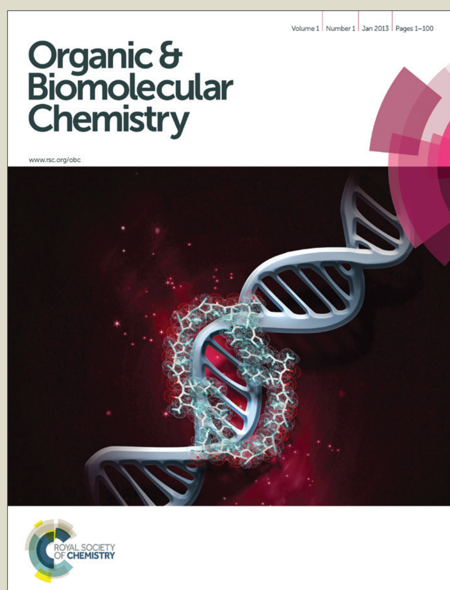
Organic & Biomolecular Chemistry

Accepted Manuscript



This is an *Accepted Manuscript*, which has been through the Royal Society of Chemistry peer review process and has been accepted for publication.

Accepted Manuscripts are published online shortly after acceptance, before technical editing, formatting and proof reading. Using this free service, authors can make their results available to the community, in citable form, before we publish the edited article. We will replace this *Accepted Manuscript* with the edited and formatted *Advance Article* as soon as it is available.

You can find more information about *Accepted Manuscripts* in the Information for Authors.

Please note that technical editing may introduce minor changes to the text and/or graphics, which may alter content. The journal's standard Terms & Conditions and the Ethical guidelines still apply. In no event shall the Royal Society of Chemistry be held responsible for any errors or omissions in this *Accepted Manuscript* or any consequences arising from the use of any information it contains.

ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/obc

Organic & Biomolecular Chemistry RSCPublishing

COMMUNICATION

Synthesis of nucleobase-caged peptide nucleic acids having improved photochemical properties†

Cite this: DOI: 10.1039/x0xx00000x

Received 00th January 2012,
Accepted 00th January 2012

DOI: 10.1039/x0xx00000x

www.rsc.org/

Takayoshi Watanabe,[a,b] Tomoko Hoshida,[a] Jun Sakyo,[a] Mariko Kishi,[a] Satoshi Tanabe,[a] Junichi Matsuura,[a] Shingo Akiyama,[a] Makiko Nakata,[a] Yasuaki Tanabe,[a] Akinobu Z. Suzuki,[a] Soichiro Watanabe[a] and Toshiaki Furuta*[a]

A nucleobase-caged peptide nucleic acid (PNA) having a (6-bromo-7-methoxycoumarin)-4-ylmethoxycarbonyl (Bmcmoc) caging group was newly synthesized. The Bmcmoc-caged PNAs were photolyzed to produce parent PNAs with high photochemical efficiency. Introduction of a single Bmcmoc group was sufficient to suppress polymerase chain reaction (PCR) clamping activity and triplex invasion complex formation. Photo-mediated restoration of the PCR clamping activity was also demonstrated.

Caged compounds are molecules designed such that their biological functions are temporarily masked and reactivated by the application of external triggers such as ultraviolet light.[1] N-(2-aminoethyl)glycine peptide nucleic acid (aeg-PNA) is a DNA mimetic in which the sugar-phosphate backbone is replaced by a 2-aminoethylglycine linkage.[2] Because aeg-PNA molecules can bind to their complementary oligonucleotides with high affinity and improved specificity, PNAs can be potentially antisense and antigene agents.[3] PNAs have also been used to create biochemical tools[4] used in transcription activation,[5] polymerase chain reaction (PCR) clamping,[6] target detection[7], and vector tagging[8]. If produced correctly, caged PNA molecules can be used as photochemically controllable chemical tools for the manipulation of oligonucleotide functions both in cells and *in vitro*. To prepare caged PNAs, a caging group should be introduced into a functional group of nucleobases as a steric mask or should be used as a photocleavable linker incorporated into a hairpin-shaped PNA/oligonucleotide duplex. The latter approach was demonstrated by Dmochowski's group, who reported synthesis of hairpin-shaped caged negatively charged PNAs (ncPNAs) as photochemically controllable antisense molecules and who used the molecules to induce translation inhibition in live zebrafish embryos.[9] Very recently, nucleobase-caged PNAs were reported and used in light-triggered PNA/PNA recognition by Diederichsen's group.[10] Introduction of two or three 2-(2-nitrophenyl)propyl (NPP) caging groups to each strand of complementary PNAs was necessary to inhibit duplex formation. Modification of a single nucleobase in one PNA strand partially prevented PNA/PNA duplex formation, which is consistent with observations of nucleobase-caged DNAs.[11]

