower due to the PNA-independent nonspecific binding between TO and DNA. Nevertheless, the use of TO-labeled acpcPNA in combination with S1 nuclease enzymatic digestion could effectively differentiate complementary from noncomplementary (including single-mismatched) DNA targets with a high degree of specificity in a broad sequence context. Although an additional enzymatic digestion step is required, this could be conveniently carried out by simply adding the enzyme at ambient temperature before measuring the fluorescence after a certain short period of time (~10 to 60 min). In principle, it should also be possible to improve the specificity further without using S1 nuclease digestion by modifying the label to eliminate the positive charge, as shown in the pyrene case, or by adjusting the length and/or rigidity of the linker between the label and the probe. However, the main focus of this work was to demonstrate a new and general strategy for on-solid-support postsynthetic modification of acpcPNAs by reductive alkylation and click chemistry, and the data presented here accordingly serves to illustrate this point.

## ASSOCIATED CONTENT

### Supporting Information

$^1$H and $^{13}$C NMR spectra of **aLP** and **aTO**, analytical HPLC and MALDI-TOF mass spectra of labeled acpcPNAs and representative fluorescence spectra. This material is available free of charge via the Internet at http://pubs.acs.org.

## AUTHOR INFORMATION

### Corresponding Author

*E-mail: vtirayut@chula.ac.th; Fax: +66-2-2187598; Tel.: +66-2-2187627.

### Author Contributions

Preliminary forms of this work have been communicated as (i) a proceedings paper in Pure and Applied Chemistry International Conference 2012 (PACCON 2012), Chiang Mai, Thailand and (ii) as an abstract in the 37th Congress on Science and Technology of Thailand (STT37), Bangkok, Thailand.

### Notes

The authors declare no competing financial interest.

## ■ ACKNOWLEDGMENTS

Financial support from the Thailand Research Fund (RTA5280002 to T.V. and MRG5180025 to C.S.), the Integrated Innovation Academic Center: IIAC Chulalongkorn University Centenary Academic Development Project (CU56-AM02), the Higher Education Research Promotion and National Research University Project of Thailand, Office of the Higher Education Commission (AM1006A), the Thai government stimulus package 2 (TKK2555, SP2) under the Project for Establishment of Comprehensive Center for Innovative Food, Health Products and Agriculture, and the Center of Excellence for Innovation in Chemistry (PERCH–CIC), Commission on Higher Education, Ministry of Education are gratefully acknowledged.

