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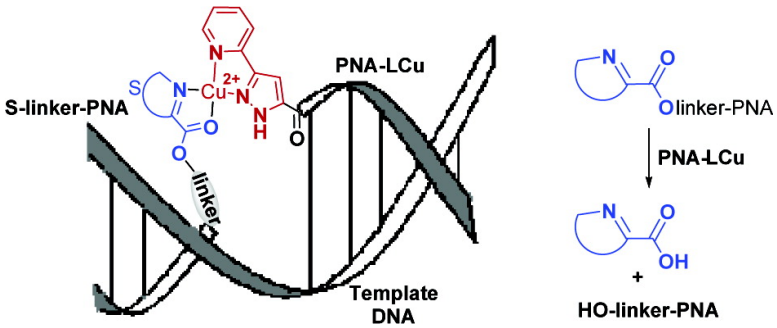

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Templated Metal Catalysis for Single Nucleotide Specific DNA Sequence Detection

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Abstract: A catalytic DNA-templated reaction of hydrolysis of an ester group in an N-modified peptide nucleic acid, which is activated by a Cu^{2+} complex−PNA, has been discovered and optimized. Both the ester-containing PNA and the metal complex PNA bind neighboring sites on a template DNA. This brings the reacting groups (the ester and the Cu^{2+} complex) in proximity to each other and accelerates the hydrolysis of the ester ~500 times in comparison with its hydrolysis in the absence of the template. The hydrolysis reaction provides $>10^2$-fold kinetic discrimination between DNAs that are different from each other at a single nucleotide position. Natural enzyme T4 DNA ligase is slightly less selective. On the basis of this reaction a fully homogeneous and sensitive assay for sequence-specific DNA detection has been developed (10 fmol DNA). Identification of one of four DNAs (variation at one position) can be done in a single experiment. Since the Cu^{2+} ion is tightly bound in an associate containing the ester PNA, the metal complex PNA, and the template DNA, application of this method in buffers containing other Cu^{2+}-binding ligands, e.g., PCR buffer and physiological buffer, is possible.

Introduction

Sequence-specific detection of nucleic acids is a crucial research aspect in biological sciences and is becoming increasingly important in diagnostics and genomics.[1] Since the amount of nucleic acids in biological samples is limited, a hybridization event is usually used as a trigger of some catalytic reactions to amplify a nucleic acid target. Practically important homogeneous catalytic assays, which are triggered by hybridization between a probe and a nucleic acid target, include, for example, polymerase chain reaction (PCR), Invader assay, ligase chain reaction (LCR), strand displacement amplification (SDA), and rolling cycle amplification (RSA).[1] In all of them enzyme-catalyzed reactions are used, since they have quick turnover rates and are highly processive. Although they are quite useful, the enzymatic methods have several limitations. In particular, enzymes are often inefficient with short probes, for which the highest sequence specificity is achieved, they do not recognize non-natural or heavily modified probes, and enzyme-based assays are unlikely to be used in vivo, because of difficulties of enzyme delivery into cells.

Some recent studies have been devoted to coupling a hybridization event with nonenzymatic reactions in an attempt to develop methods lacking the mentioned drawbacks.[2] For example, Kool et al. have described phosphothioate−iodide DNA ligation on template DNAs and RNAs.[3] This reaction is more specific in terms of sequence fidelity than analogous enzymatic ligation catalyzed by T4 DNA ligase. However, since it is strongly inhibited by its products, catalytic turnovers can be achieved only in the presence of enormous excess (10^4-fold) of DNA probes. Sensitivity of this method is further decreased by slow turnover rates. An alternative strategy has been reported by Taylor et al.[4] He has described DNA- or RNA-templated hydrolysis of activated esters in the presence of imidazole derivatives and DNA-templated Staudinger reaction. An important advantage of these templated cleavage reactions is that the problem of product inhibition is much less pronounced, since affinities of products and substrates to the template DNA are not very different. Therefore, sensitivity of DNA detection can be improved due to catalytic turnover.

We have recently communicated a DNA-templated reaction of hydrolysis of picolinate ester (S) in an N-modified peptide

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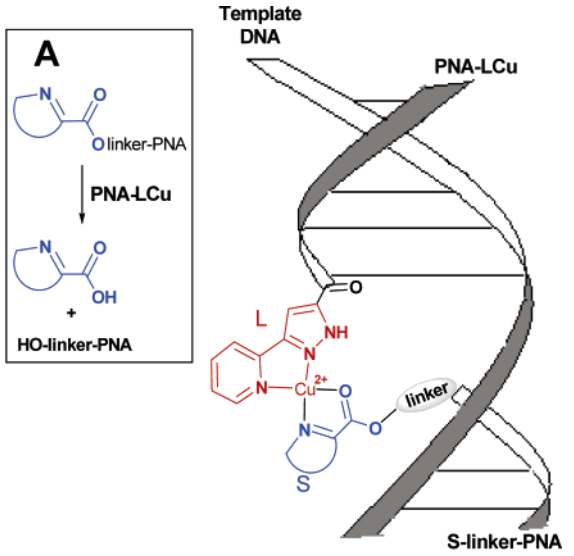