We have been studying the preparation, photochemical properties and biological use of caged nucleotides.[12] We reported the synthesis and photochemistry of (6-bromo-7-methoxycoumarin-4-yl)methoxy carbonyl (Bmcmoc) group as a new photo-removable protecting group for nucleobases.[12d] Bmcmoc-caged nucleosides have 10–100 times better photolytic efficiency than those of other caging groups, including the NPP group. Therefore the Bmcmoc group is a good candidate for preparing nucleobase-caged PNAs. For this study, we elucidated whether the oxycarbonyl linkage of Bmcmoc group can tolerate through coupling, deprotection, and cleavage conditions of a standard solid-phase PNA synthesis. In addition, we tested whether the high photochemical efficiency of Bmcmoc group is maintained when the group is incorporated into a rather hydrophobic oligo PNA strand. Furthermore, we examined whether more than two caging groups are always necessary to suppress the original function of PNA. Finally, we examined the introduction of a single Bmcmoc group to ascertain whether it can suppress a strand-invasion type $(PNA)_2$/DNA triplex formation between a 16-mer PNA and a dsDNA.[13]

Organic & Biomolecular Chemistry Accepted Manuscript

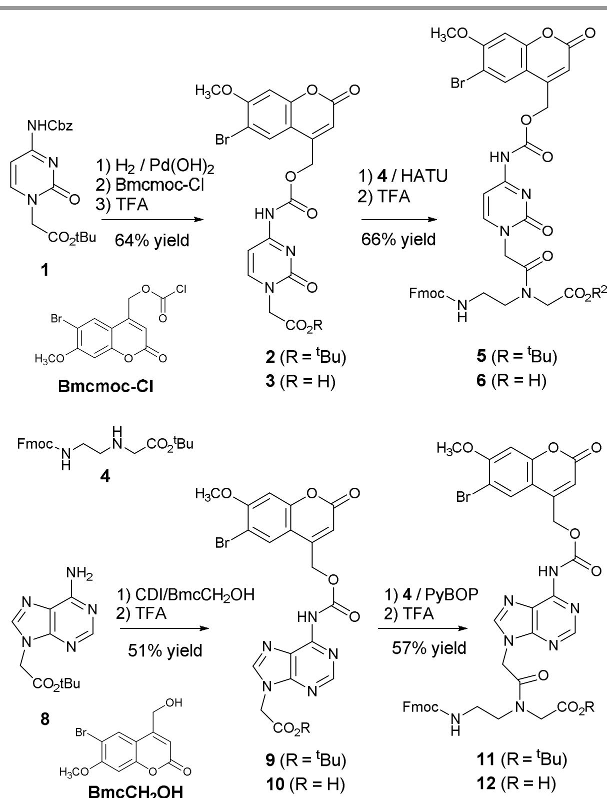
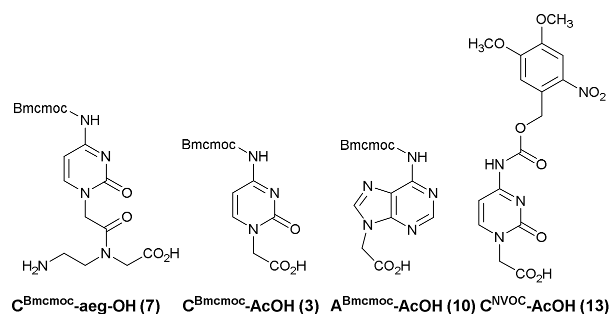


Fig. 1 Synthesis of a Bmcmoc-caged PNA monomers.