## ■ REFERENCES

(1) Tyagi, S., and Kramer, F. R. (1996) Molecular beacons: Probes that fluoresce upon hybridization. *Nat. Biotechnol. 14*, 303–308.
(2) Marras, S. E. (2008) Interactive fluorophore and quencher pairs for labeling fluorescent nucleic acid hybridization probes. *Mol. Biotechnol. 38*, 247–255.
(3) Venkatesan, N., Seo, Y. J., and Kim, B. H. (2008) Quencher-free molecular beacons: A new strategy in fluorescence based nucleic acid analysis. *Chem. Soc. Rev. 37*, 648–663.
(4) Østergaard, M. E., and Hrdlicka, P. J. (2011) Pyrene-functionalized oligonucleotides and locked nucleic acids (LNAs): Tools for fundamental research, diagnostics, and nanotechnology. *Chem. Soc. Rev. 40*, 5771–5778.
(5) Amblard, F., Cho, J. H., and Schinazi, R. F. (2009) Cu(I)-catalyzed Huisgen azide-alkyne 1,3-dipolar cycloaddition reaction in nucleoside, nucleotide, and oligonucleotide chemistry. *Chem. Rev. 109*, 4207–4220.
(6) El-Sagheer, A. H., and Brown, T. (2010) Click chemistry with DNA. *Chem. Soc. Rev. 39*, 1388–1405.
(7) Seela, F., Suresh, S., and Pujar, S. S. (2010) Azide-alkyne "Click" conjugation of 8-aza-7-deazaadenine-DNA: Synthesis, duplex stability, and fluorogenic dye labeling. *Bioconjugate Chem. 21*, 1629–1641.
(8) Beyer, C., and Wagenknecht, H.-A. (2010) In situ azide formation and "click" reaction of nile red with DNA as an alternative postsynthetic route. *Chem. Commun. 46*, 2230–2231.
(9) Nielsen, P. E. (2010) Peptide nucleic acids (PNA) in chemical biology and drug discovery. *Chem. Biodiversity 7*, 796–804.
(10) Kuhn, H., Demidov, V. V., Coull, J. M., Fiandaca, M. J., Gildea, B. D., and Frank-Kamenetskii, M. D. (2002) Hybridization of DNA and PNA molecular beacons to single-stranded and double-stranded DNA targets. *J. Am. Chem. Soc. 124*, 1097–1103.
(11) Seitz, O. (2000) Solid phase synthesis of doubly labeled peptide nucleic acids as probes for the real-time detection of hybridization. *Angew. Chem., Int. Ed. 39*, 3249–3252.
(12) Grossmann, T. N., Röglin, L., and Seitz, O. (2007) Triplex molecular beacons as modular probes for DNA detection. *Angew. Chem., Int. Ed. 46*, 5223–5225.
(13) Socher, E., Bethge, L., Knoll, A., Jungnick, N., Herrmann, A., and Seitz, O. (2008) Low-noise stemless PNA beacons for sensitive DNA and RNA detection. *Angew. Chem., Int. Ed. 47*, 9555–9559.
(14) Knemeyer, J.-P., Marmé, N., and Sauer, M. Probes for detection of specific DNA sequences at the single-molecule level. *Anal. Chem. 72*, 3717–3724.
(15) Dobson, N., McDowell, D. G., French, D. J., Brown, L. J., Mellor, J. M., and Brown, T. (2003) Synthesis of HyBeacons and dual-labelled probes containing 2′-fluorescent groups for use in genetic analysis. *Chem. Commun.*, 1234–1235.
(16) Hwang, G. T., Seo, Y. J., and Kim, B. H. (2004) A highly discriminating quencher-free molecular beacon for probing DNA. *J. Am. Chem. Soc. 126*, 6528–6529.
(17) Nazarenko, I., Lowe, B., Darfler, M., Iknonmi, P., Schuster, D., and Rashtchian, A. (2002) Multiplex quantitative PCR using self-quenched primers labeled with a single fluorophore. *Nucleic Acids Res. 30*, e37.