Figure 1. Suggested structure of a complex S/CuL formed upon mixing S-linker-PNA, PNA-LCu, and a template DNA, which is responsible for activation of the ester group (S) toward hydrolysis. DNA-templated ester hydrolysis catalyzed by the Cu^{2+} complex is shown in inset A.

nucleic acid (S-linker-PNA) by a Cu^{2+} complex−PNA (PNA-LCu, Figure 1).[5]

Metal catalysis is generally more efficient and more versatile than organocatalysis. PNA is a neutral DNA analogue, which binds nucleic acids strongly and sequence specifically.[6] Both the S-linker-PNA and the PNA-LCu bind neighboring sites on a template DNA, which brings the reacting groups (S and LCu) in proximity to each other and accelerates the hydrolysis of the substrate ester (S). We have found that shortening the length of the linker between the ester group and the PNA fragment from four to two atoms accelerates templated hydrolysis significantly. In this paper we present results of further variation of the S-linker-PNA structure. In particular, direct conjugation of ester groups to PNA (no linker) and substitution of Cu^{2+}-anchoring group (2-pyridyl, S) for a stronger Cu^{2+} binder (*N*-methyl-2-imidazolyl)[7] have been studied. The latter modification could lead to stabilization of the complex S/CuL, which is responsible for catalytic ester hydrolysis, and acceleration of the templated hydrolysis reaction (Figure 1). A MALDI-TOF mass spectrometric assay for indirect DNA analysis has been developed based on templated hydrolysis of the optimized S-linker-PNAs. MALDI-TOF mass spectrometry is an accurate, sensitive, and rapid method of analysis, which allows multiplexing and can be used in high-throughput genotyping.[8] The indirect DNA analysis via mass spectrometric detection of PNA rather than DNA probes has two important advantages: a higher sensitivity and the possibility of analysis of samples in biological buffers containing usually high concentrations of metal ion salts. Methods of DNA analysis using either PNA−DNA binding or stoichiometric DNA-templated reactions of PNA probes[9] have been reported. Catalytic reactions of PNA probes, which could offer additional sensitivity due to catalytic turnover, have not yet been applied for DNA analysis.

Results and Discussion

Synthesis. The structure of the linker between the picolinate fragment and the PNA N-terminus affects the rate of S-linker-PNA hydrolysis in the DNA-templated reaction. In our previous communication four- and two-atom linkers have been studied.[5] To test a system with the ester fragment directly attached to the PNA terminus (no linker), we have synthesized a PNA that has an alcohol group in place of the N-terminal amino group, PNA **5** (Scheme 1). Synthesis has been started from the preparation of PNA **2** using a standard solid phase synthesis protocol. Then the N-terminus of PNA **2** has been reacted with bromoacetyl bromide to attach a bromoalkyl group, which was further aminated using aminoethanol. The resulting alkylamino alcohol (PNA **4**) has been O- and N-acylated by 1-*N*-thymine-acetic acid, which was preactivated by a HBTU, HOBT, DIEA mixture.[10] The O-acyl group of the diacylated product has been selectively hydrolyzed using concentrated aqueous NH_3 to give PNA **5**, >90% yield over steps b−d starting from PNA **2**. Coupling of picolinic acid, which was activated by DIC, cleavage, deprotection of the resulting conjugate using a TFA/*m*-cresol mixture, and final HPLC purification has afforded a PNA with the ester group directly attached to the modified PNA terminus (PNAs **6a** and **6b**). Additionally, the picolyl fragment of previously reported conjugates **12** and **13** has been substituted for a 1-*N*-methyl-2-imidazolyl fragment to test whether donor properties of the Cu^{2+} anchoring group affect the rate of the templated ester hydrolysis. These derivatives (PNAs **9**, **11a**,**b**) have been synthesized by acylation of HO-linker-PNAs **8** and **10** using DIC-activated 1-*N*-methyl-2-imidazolylcarboxylic acid (Schemes 1, 2). The obtained conjugates have been cleaved from the solid support and deprotected using a TFA/*m*-cresol mixture and finally purified by HPLC.