We synthesized two caged PNA monomers having a Bmcmoc protecting group on nucleo-bases (Fig. 1). A Bmcmoc group was introduced into the amino moiety of *tert*-butyl cytosine acetate via the corresponding chloroformate intermediate (Bmcmoc-Cl[12d]). Modification of adenine base was achieved by the reaction of $BmcCH_2OH$[12d] with an isocyanate generated from **8**[14] and CDI.[15] The Bmcmoc protected cytosin-1-yl acetic acid (**3**) and adenin-9-yl acetic acid (**10**) were coupled to a (2-aminoethyl)glycine unit (**4**) followed by deprotection of the tBu groups to yield Fmoc-protected caged PNA monomers, Fmoc-C^{Bmcmoc}-aeg-OH (**6**) and Fmoc-A^{Bmcmoc}-aeg-OH (**12**). The Fmoc protecting group of **6** was deprotected by 20% piperidine to give C^{Bmcmoc}-aeg-OH (**7**), which was used in a photolysis reaction for comparison. Similarly, C^{NVOC}-AcOH (**13**) was synthesized by the reaction of compound **1**[16] with NVOC-Cl (82% yield), followed by deprotection of the tBu group (87% yield).

Caged PNA oligomers were synthesized in 0.02 mmol scale by following an Fmoc SPPS protocol using HATU as a condensation agent. We synthesized 5-mer, 10-mer, and 16-mer caged PNAs having a single Bmcmoc-caged cytosine (C^{Bmcmoc}) (Fig. 2). The 10-mer sequence (TAGCTGTTTC) was chosen to target the start codon region of *E. coli* lac Z gene.[17] The 16-mer homopyrimidine sequence (TTCTCTTCCTTCTCTT) was designed to produce a $(PNA)_2$/DNA triplex using a strand invasion mechanism.[18] The C-terminus glycine was introduced as a spacer for improvement of the yields of the coupling reactions. After cleavage from the Alko-PEG resin, the desired products were purified using reversed-phase HPLC and were characterized using ESI-MS. The results show that the Bmcmoc group tolerates through coupling, deprotection, and cleavage conditions of the Fmoc SPPS.



5-mer cPNA (14): H_2N-TTC^{Bmcmoc}TT-Gly-COOH
10-mer cPNA (15): H_2N-TAGC^{Bmcmoc}TGTTTC-Gly-COOH
16-mer cPNA (16): H_2N-TTCTCTTC^{Bmcmoc}CTTCTCTT-Gly-COOH

Fig. 2 Chemical structures of the caged compounds used in this study.

Photophysical and chemical properties of the Bmcmoc-caged PNAs were measured in a simulated physiological saline solution. The photolytic efficiency of the Bmcmoc caging group was compared with that of the NVOC group. The molar absorptivities of the caged C^{Bmcmoc}-AcOH (**3**) and C^{NVOC}-AcOH (**13**) were almost identical at 350 nm (ε_{350} = 5,100 M^{-1} cm^{-1} for **3** and 5,200 M^{-1} cm^{-1} for **13**). Photolysis was performed at 350 nm (two RPR 350 nm lamps, 4 mJ/s). The photolytic consumption of the starting compounds followed single exponential decay, from which we obtained a photolysis quantum yield of 0.24 for **3** and 0.0014 for **13** (Fig. S3). Consequently, the photolytic efficiency, $\varepsilon\Phi$ value of the disappearance of **3** is calculated to be 1,220 M^{-1} cm^{-1}, which is comparable to that of the Bmcmoc-caged cytosine[12d] and is more than 170 times larger than that of **13**. The Bmcmoc-caged PNA monomer **7** and the A^{Bmcmoc}-AcOH (**10**) have similar photophysical and photochemical properties to those of **3** (Table 1). When incorporated into PNA oligomers, the C^{Bmcmoc} group showed slightly decreased photolytic quantum yields. In the PNA oligomers, one would expect the Bmcmoc to experience a more hydrophobic environment; this could explain the decrease in the photolysis quantum yields.[19] The observed $\varepsilon\Phi$ values of 1,090 M^{-1} cm^{-1} for 10-mer and 760 M^{-1} cm^{-1} for 16-mer are still two orders of magnitude larger than that of C^{NVOC}-AcOH (Table 1). Moreover, almost quantitative production of the parent PNA was observed from photolysis of the 5-mer cPNA (**14**) with a quantum yield of 0.16 (Fig. 3). The molar absorptivities of the Bmcmoc-caged PNAs (**14** - **16**) are approximately 10,000 M^{-1} cm^{-1} at 330 nm and 200 M^{-1} cm^{-1} at 385 nm (Fig. S1). The compounds can be photolyzed at wavelengths of up to 385 nm.