(18) Seo, Y. J., Ryu, J. H., and Kim, B. H. (2005) Quencher-free, end-stacking oligonucleotides for probing single-base mismatches in DNA. *Org. Lett. 7*, 4931–4933.
(19) Okamoto, A., Kanatani, K., and Saito, I. (2004) Pyrene-labeled base-discriminating fluorescent DNA probes for homogeneous SNP typing. *J. Am. Chem. Soc. 126*, 4820–4827.
(20) Svanvik, N., Westman, G., Wang, D., and Kubista, M. (2000) Light-up probes: Thiazole orange-conjugated peptide nucleic acid for detection of target nucleic acid in homogeneous solution. *Anal. Biochem. 281*, 26–35.
(21) Svanvik, N., Nygren, J., Westman, G., and Kubista, M. (2001) Free-probe fluorescence of light-up probes. *J. Am. Chem. Soc. 123*, 803–809.
(22) Köhler, O., and Seitz, O. (2003) Thiazole orange as fluorescent universal base in peptide nucleic acids. *Chem. Commun*, 2938–2939.
(23) Köhler, O., Jarikote, D. V., and Seitz, O. (2005) Forced intercalation probes (FIT probes): Thiazole orange as a fluorescent base in peptide nucleic acids for homogeneous single-nucleotide-polymorphism detection. *ChemBioChem 6*, 69–77.
(24) Socher, E., Jarikote, D. V., Knoll, A., Röglin, L., Burmeister, J., and Seitz, O. (2008) FIT probes: Peptide nucleic acid probes with a fluorescent base surrogate enable real-time DNA quantification and single nucleotide polymorphism discovery. *Anal. Biochem. 375*, 318–330.
(25) Englund, E. A., and Appella, D. H. (2005) Synthesis of γ-substituted peptide nucleic acids: A new place to attach fluorophores without affecting DNA binding. *Org. Lett. 7*, 3465–3467.
(26) Vilaivan, T., and Srisuwannaket, C. (2006) Hybridization of pyrrolidinyl peptide nucleic acids and DNA: Selectivity, base-pairing specificity, and direction of binding. *Org. Lett. 8*, 1897–1900.
(27) Vilaivan, C., Srisuwannaket, C., Ananthanawat, C., Suparpprom, C., Kawakami, J., Yamaguchi, Y., Tanaka, Y., and Vilaivan, T. (2011) Pyrrolidinyl peptide nucleic acid with α/β-peptide backbone. *Artificial DNA: PNA XNA 2*, 50–59.
(28) Ananthanawat, C., Vilaivan, T., Hoven, V. P., and Su, X. D. (2010) Comparison of DNA, aminoethylglycyl PNA and pyrrolidinyl PNA as probes for detection of DNA hybridization using surface plasmon resonance technique. *Biosens. Bioelectron. 25*, 1064–1069.
(29) Vilaivan, T., and Lowe, G. (2002) A novel pyrrolidinyl PNA showing high sequence specificity and preferential binding to DNA over RNA. *J. Am. Chem. Soc. 124*, 9326–9327.
(30) Taechalertpaisarn, J., Sriwarom, P., Boonlua, C., Yotapan, N., Vilaivan, C., and Vilaivan, T. (2010) DNA-, RNA- and self-pairing properties of a pyrrolidinyl peptide nucleic acid with a (2′*R*, 4′*S*)-prolyl-(1*S*,2*S*)-2-aminocyclopentanecarboxylic acid backbone. *Tetrahedron Lett. 51*, 5822–5826.
(31) Mansawat, W., Vilaivan, C., Balázs, A., Aitken, D. J., and Vilaivan, T. (2012) Pyrrolidinyl peptide nucleic acid homologues: Effect of ring size on hybridization properties. *Org. Lett. 14*, 1440–1443.
(32) Boonlua, C., Vilaivan, C., Wagenknecht, H.-A., and Vilaivan, T. (2011) 5-(Pyren-1-yl)uracil as a base-discriminating fluorescent nucleobase in pyrrolidinyl peptide nucleic acids. *Chem. Asian J. 6*, 3251–3259.