Optimization of the Templated Hydrolysis Reaction. Some transition metal ions are known to accelerate hydrolysis of esters containing metal-anchoring donor groups, e.g., picolinic and amino acid esters.[11] We have tested DNA-templated hydrolysis of PNA **12** (1 μM) by M^{2+}-PNA **14a** (1 μM) at pH 7 (MOPS 10 mM, NaCl 50 mM) in the presence of different metal ions (1 μM): $M^{2+} = Cu^{2+}$ ($CuSO_4$), Ni^{2+} ($NiSO_4$), Zn^{2+} ($ZnCl_2$), and Pd^{2+} ($Pd(O_2CCH_3)_2$). Except for Zn^{2+}, all listed ions are expected to form stable complexes with PNA **14a** at micromolar concentration, since at these conditions the corresponding M^{2+}-PNA **14a** complexes can be detected by MALDI-TOF mass

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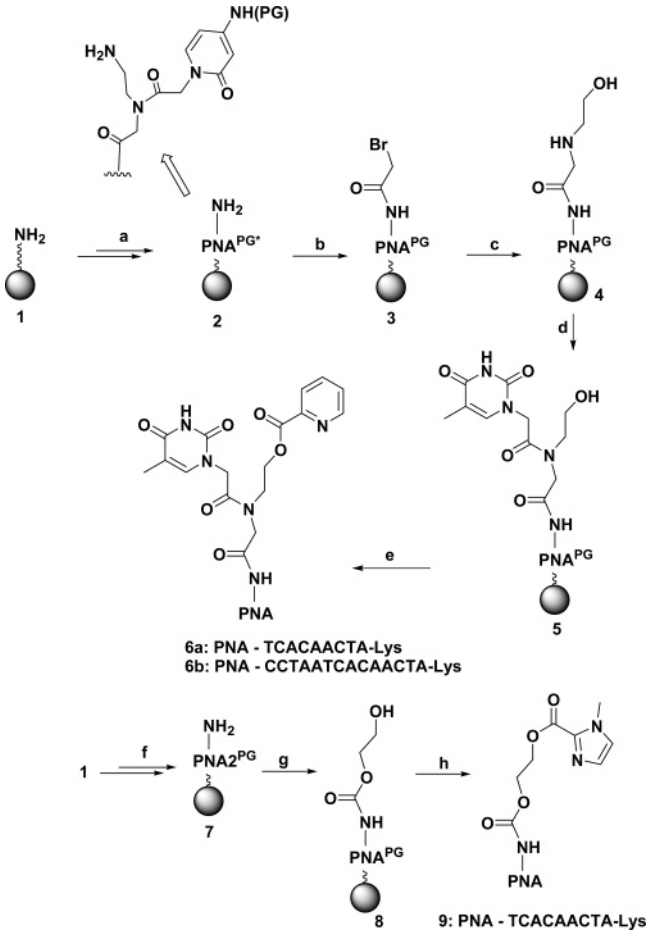
Scheme 1. Synthesis of Peptide Nucleic Acids Modified with Ester Groups at the N-Terminus[a]



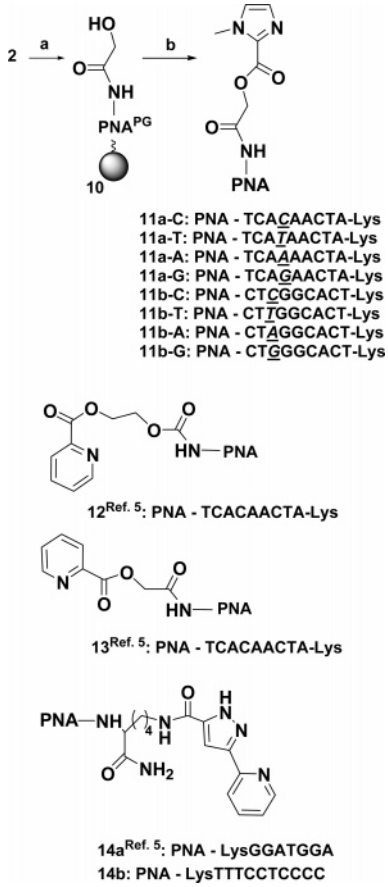
[a] (a), (f) PNA synthesis; (b) $BrCH_2C(O)Br$, DIEA; (c) $HO(CH_2)_2NH_2$, DIEA; (d) 1. 1-*N*-thymineacetic acid, HBTU, HOBT, DIEA, 2. NH_3 aq; (e) 1. picolinic acid, DIC, DMAP, 2. TFA, *m*-cresol; (g) 1. CDI, 2. $HO(CH_2)_2OH$, NaH; (h) 1. 1-*N*-methyl-2-imidazolyl carboxylic acid, DIC, DMAP, 2. TFA, *m*-cresol.

spectrometry. PNA probes, complementary DNA, buffer, and metal ions were incubated at room temperature for 60 min, 120 min, and 24 h before analysis.