Organic & Biomolecular Chemistry Accepted Manuscript

This journal is © The Royal Society of Chemistry 2012

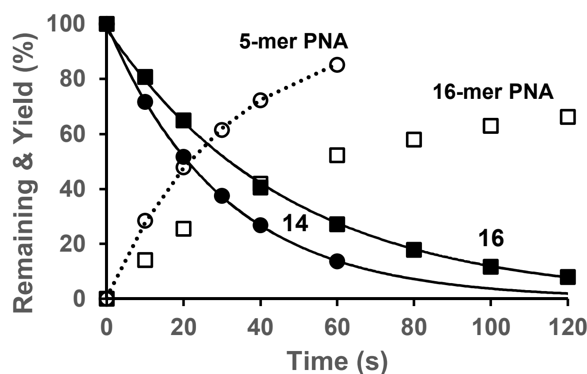


Fig. 3 Time course for photolysis of the caged PNAs. Samples (10^{-5} M) were irradiated at 350 nm (4 mJ/s) under simulated physiological conditions (10 mM K-MOPS buffer at pH 7.2). Closed circles show the consumption of **14**. Open circles show the yield of 5-mer PNA. Closed squares show the consumption of **16**. Open squares show the yield of 16-mer PNA. Solid lines show the least-squares curve fit to a simple decaying exponential for **14** and **16**. The dotted line shows the rising exponential for 5-mer PNA.

Table 1 Selected Photophysical and Chemical Properties of Nucleobase-caged Peptide Nucleic Acids

	λ_{max} (nm) [a]	Φ_{dis} [b]	$\varepsilon\Phi_{dis}$ [c]	Φ_{app} [d]	$\varepsilon\Phi_{dis}$ [e]
C^{Bmcmoc}-AcOH (3)	294, 328	0.24	1220		
C^{NVOC}-AcOH (13)	295, 349	0.0014	7.3		
A^{Bmcmoc}-AcOH (10)	270, 329	0.18	1140		
C^{Bmcmoc}-aeg-OH (7)	291, 329	0.22	1250		
5-mer cPNA (14)	268, 329	0.18	1020	0.16	920
10-mer cPNA (15)	260, 331	0.14	1090		
16-mer cPNA (16)	268, 331	0.13	760	0.075	460

[a] Absorption maximum (nm) measured in K-MOPS (pH 7.2). [b] Quantum yields for disappearance of starting materials upon irradiation (350 nm). Samples (10 μM) in K-MOPS (pH 7.2) were photolyzed with two RPR 350 nm lamps. [c] Product of the photolysis quantum yield for disappearance and molar absorptivity at 350 nm (M^{-1} cm^{-1}). [d] Quantum yields for appearance of the PNAs upon irradiation (350 nm). [e] Product of the photolysis quantum yield for appearance and molar absorptivity at 350 nm.

To determine the extent of destabilization of a PNA/DNA duplex from the introduction of a single Bmcmoc group, the melting temperature (T_m) difference of 10-mer PNA/DNA between the non-caged and the caged PNAs was measured. The T_m value of the non-caged 10-mer PNA (H_2N-TAGCTGTTTC-COOH) and its complementary DNA was 36.7 °C. The stability of the duplex was decreased by 7.7 °C for the 10-mer cPNA (**15**)/DNA (T_m = 29.0 °C). This decrease is consistent with the fact that introduction of a single mismatch caused a decrease in T_m values by 8–15 °C in PNA/DNA duplexes.[20] The results show that the nucleobase-caged Bmcmoc-cytosine corresponded to a mismatched base. Therefore complete suppression of PNA/DNA duplex formation was not achieved by the introduction of a single Bmcmoc group on a nucleobase. After exposure to 350-nm light (4 mJ/s, 120 s), an increase of T_m value (33.2 °C) observed in **15**/DNA mixture confirmed photo-deprotection of the Bmcmoc group to give the parent 10-mer PNA.