(33) Mansawat, W., Boonlua, C., Siriwong, K., and Vilaivan, T. (2012) Clicked polycyclic aromatic hydrocarbon as a hybridization-responsive fluorescent artificial nucleobase in pyrrolidinyl peptide nucleic acids. *Tetrahedron 68*, 3988−3995.
(34) Reenabthue, N., Boonlua, C., Vilaivan, C., Vilaivan, T., and Suparpprom, C. (2011) 3-Aminopyrrolidine-4-carboxylic acid as versatile handle for internal labeling of pyrrolidinyl PNA. *Bioorg. Med. Chem. Lett. 21*, 6465−6469.
(35) Bethge, L., Jarikote, D. V., and Seitz, O. (2008) New cyanine dyes as base surrogates in PNA: Forced intercalation probes (FIT-probes) for homogeneous SNP detection. *Bioorg. Med. Chem. 16*, 114−115.
(36) Bates, R. W., and Dewey, M. R. (2009) A formal synthesis of swainsonine by gold-catalyzed allene cyclization. *Org. Lett. 11*, 3706−3708.
(37) Chan, T. R., Hilgraf, R., Sharpless, K. B., and Fokin, V. V. (2004) Polytriazoles as copper(I)-stabilizing ligands in catalysis. *Org. Lett. 6*, 2853−2855.
(38) Khan, N., Arumukam, V., and Balasubramanian, S. (1996) Solid phase reductive alkylation of secondary amines. *Tetrahedron Lett. 37*, 4819−4822.
(39) Tourwé, D., Piron, J., Defreyn, P., and van Binst, G. (1993) A new method for the solid phase synthesis of hydroxyethylamine peptide bond isosteres: Synthesis of an HIV-1 protease inhibitor and of a *β*-casomorphin-5 analogue. *Tetrahedron Lett. 34*, 5499−5502.
(40) Coy, D. H., Hocart, S. J., and Sasaki, Y. (1988) Solid phase reductive alkylation techniques in analogue peptide bond and side chain modification. *Tetrahedron 44*, 835−841.
(41) Balaji, B. S., Gallazzi, F., Jia, F., and Lewis, M. R. (2006) An efficient, convenient solid-phase synthesis of amino acid-modified peptide nucleic acid monomers and oligomers. *Bioconjugate Chem. 17*, 551−558.
(42) Spielmann, H. P., Wemmer, D. E., and Jacobsen, J. P. (1995) Solution structure of a DNA complex with the fluorescent- bis-intercalator TOTO determined by NMR spectroscopy. *Biochemistry 34*, 8542−8553.
(43) Nygren, J., Svanvik, N., and Kubista, M. (1998) The interactions between the fluorescent dye thiazole orange and DNA. *Biopolymers 46*, 39−51.
(44) Armitage, B. A. (2005) Cyanine dye−DNA interactions: Intercalation, groove binding, and aggregation. *Top. Curr. Chem. 253*, 55−76.
(45) Komiyama, M., Ye, S., Liang, X., Yamamoto, Y., Tomita, T., Zhou, J.-M., and Aburatani, H. (2003) PNA for one-base differentiating protection of DNA from nuclease and its use for SNPs detection. *J. Am. Chem. Soc. 125*, 3758−3762.
(46) Ye, S., Miyamita, Y., Ohnishi, T., Yamamoto, Y., and Komiyama, M. (2007) Combination of peptide nucleic acid beacon and nuclease S1 for clear-cut genotyping of single nucleotide polymorphisms. *Anal. Biochem. 363*, 300−302.
(47) Kerman, K., Saito, M., and Tamiya, E. (2008) Electroactive chitosan nanoparticles for the detection of single-nucleotide polymorphisms using peptide nucleic acids. *Anal. Bioanal. Chem. 391*, 2759−2767.
(48) Privat, E., and Asseline, U. (2001) Synthesis and binding properties of oligo-2′-deoxyribonucleotides covalently linked to a thiazole orange derivative. *Bioconjugate Chem. 12*, 757−769.
(49) Lartia, R., and Asseline, U. (2006) New cyanine−oligonucleotide conjugates: Relationships between chemical structures and properties. *Chem.—Eur. J. 12*, 2270−2281.
(50) Bethge, L., Singh, I., and Seitz, O. (2010) Designed thiazole orange nucleotides for the synthesis of single labeled oligonucleotides that fluoresce upon matched hybridization. *Org. Biomol. Chem. 8*, 2439−2448.
(51) Menacher, F., Rubner, M., Berndl, S., and Wagenknecht, H.-A. (2008) Thiazole orange and Cy3: Improvement of fluorescent DNA probes with use of short range electron transfer. *J. Org. Chem. 73*, 4263−4266.
(52) Englund, E. A., and Appella, D. H. (2007) *γ*-Substituted peptide nucleic acids constructed from L-lysine are a versatile scaffold for multifunctional display. *Angew. Chem., Int. Ed. 46*, 1414−1418.
(53) Snare, M. J., Treloar, F. E., Ghiggino, K. P., and Thistlethwaite, P. J. (1982) The photophysics of Rhodamine B. *J. Photochem. 18*, 335−346.