Zn^{2+} and Pd^{2+} do not substantially accelerate DNA-templated hydrolysis of PNA **12** in comparison with its hydrolysis in the absence of the template, while Cu^{2+} and Ni^{2+} provide considerable acceleration, with the former ion being ~10 times more active. Moreover, background hydrolysis was found to be considerably faster in the presence of Ni^{2+}, which probably reflects lower binding affinity of Ni^{2+} toward PNA **14a**. Therefore, all further experiments have been conducted with Cu^{2+} ions. At room temperature Cu^{2+}-promoted templated hydrolysis is very slow, producing <5% of the product in 24 h. The optimal temperature of this reaction was found to be around 35 °C. At these conditions templated hydrolysis of picolinic esters is fast, while background nontemplated hydrolysis is still very slow. Moreover, the presence of >50 mM NaCl is required to achieve sufficient rate of exchange of the hydrolysis product for the intact S-linker-PNA. In the absence of NaCl this reaction is strongly inhibited by its products, presumably due to the slow exchange.

Cleavage of S-linker-PNAs by the PNA-LCu is strongly accelerated by catalytic amounts of a DNA template (Figure

Scheme 2. Synthesis of Peptide Nucleic Acids Modified with Ester Groups at the N-Terminus[a]



[a] (a) 1. $AcOCH_2CO_2H$, HBTU, HOBT, DIEA, 2. NH_3 aq; (j) 1. 1-*N*-methyl-2-imidazolyl carboxylic acid, DIC, DMAP, 2. TFA, *m*-cresol.

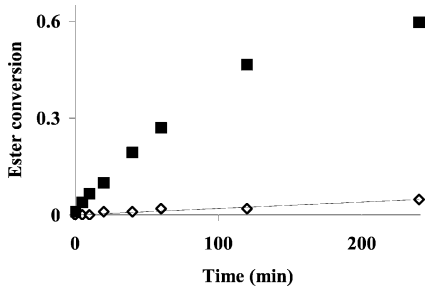


Figure 2. Hydrolysis of PNA **6a** (5 μM) by Cu^{2+}-PNA **14a** (1 μM) in the presence (filled squares) and absence (open diamonds) of DNA **15** (1 μM). 10 mM MOPS pH 7, NaCl 50 mM. DNA sequences and numbering are given in Scheme 3.

2). Ratios of catalyzed to background hydrolysis rates (V_0^{cat}/V_0^{uncat}) of PNA **6a** (no linker between ester group and PNA) and PNA **12** (four-atom linker) are similar to each other and smaller than V_0^{cat}/V_0^{uncat} found for PNA **13** (two-atom linker) (Table 1). Additionally, melting points of duplexes **6a**/**15** and **12**/**15** are 3.5−7.1 °C smaller than that of **13**/**15** (Table 1). However, these T_m's are still >12 °C above the temperature used for the templated hydrolysis reaction, which indicates that the duplexes are fully formed at these conditions. It is known that motions of molecular fragments within helical structures become more pronounced at temperatures close to melting points. This effect is especially strong at duplex termini. Such motions destabilize the structure of the S/CuL complex formed on DNA templates and, therefore, are expected to decrease the

Table 1. Optimization of Signal-to-Background Ratio for DNA-Triggered Ester−PNA Hydrolysis

S-linker-PNA/PNA-LCu/DNA	anchor[a]	linker	V_0^{cat}/V_0^{uncat} [b]	T_m (°C)[c]
11a-C/Cu-14a/15	Im	$-CH_2C(O)-$	487	55.0 ± 1.4
9a/Cu-14a/15	Im	$-(CH_2)_2OC(O)-$	145	51.2 + 1.5
6a/Cu-14a/15	Py	none	118	47.1 ± 1.3
6b/Cu-14a/27	Py	none	112	64.4 ± 0.4
6b/Cu-14a/28	Py	none	84	
6b/Cu-14a/29	Py	none	42	
6b/Cu-14a/30	Py	none	42	
6b/Cu-14a/31	Py	none	1	
13/Cu-14a/15	Py	$-CH_2C(O)-$	485[ref 5]	54.2 ± 1.6
12/Cu-14a/15	Py	$-(CH_2)_2OC(O)-$	150[ref 5]	50.7[ref 5]

[a] Anchors: Im, *N*-methyl-2-imidazolyl. Py, 2-pyridyl. [b] Initial rates have been used in the calculation of V_0^{cat}/V_0^{uncat} values; standard deviations of these values are less than ±10%. [c] [S-linker-PNA] = [PNA-LCu] = [DNA] = 2 μM, 10 mM MOPS pH 7, NaCl 50 mM. PNA and DNA sequences and numbering are given in Schemes 1−3.