We hypothesized that nucleobase-caged PNAs having a single Bmcmoc caging group are useful as caged PNAs in applications in which a smaller affinity difference in PNA/DNA duplexes between an intact PNA and a cPNA would be amplified or integrated. To test this hypothesis, we conducted a study using PCR clamping. PNA-mediated PCR clamping is achieved by annealing of a PNA to its complementary template DNA and inhibition of primer extension reactions. The method is based on the higher selectivity and specificity of duplex formation between complementary PNA and DNA than that of a DNA/DNA duplex. Therefore, the residual inhibitory activity of a caged PNA on DNA polymerization would be detected efficiently using PCR clamping experiments. We used PCR as a simple model of DNA polymerization in cells: DNA replication and DNA repair.

The effects of the non-caged PNA and the cPNA were measured in standard PCR reactions containing Blend-Taq DNA polymerase, *p*UC18 as a template, and a set of primers (398 bp product). Either the 10-mer PNA or the 10-mer cPNA (**15**) was added to the PCR mixture. After 20 cycles of amplification, the amplicons were analyzed using agarose gel electrophoreses (Figs. 4a, 4b). The amount of the 398-bp product was decreased by 80% in the presence of the non-caged PNA (120 times molar excess to the primer). The affinity of the primer (15-mer, T_m = 36°C) is almost identical to that of the competitive 10-mer PNA (T_m = 36.7°C). Therefore, partial clamping activity was observed. However, the amount of the amplified product (398 bp) was restored to 90% when compound **15** was added to the PCR mixtures. The same amount of reduction was observed when a different primer that is not competitive to the 10-mer PNA was used. Therefore, the 10% reduction in the product amount is expected to be attributable to the sequence-independent suppression of the amplification in the presence of PNA molecules. Exposure to 350-nm UV light restored the clamping activity of the 10-mer cPNA (**15**) by a comparable level to that of the non-caged PNA (Fig. 4b). The results indicate that the introduction of a single nucleobase-caged cytosine enabled the suppression of sequence selective inhibitory activity of the 10-mer PNA on a DNA polymerization reaction.

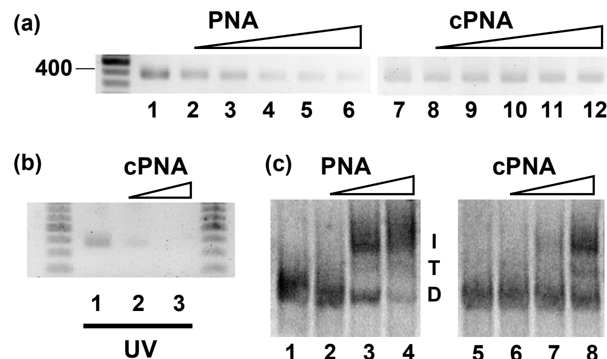


Fig. 4 PCR clamping and triplex invasion study. Agarose gel electrophoresis of the PCR products (a, b). PCRs were performed in the presence of 10-mer PNA or 10-mer cPNA (**15**):

Organic & Biomolecular Chemistry Accepted Manuscript

(a) lanes 1 and 7, no PNAs; lane 8, 12 μM; lanes 2 and 9, 18 μM; lanes 3 and 10, 24 μM; lanes 4 and 11, 30 μM; lanes 5 and 12, 36 μM; lane 6, 42 μM; (b) PCR mixtures containing **15** were exposed to UV light (350 nm, 2 mJ/s, 120 s). Lane 1, no cPNA; lane 2, 24 μM; lane 3, 48 μM; (c) Gel-shift analysis of triplex invasion complexes between the 50-mer dsDNA and the 16-mer PNA or 16-mer cPNA (**16**). Lanes 1 and 5, no PNAs; lanes 2 and 6, 0.2 μM; lanes 3 and 7, 1 μM; lanes 4 and 8, 2 μM); I, triplex invasion complex; T, triplex; D, duplex.

