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Convergent Synthesis of Peptide Nucleic Acids by Native Chemical Ligation

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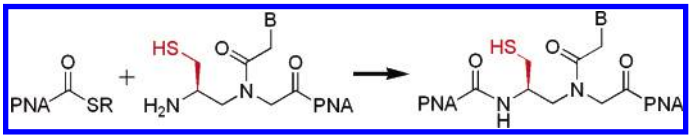
ABSTRACT



A convergent strategy for synthesizing long contiguous PNA by a native chemical ligation-like technique of PNA segment couplings is presented. This approach required the synthesis of a new PNA-monomer featuring a 1-amino-2-thiol group. It is shown that the additional mercaptomethyl group leaves the hybridization properties of PNA ligation products unaffected. Furthermore, rapid and efficient fluorescence labeling of the ligation products is demonstrated.

Peptide nucleic acids (PNA) are achiral, uncharged DNA analogues in which the nucleobases are attached to a 2-aminoethylglycine scaffold.[1] The ability of PNA to bind to complementary nucleic acids with very high affinity and selectivity and the resistance to chemical and biological degradation reactions has led to interesting applications in DNA diagnostics,[2] in antisense/antigene technology,[3] and in detection of RNA and DNA in cells.[4] In particular, PNA-oligomers spanning 18−25 nucleobases in length have been used to monitor telomere dynamics,[5] to inhibit human telomerase,[6] or to immobilize nucleic acids.[7] PNA typically is synthesized on the solid phase according to Fmoc or Boc peptide synthesis protocols by iterative steps of protecting group removal and coupling of Fmoc/Bhoc- or Boc/Cbz-protected building blocks.[8] However, the linear solid-phase synthesis of PNAs longer than 20 nucleobases and/or of PNA containing purine-rich sequences can result in low product yield and/or low purity.[9] In peptide chemistry, convergent ligation techniques have been developed in order to overcome the statistical accumulation of byproducts in the stepwise synthesis of long peptides. The native chemical ligation (NCL) reaction is a powerful method to join two unprotected peptides in aqueous solution.[10] In this reaction, a peptide thioester is allowed to react with a cysteinyl peptide to first form a thioester intermediate which, through a spontaneous $S \rightarrow N$ acyl shift, furnishes the peptide bond in the ligation product.[11]

The native chemical ligation chemistry has also been used to conjugate peptidic and nonpeptidic fragments to other

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biomolecules including DNA or PNA.[12] In this work, cysteine served as the ligation handle. However, the incorporation of cysteine in a convergent PNA−PNA fragment coupling reaction would significantly distort the PNA backbone and would hence reduce the affinity for target nucleic acids. We hereby report the synthesis of a new mercaptomethyl-modified PNA-monomer that allows the application of native chemical ligation-like fragment coupling reactions without detriment to backbone geometry. It is shown that the DNA affinity of the PNA ligation products is as high as the affinity of nonmodified PNA obtained by linear solid-phase synthesis. Furthermore, rapid and efficient fluorescence labeling of the ligation products is demonstrated. The presented convergent approach enables the concise and high-yielding synthesis of long fluorescently labeled PNA.

To enable PNA-fragment condensations by NCL-like reactions a PNA-monomer is required that contains a 1-amino-2-thiol structure analogous to cysteine. We designed the new PNA-monomer **1** as a structure analogue of a PNA adenine monomer **2** (Figure 1). The introduction of amino

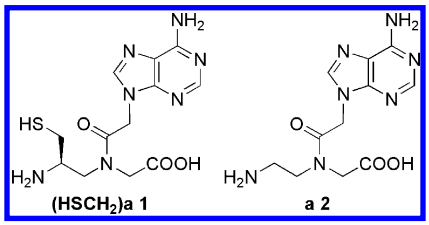
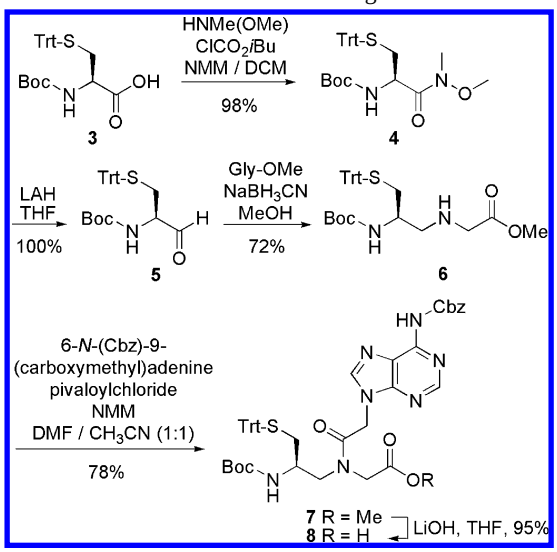