Scheme 3. Sequences and Numbering of DNAs

DNA 15: TAGTT*G*TGATCCATCC
DNA 16: TAGTT*A*TGATCCATCC
DNA 17: TAGTT*T*TGATCCATCC
DNA 18: TAGTT*C*TGATCCATCC
DNA 19: AGTGCC*G*AGTCCATCC
DNA 20: AGTGCC*A*AGTCCATCC
DNA 21: AGTGCC*T*AGTCCATCC
DNA 22: AGTGCC*C*AGTCCATCC
DNA 23: TAGTT*G*TGAGGGGAGGAAA
DNA 24: TAGTT*A*TGAGGGGAGGAAA
DNA 25: TAGTT*T*TGAGGGGAGGAAA
DNA 26: TAGTT*C*TGAGGGGAGGAAA
DNA 27: TAGTTGTGATTAGGTCCATCC
DNA 28: TAGTT*T*TGATTAGGTCCATCC
DNA 29: TAGTTGT*T*ATTAGGTCCATCC
DNA 30: TAGTTGTGAT*G*AGGTCCATCC
DNA 31: TAGTTGTGATTAGGTCC*C*TCC

rate of the templated hydrolysis reaction. Due to lower melting points, the mentioned destabilization is expected to be stronger for duplexes **6a**/**15** and **12**/**15**. Another reason for the observed differences in V_0^{cat}/V_0^{uncat} values may be that the two-atom linker of PNA **13** is optimally suited for formation of the S/CuL complex on the DNA template, whereas this complex is destabilized in case of the longer linker of PNA **12** due to entropy loss and in case of the shorter linker of PNA **6a** due to enthalpy loss. T_m of the **6a**/**15** duplex can be tuned by choosing another PNA sequence. We have selected a 14-mer PNA, PNA **6b**, to achieve a substantial increase of T_m of the corresponding PNA/DNA duplex, +17.3 °C (Table 1). Surprisingly, the rates of templated hydrolysis of PNA **6b** and shorter PNA **6a** have been found to be practically the same (Table 1). This suggests that the linker in the S-linker-PNA affects the templated hydrolysis reaction directly via stabilization/destabilization of the complex S/CuL rather than indirectly via stabilization/destabilization of S-linker-PNA/DNA duplexes. A substitution of the 2-pyridyl fragment in PNA **12** and **13** for the *N*-methyl-2-imidazolyl fragment (PNAs **9**, **11a**, **11b**) does not significantly alter the rates of the DNA-templated reaction.

In our preliminary communication we have reported that a single mismatch within duplexes **12**/**15** and **14a**/**15** reduces the initial cleavage rate 7−15-fold, which is significantly worse than the difference between hydrolysis in the presence and absence of the template, $V_0^{cat}/V_0^{uncat} > 100$.[5] This indicates that at least some part of the mismatched DNAs bind the catalyst and the substrate. Though a single mismatch in PNA/DNA duplexes leads usually to a significant decrease of their stability, the magnitude of this effect is dependent on the position of the mismatch.[12] Potentially it should be possible to achieve the single mismatch discrimination equal to V_0^{cat}/V_0^{uncat} values depending on the mismatch position. This would correspond to the situation when all mismatched DNA is not bound to its target PNA, while all complementary DNA is bound. We have demonstrated that such selectivity can be achieved in the templated metal-catalyzed reaction by variation of the mismatch position in the template DNA. Hydrolysis of PNA **6b** in the presence of DNAs **28**−**31** containing single mismatches in different positions has been conducted (Table 1). The most duplex-destabilizing mutation in DNA **27**, A18→C18, was found to be positioned opposite the PNA-LCu, and the corresponding mutated DNA **31** does not act at all as a template in the hydrolysis of PNA **6b** ($V_0^{cat}/V_0^{uncat} = 1$). This corresponds to a 112-fold single mismatch discrimination. Natural enzyme T4 DNA ligase reaches 10^2-fold discrimination at optimal conditions[13] and more selective *Tth* ligase from *Thermus thermophilus* $\sim 10^3$-fold.[14] In chemical ligations of DNA probes on nucleic acid templates $\sim 10^4$-fold discrimination of single mismatches has been achieved,[2] while ligations of PNA probes provide $\sim 10^2$-fold discrimination.[9c]