Oligopyrimidinyl PNAs are known to interact with dsDNAs having homopurine target sequences to form triple helixes, conventional PNA-dsDNA Hoogsteen type triplexes, or triplex invasion $(PNA)_2DNA$ complexes involving an unbound DNA strand in the P-loop structure.[18a] Wang et al. reported that homopyrimidine PNAs longer than 12-mer formed triplex invasion complexes with dsDNAs and induced transcription initiation both *in vitro* and in human cultured cells.[18b] The triplex invasion complex includes combined Hoogsteen and Watson–Crick type base pairings. Therefore, the affinity difference between the intact PNAs and the cPNAs to their complementary dsDNA targets would be integrated and enhanced. To test this hypothesis, effects of the single Bmcmoc group were investigated in the reported triplex invasion complex between the 16-mer PNA and the 50-mer dsDNA.[18b] The 16-mer PNA (H_2N-TTCTCTTCCTTCTCTT-CO_2H) or the 16-mer cPNA (**16**) was mixed with the 50-mer dsDNA having a PNA binding sequence (AAGAGAAGGAAGAGAA). Results were analyzed using polyacrylamide gel electrophoresis. Two slower-migrating bands appeared at the expense of the dsDNA (D in Fig. 4c) when more than five-fold molar excess of the 16-mer PNA (1 μM) was used (lanes 3 and 4 in Fig. 4c). Incubation was carried out in low ionic strength solutions. Therefore, the intense band should be assigned as the triplex invasion complex (I) containing two PNA molecules.[21] Another band must be the conventional PNA-dsDNA triplex (T). However, formation of the triplex invasion complex was markedly suppressed when the dsDNA was incubated with 1 μM of the 16-mer cPNA (lane 7 in Fig. 4c). Use of ten-fold excess of the cPNA (2 μM) engenders formation of a substantial amount of the triplex and the invasion complex. These results indicate that the 16-mer cPNA having a single Bmcmoc-caged cytosine base can be used as a caged compound of triplex invasion forming PNA when the appropriate amount of the compound is used.

In conclusion, new nucleobase-caged PNA oligomers having a Bmcmoc caging group on a single cytosine base were synthesized following a standard Fmoc SPPS protocol. The Bmcmoc-caged PNAs were photolyzed to produce the parent PNAs with high photochemical efficiencies ($\varepsilon\Phi_{350}$ is 1090 M^{-1} cm^{-1} for 10-mer and 760 M^{-1} cm^{-1} for 16-mer). Although the suppression of a PNA/DNA duplex formation could not be achieved completely by introduction of a single Bmcmoc group, the PCR clamping activity of the 10-mer caged PNA was suppressed completely and restored upon photo-irradiation. The formation of a triplex invasion complex between the 50-mer dsDNA having a homopyrimidine PNA binding sequence and the complementary 16-mer PNA was inhibited when the five-fold molar excess of the caged PNA was used. The results imply that nucleobase-caged PNAs having a single caging group are useful as caged PNAs in certain applications in which an affinity difference between an intact and a caged PNA would be amplified or integrated.

Acknowledgements

We thank MEXT KAKENHI (221S0003, 22121520, 22310141 and 24121724) for financial support of this work.

Notes and references

[a] *Department of Biomolecular Science, Faculty of Science, Toho University, 2-2-1 Miyama, Funabashi, Chiba 274-8510, Japan. E-mail: furuta@biomol.sci.toho-u.ac.jp; Fax: +81-47-472-1169; Tel: +81-47-472-1169*
[b] *Present address: School of Materials Science, Japan Advanced Institute of Science and Technology, 1-1 Asahidai, Nomi, Ishikawa 923-1292.*

† Electronic Supplementary Information (ESI) available: [details of any supplementary information available should be included here]. See DOI: 10.1039/c000000x/