Figure 1. Structures of the PNA adenine monomer **2** and the analogue **1** containing the 1-amino-2-thiol structure needed for native chemical ligation-like reactions.

acid side chains such as from alanine, lysine, or arginine into the PNA backbone has been reported.[13] Surprisingly, thiol groups have not been connected to the aminoethylglycine scaffold. The envisaged application called for *N*-terminal coupling of mercaptomethyl-modified PNA monomer **1**. It was therefore planned to employ acid-labile protecting groups shown in Boc/Trt/Cbz-adenine monomer **8** which can be removed during the acid cleavage step of Boc-based solid-phase PNA synthesis (Scheme 1). The synthesis commenced from Boc/Trt-cysteine **3** which was activated as a mixed anhydride by addition of isobutylchloroformate to enable the formation of the "Weinreb amide" **4**. Reduction with lithium aluminum hydride furnished the corresponding aldehyde **5** in nearly quantitative overall yield.[14] This aldehyde was used in a reductive amination reaction with glycine methyl ester hydrochloride, which delivered the desired PNA-backbone building block **6** in 72%

Scheme 1. Synthesis of Protected PNA Monomer **8** Suitable for Native Chemical Ligation



yield. We next sought a method to couple 6-*N*-(benzyloxycarbonyl)-9-(carboxymethyl)adenine[15] to the unreactive secondary amine in **6**. Here, the use of pivaloyl chloride under dry conditions proved superior to peptide couplings promoted by carbodiimides, PyBOP, or HATU. The fully protected mercaptomethyl adenine monomer **7** was obtained in 78% yield. Finally, the methyl ester was saponified by treatment with aqueous LiOH in THF, which completed a five-step sequence and provided the optically active mercaptomethyl-PNA monomer **8** in 52% overall yield.

The 1-amino-2-thiol-protected PNA-monomer **8** was next employed in the solid-phase synthesis of PNA. Starting from Fmoc-Gly-loaded MBHA-resin, Boc/Cbz-protected PNA building blocks were assembled according to the Boc-strategy (Scheme 2). After coupling of Boc/Trt/Cbz-protected adenine building block **8** the Boc- and Trt-groups were removed by adding TFA/*m*-cresol prior to treatment with TFMSA/TFA/*m*-cresol required for Cbz and resin cleavage. Application of this two-step protocol prevented the formation of tritylated crude products observed in global deprotections with TFMSA/TFA mixtures containing *m*-cresol, thioanisol, or triisopropylsilane. The desired PNA conjugates **11**, **12**, and **13** were obtained in 26%, 9%, and 4% overall yield, respectively, after HPLC purification.

The solid-phase synthesis of PNA-thioesters such as **14** presents a challenge.[16] Most commonly, peptide thioesters are prepared according to the Boc-strategy by using mercaptopropionyl-based linkers.[17] However, PNA-thioesters are

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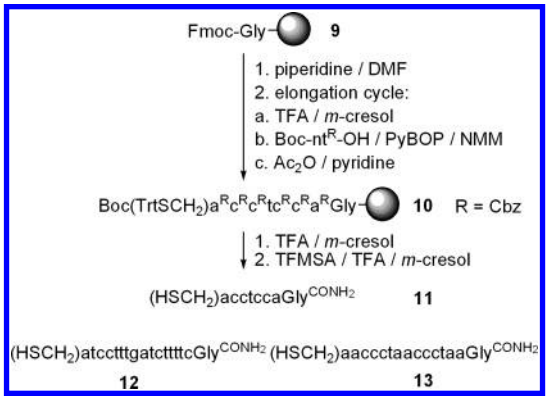
Table 1. PNA Conjugates, Ligation Products, and Yields of the Ligation Reactions[a]

nucleophile	electrophile		ligation product		yield[b]/%
11	^{Ac}ttccccacS$(CH_2)_2$COGly^{CONH_2}	**14**	^{Ac}ttccccac$(HSCH_2)$acctccaGly^{CONH_2}	**16**	72
12	^{Ac}ttccccacS$(CH_2)_2$COGly^{CONH_2}	**14**	^{Ac}ttccccac$(HSCH_2)$atccttgatcttttcGly^{CONH_2}	**17**	67
13	^{Ac}ccctaaccctS$(CH_2)_2$COGly^{CONH_2}	**15**	^{Ac}ccctaaccct$(HSCH_2)$aaccctaaccctaaGly^{CONH_2}	**18**	69