The DNA-templated hydrolysis reaction may be potentially inhibited by additional Cu^{2+} binding ligands, which compete with the ester substrate for free sites of the metal ion. This could become a significant limitation of metal complex based methods of detection of unpurified nucleic acids, which were obtained by PCR or directly from biological samples. Such samples may contain nucleotide triphosphate, phosphate, and chloride anions, all of which bind and inhibit catalytic activity of Cu^{2+} when present at high concentrations. Additionally, high concentration of sodium and potassium ions in the mentioned media may hinder direct analysis of probes by mass spectrometry. Therefore, a prepurification step is usually used in known methods of mass spectrometric detection of DNA.[1,9c,15] Surprisingly, the DNA-templated ester hydrolysis reaction reported here does work in a typical PCR buffer (buffer B, Experimental Section) as well as in a physiological buffer (buffer C, Experimental Section). This may be attributed to the exceptionally high stability of the complex S/CuL, which is probably a result of the preorganization of the corresponding ligands on a DNA template (Figure 1). This is in agreement with the fact that the DNA-templated reaction is not substantially inhibited by up to

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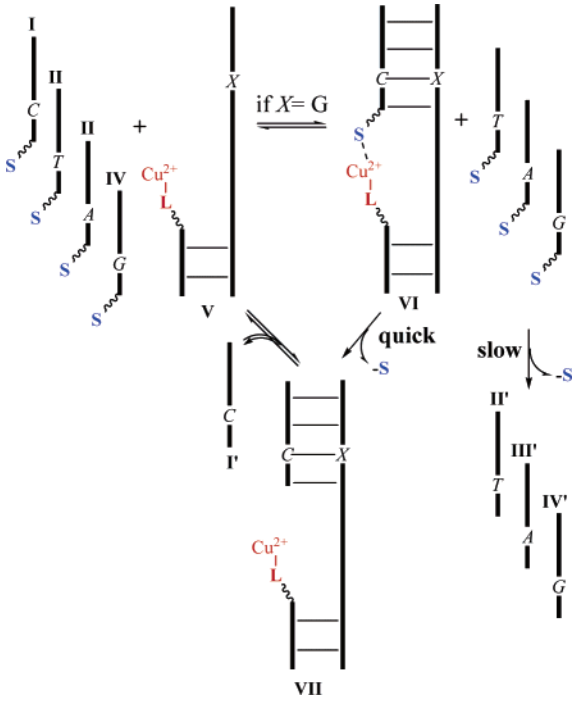

Figure 3. Application of DNA-templated ester hydrolysis in sequence-specific DNA detection. Compounds **I**−**IV**: S-linker-PNAs, which differ from one another at a single position in a PNA strand (C, T, A, or G nucleaobase); an ester group is marked with **S** and the PNA strand is shown as a stick. Associate **V** is a duplex between a template DNA (shown as a stick with **X**, which represents an unknown nucleotide) and a PNA-LCu (catalyst). The template is complementary to only one of four PNAs **I**−**IV**. The complementary PNA forms associate **VI** with **V** (shown on example of PNA **I**), in which the ester (**S**) is activated due to coordination to Cu^{2+}-L and is quickly hydrolyzed by water.

2 equiv of the hydrolysis products, picolinic acid and *N*-methyl-2-imidazolylcarboxylic acid (Figure 2), which have considerably higher affinity toward Cu^{2+} than their esters do. Moreover, substitution of the 2-pyridyl fragment for the stronger Cu^{2+} binder, *N*-methyl-2-imidazolyl fragment,[7] in the substrate ester does not alter the rate of the templated ester hydrolysis. This may indicate that Cu^{2+} is already fully bound in the associate **VI** (Figure 3) containing the picolyl ester−PNA at concentrations used for the assays.

MALDI-TOF Mass Spectrometric Assay for Detection of Single Nucleotide Variations in DNA. MALDI-TOF mass spectrometry is a highly sensitive and mild method of analysis of short DNA and PNA probes. A time-of-flight (TOF) detector provides excellent resolution. Monocharged ions are usually detected, while peaks corresponding to doubly charged species as well as gas phase monocharged dimers have very low intensity. Therefore, each component of the mixture gives practically a single peak in the mass spectrum.[16] In contrast, in ESI mass spectra one can detect several charged forms per single compound. MALDI-TOF mass spectrometry has been recently applied in quantitative analysis of DNA,[17] PNA,[5] and antibiotics.[18] These factors make MALDI-TOF mass spectrometry a method of choice for analysis of complicated mixtures.

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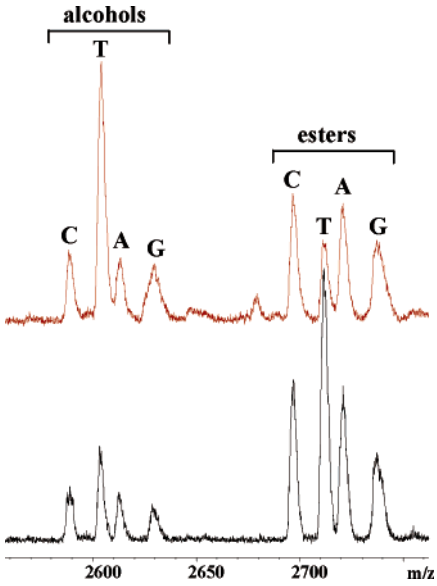