1 P. Klan, T. Solomek, C. G. Bochet, A. Blanc, R. Givens, M. Rubina, V. Popik, A. Kostikov, and J. Wirz, *Chem. Rev.* 2013, **113**, 119.
2 P. E. Nielsen, M. Egholm, R. H. Berg, and O. Buchardt, *Science* 1991, **254**, 1497.
3 (a) J. Hu, and D. R. Corey, *Biochemistry* 2007, **46**, 7581. (b) J. C. Hanvey, N. J. Peffer, J. E. Bisi, S. A. Thomson, R. Cadilla, J. A. Josey, D. J. Ricca, C. F. Hassman, M. A. Bonham, K. G. Au, S. G. Carter, D. A. Bruckenstein, A. L. Boyd, S. A. Noble, and L. E. Babiss, *Science* 1992, **258**, 1481. (c) T. Shiraishi, and P. E. Nielsen, *Bioconjugate Chem.* 2012, **23**, 196.
4 S. Shakeel, S. Karim, A. and Ali, A., *J. Chem. Technol. Biotechnol.* 2006, **81**, 892.
5 (a) N. E. Mollegaard, O. Buchardt, M. Egholm, and P. E. Nielsen, *Proc. Natl. Acad. Sci. U.S.A.* 1994, **91**, 3892. (b) G. Wang, X. S. Xu, *Cell Res.* 2004, **14**, 111.
6 H. Orum, P. E. Nielsen, M. Egholm, R. H. Berg, O. Buchardt, and C. Stanley, *Nucleic Acids Res.* 1993, **21**, 5332.
7 E. Ortiz, G. Estrada, and P. M. Lizardi, *Mol. Cell. Probes* 1998, **12**, 219.
8 A. G. Rebuffat, A. R. Nawrocki, P. E. Nielsen, A. G. Bernasconi, E. Bernal-Mendez, B. M. Frey, and F. J. Frey, *FASEB J.* 2002, **16**, 1426.
9 (a) X. Tang, S. Maegawa, E. S. Weinberg, and I. J. Dmochowski, *J. Am. Chem. Soc.* 2007, **129**, 11000. (b) I. J. Dmochowski, X. Tang, *BioTechniques* 2007, **43**, 161.
10 S. Guha, J. Graf, B. Goricke, and U. Diederichsen, *J. Pept. Sci.* 2013, **19**, 415.
11 L. Krock, and A. Heckel, *Angew. Chem. Int. Ed.* 2005, **44**, 471.
12 (a) H. Ando, T. Furuta, R. Y. Tsien, and H. Okamoto, *Nat. Genet.* 2001, **28**, 317. (b) H. Ando, T. Furuta, and H. Okamoto, *Methods Cell Biol.* 2004, **77**, 159. (c) H. Ando, M. Kobayashi, T. Tsubokawa, K. Uyemura, T. Furuta, and H. Okamoto, *Develop. Biol.* 2005, **287**, 456. (d) T. Furuta, T. Watanabe, S. Tanabe, J. Sakyo, and C.

Organic & Biomolecular Chemistry Accepted Manuscript

 This journal is © The Royal Society of Chemistry 2012

Matsuba, *Org. Lett.* 2007, **9**, 4717. (e) T. Furuta, K. Manabe, A. Teraoka, K. Murakoshi, A. Ohtsubo, and A. Suzuki, *Org. Lett.* 2012, **14**, 6182. (f) T. Furuta, *J. Synth. Org. Chem. Jpn* 2012, **69**, 1164. (g) A. Teraoka, K. Murakoshi, K. Fukamauchi, A. Z. Suzuki, S. Watanabe, T. Furuta, *Chem. Commun.* 2014, **50**, 664.

13 Part of this work was presented at 20th International Conference on Physical Organic Chemistry, Busan, Korea, Aug. 22-28, 2010.

14 G. Cuelemans, A. V. Aerschot, B. Wroblowski, J. Rozenski, C. Hendrix, and P. Herdewijn, *Chem. Euro. J.* 1997, **3**, 1997.

15 F. Debaene, N. Winssinger, *Org. Lett*. 2003, **5**, 4445.

16 S. A. Thomson, J. A. Josey, R. Cadilla, M. D. Gaul, C. F. Hassman, M. J. Luzzio, A. J. Pipe, K. L. Reed, D. J. Ricca, R. W. Wiethe, and S. A. Noble, *Tetrahedron* 1995, **51**, 6179.

17 L. Good, S. K. Awasthi, R. Dryselius, O. Larsson, and P. E. Nielsen, *Nat. Biotech.* 2001, **19**, 360.

18 (a) P. E. Nielsen, and M. Egholm, O. Buchardt, *J. Mol. Recognit.* 1994, **7**, 165. (b) G. Wang, K. Jing, R. Balczon, and X. Xu, *J. Mol. Biol.* 2001, **313**, 933.

19 A. Z. Suzuki, T. Watanabe, M. Kawamoto, K. Nishiyama, H. Yamashita, M. Ishii, M. Iwamura, and T. Furuta, *Org. Lett.* 2003, **5**, 4867.

20 P. E. Nielsen, and M. Egholm in "Peptide Nucleic Acids Protocols and Applications," P. E. Nielsen Eds. Horizon Bioscience, Norfolk, 2004, pp. 1-36.

21 T. Bentin, G. I. Hansen, and P. E. Nielsen, *Nucleic Acids Res.* 2006, **34**, 5790.

Organic & Biomolecular Chemistry Accepted Manuscript