[a] Reaction conditions: 0.1 mM reactants in a buffer containing 10 mM tris(2-carboxyethyl)phosphine hydrochloride, 10 mM MESNA, and 100 mM NaH_2PO_4 adjusted to pH 7.4. [b] Product yields after 14 h determined by HPLC analysis.

subject to cyclization reactions which furnish six-membered lactams. Indeed, the linear solid-phase synthesis of PNA-thioester **14** proceeded with low yield due to the formation of *C* → *N*-terminally truncated PNA byproducts (Figure S1, Supporting Information). The formation of these so-called inverted truncation products can be explained by assuming that the reactive thiol group obtained after cyclization experienced repeated cycles of acylation and lactamization. Presumably, cyclative PNA-cleavage predominantly occurred during coupling of the second monomer. To reduce the amount of the undesired lactamization reaction, the usual coupling procedure (0.1 M PNA building block, 0.15 M *N*-methylmorpholine, 0.1 M PyBOP) was changed to less basic conditions. The second PNA building block was coupled at 0.125 M concentration by performing the *in situ* neutralization with 1.1 equiv (based on acyl donor) of 2,6-lutidine (pK_a 6.65) instead of 1.5 equiv of *N*-methylmorpholine (pK_a 7.38). After the critical extension of resin-bound PNA-monomer, the subsequent coupling reactions proceeded as previously described. Ultimately, acidolytic release provided PNA-thioesters **14** and **15** as the main products in 11% and 3% yield after HPLC purification.

Scheme 2. Solid Phase Synthesis.



The reactivity of PNA-thioester **14** was studied in exploring the native chemical ligation-like reaction with 1-amino-2-thiol PNA **11**. The reaction was carried out in the presence of 10 mM tris(2-carboxyethyl)phosphine hydrochloride to maintain a reducing environment. Sodium 2-mercaptoethanesulfonate (MESNA) was added for *in situ* generation of a more reactive thioester **19**. HPLC analysis of aliquots showed a new peak that appeared at 15.6 min with a mass that corresponded to ligation product **16** (Figure 2). After 14 h reaction time PNA ligation product **16** was formed in 72% yield.

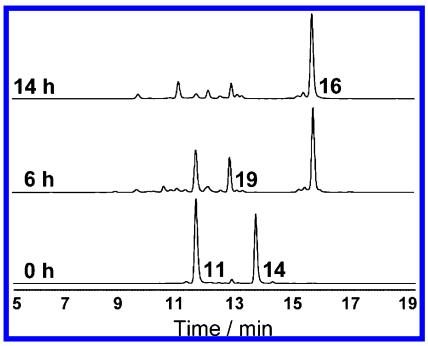


Figure 2. HPLC of ligation between PNA−thioester **14** and PNA−thiol conjugate **11**. Reaction conditions: see Table 1.

The feasibility of the synthesis of large PNA was investigated by allowing PNA thioester **14** to react with 16mer PNA **12** (Table 1). The ligation proceeded smoothly and provided 24mer PNA in 67% yield after 14 h reaction time. Furthermore, the ligation of 10mer PNA-thioester **15** with 14mer PNA **13** was explored. The sequence of PNA **18** corresponds to a tetrad of the C_3TA_2 repeat that should allow the probing of telomer length in human cells.[18] The ligation of **15** with **13** succeeded just as well as the previous ones supplying product **18** in 69% yield. These results provided clear evidence that the synthesized PNA-monomer **8** enables fragment coupling of PNA-oligomers by an efficient NCL-like reaction.