Figure 4. MALDI-TOF mass spectra of a mixture of S-linker-PNAs **11a-C**, **-T**, **-A**, **-G** (each 5 μM) and the catalyst Cu^{2+}-PNA **14a** (1 μM) with (orange spectrum) and without (black spectrum) template DNA **16** (1 μM) taken 60 min after component mixing. During the hydrolysis reaction the S-linker-PNAs (peaks in MS are labeled 'esters') are transformed to alcohols (peaks are labeled 'alcohols'). 10 mM MOPS, pH 7, NaCl 50 mM. PNA and DNA sequences and numbering are given in Schemes 1−3.

Among all studied S-linker-PNAs the best selectivity (V_0^{cat}/V_0^{uncat}) in the templated hydrolysis reaction has been observed for PNA **11a-C**, which contains an *N*-methyl-2-imidazolyl fragment attached to the N-terminus of PNA via the two-atom linker. This PNA conjugate and its analogues have been further tested in mass spectrometric assays. In the typical experiment a mixture of four S-linker-PNAs (**I**−**IV**, Figure 3), which differ from one another at a single position in a PNA strand (C, T, A, or G nucleobase), was allowed to react with catalytic amounts (0.2 equiv) of associate **V**. The latter is a duplex between the PNA-LCu and a DNA template and is complementary to one of the S-linker-PNAs. This complementary S-linker-PNA binds the associate **V**, forming **VI**, and its ester group is hydrolyzed due to intramolecular activation by the Cu^{2+} complex of the PNA-LCu. The mismatched S-linker-PNAs are hydrolyzed more slowly due to less efficient intermolecular activation by the Cu^{2+} complex. On the basis of these different reactivities one can identify a sequence of the analyte (template) DNA.

MALDI-TOF mass spectra of mixtures containing four S-linker-PNAs **11a-C**, **-T**, **-A**, **-G**, and the catalyst Cu^{2+}-PNA **14a** with and without DNA **16**, which is complementary to PNA **11a-T**, are shown in Figure 4. These spectra were acquired 60 min after mixing the mentioned components. As expected PNA **11a-T** is cleaved significantly faster than the other PNAs in the presence of the template, while all substrates are cleaved at a similar rate in the absence of the template. When other template DNAs (**15**, **17**, **18**) are used in place of DNA **16**, the corresponding complementary PNAs are cleaved preferentially. This assay could be repoduced with other S-linker-PNAs (**11b-C**, **-T**, **-A**, **-G**) and corresponding DNA templates **19**−**22**, which suggests its generality. It should be noted that the PNA sequence in the PNA-LCu conjugate seems to be unimportant as long as the conjugate fully binds the template DNA. In particular, hydrolysis of PNA **11a** mixture by Cu^{2+}-PNA **14b** in the presence of any of DNA templates **23**−**26** is as efficient and

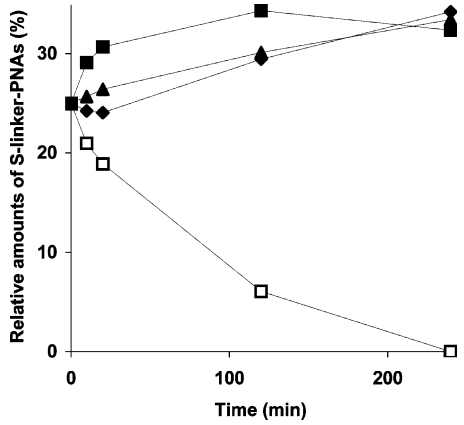

Figure 5. Relative amounts (in %) of individual S-linker-PNAs **11a-C**, **-T**, **-A**, **-G** in their mixture. Initially an equimolar mixture of S-linker-PNAs (5 μM) is present, which corresponds to 25% of each PNA at time = 0. The mixture also contains the catalyst Cu^{2+}-PNA **14a** (1 μM) and the template DNA **16** (1 μM), MOPS 10 mM pH 7, NaCl 50 mM. Filled diamond: **11a-C**, open squares: **11a-T**, filled triangles: **11a-A**, filled squares: **11a-G**. PNA and DNA sequences and numbering are given in Schemes 1−3.