There are numerous applications in DNA diagnostics and molecular biology which rely on the introduction of fluorophores to PNA and DNA. In addition to its usefulness as ligation handle in PNA nucleophiles **11**, **12**, and **13**, the mercaptomethyl groups in PNA ligation products **16**, **17**, and **18** also offer conjugation sites for the selective reaction with soft electrophiles. Ligation product **16** was allowed to react with the thiol reactive dye 7-diethylamino-3-(4′-maleimidyl phenyl)-4-methylcoumarin (CPM) (Figure 3). Near-quantitative labeling of PNA **16** was accomplished by performing

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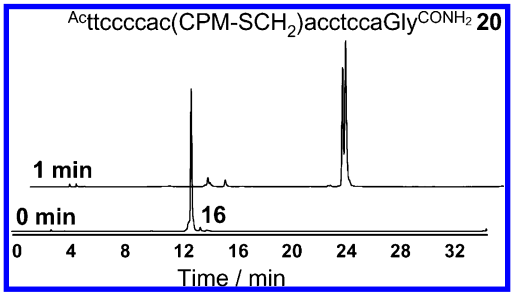

Figure 3. HPLC analysis of thiol specific labeling of PNA conjugate **16** with 7-diethylamino-3-(4′-maleimidylphenyl)-4-methylcoumarine (CPM). Reaction conditions: 0.1 mM of **16** in a buffer containing 100 mM NaH_2PO_4 and 1 mM CPM adjusted to pH 7.4.

the alkylation at 0.1 mM ligation product **16** and 10-fold excess of CPM. The reaction went to completion after only 1 min reaction time, illustrating the high reactivity of the mercaptomethyl group. HPLC analysis showed that product **20** at 23.2 min (m/z = 4451) exists as mixture of diastereomers due to the use of optically active PNA-monomer **8**.

Long PNA is required in intracellular applications such as in fluorescence *in situ* hybridization (FISH) in which the stability of probe-target complexes must be high.[19] The influence of the mercaptomethyl and CPM modifications was investigated by comparing the thermal stability of PNA·DNA duplexes containing modified PNAs **16**, **17**, and **20** with duplexes containing nonmodified PNA ^{Ac}ttccccacacctccaGly^{CONH2} **21** and ^{Ac}ttccccacatcctttgatcttttcGly^{CONH2} **22** (Table 2). Note that the linear solid-phase synthesis of 24mer PNA **22** was cumbersome and required 13 double couplings. The melting analyses revealed melting temperatures T_M of 71 and 76 °C for mercaptomethyl-PNA·DNA duplexes **16·23** and **17·24**, respectively. A comparison with the T_M of 70 and 75 °C determined for the corresponding nonmodified PNA·DNA duplexes **21·23** and **22·24**, respectively, indicated that the introduction of mercaptomethyl groups in duplexes **16·23** and **17·24** had no negative effect on the DNA affinity of PNA probes. Likewise, the presence of the CPM fluorophore in CPM-PNA·DNA duplex **20·23** (T_M = 69 °C) led to a minor decrease of duplex stability. The results from CPM labeling and hybridization studies suggest that the mercaptomethyl group in PNAs **16**, **17**, and **18** are directed to the exterior of the PNA·DNA duplex where unhindered access for the attachment of reporter groups is granted.

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Table 2. Melting Temperatures of DNA·PNA Duplexes[a]

	15 mer PNA			24 mer PNA	
	$(HSCH_2)$PNA	PNA	CPM-PNA	$(HSCH_2)$PNA	PNA
PNA•DNA	**16•23**	**21•23**	**20•23**	**17•24**	**22•24**
T_M/°C	71	70	69	76	75

[a] DNA-oligomers: 5′TGGAGGTGTGGGGAA3′ **23** and 5′GAAAAGATCAAAGGATGTGGGGA3′ **24**. Measurements were performed in a buffer containing 1 μM DNA/PNA oligomers, 10 mM NaCl, and 10 mM NaH_2PO_4 at pH 7.0. Denaturation: 15−90 °C, 1 °C/min, monitored at 260 nm.

In conclusion, we have introduced convergent access to long PNA by developing a native chemical ligation-like technique of PNA segment couplings. The required PNA-monomer features a 1-amino-2-thiol function and was synthesized in five steps starting from commercially available starting materials. The additional mercaptomethyl group in PNA ligation products such as **16** has no effect on DNA affinity. In addition to its utility in PNA fragment ligations the mercaptomethyl group also provides a selectively addressable conjugation site. The presented convergent approach may find applications in the synthesis and fluorescence labeling of difficult PNA-oligomers (>20 bases, purine rich, repetitive nucleotides) and in DNA-template-controlled ligation reactions.

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Supporting Information Available: Experimental procedures and characterization data for all new compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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