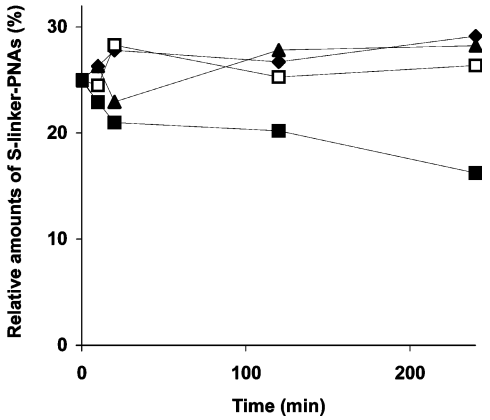

Figure 6. Relative amounts (in %) of individual S-linker-PNAs **11a-C**, **-T**, **-A**, **-G** in their mixture. Initially an equimolar mixture of S-linker-PNAs (5 μM) is present, which corresponds to 25% of each PNA at time = 0. The mixture also contains the catalyst Cu^{2+}-PNA **14a** (1 μM) and MOPS 10 mM pH 7, NaCl 50 mM. Filled diamond: **11a-C**, open squares: **11a-T**, filled triangles: **11a-A**, filled squares: **11a-G**. PNA sequences and numbering are given in Schemes 1−3.

selective as that by Cu^{2+}-PNA **14a** in the presence of any of DNAs **15**−**18**. We have determined changes in relative concentrations of S-linker-PNAs **11a-C**, **-T**, **-A**, **-G** during the templated ester hydrolysis using areas of the corresponding peaks in the mass spectra. Templated hydrolysis of PNA **11a-T** is complete after 4 h, which corresponds to 5 turnovers (Figure 5). As expected in the absence of any template DNA, relative amounts of the S-linker-PNAs are almost constant over first 4 h of hydrolysis, except that a slight decrease of the PNA **11a-G** amount is observed (Figure 6). The latter effect is due to precipitation of the mentioned PNA.

In dilute solutions the time of the templated hydrolysis reaction increases and, therefore, precipitation of PNA **11a-G** becomes a serious problem. However, DNA detection is still possible by analysis of intensities of products of the hydrolysis reaction, since they are more soluble in water (Figure 7). Sensitivity of the DNA-templated hydrolysis reaction in DNA detection has been tested using decreasing concentrations of the analyte DNA, the corresponding S-linker-PNA, and the PNA-

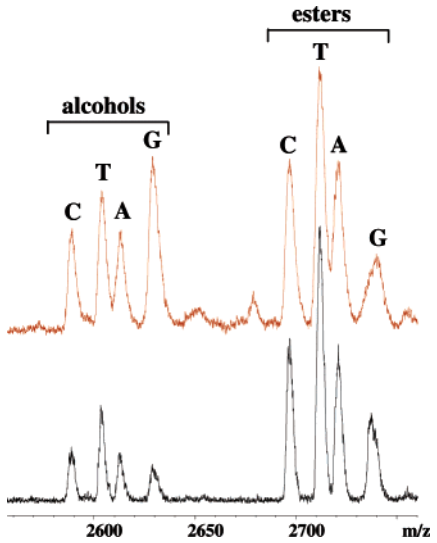

Figure 7. MALDI-TOF mass spectra of a mixture of S-linker-PNAs **11a-C**, **-T**, **-A**, **-G** (each 5 μM) and the catalyst Cu^{2+}-PNA **14a** (1 μM) with (orange) and without (black spectrum) the template DNA **18** (1 μM) taken 60 min after component mixing. 10 mM MOPS pH 7, NaCl 50 mM.

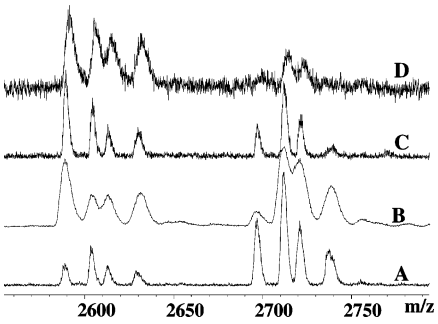

Figure 8. MALDI-TOF mass spectra of mixtures of S-linker-PNAs **11a**−**C**, **-T**, **-A**, **-G** (5 equiv each), Cu^{2+}-PNA **14a** (1 equiv), and different amounts of DNA **15** (A: no DNA, B: 1 pmol, C: 100 fmol, D: 10 fmol) acquired correspondingly 0, 1, 5, and 9 h after component mixing. 10 mM MOPS pH 7, NaCl 50 mM. PNA and DNA sequences and numbering are given in Schemes 1−3.

LCu (Figure 8). Even in the presence of only 10 fmol of analyte DNA the highest intensity peak in the region of the spectrum, where products appear, corresponds to the product of hydrolysis of the S-linker-PNA complementary to the analyte DNA.

In reported methods of indirect analysis of DNA sequences based on detection of PNA probes by MALDI-TOF mass spectrometry sensitivity is mainly defined by the instrument intrinsic sensitivity as well as efficiency of the analyte recovery after prepurification step(s). PNA−DNA binding as well as stoichiometric reactions of PNA probes have been used in such assays. Sensitivity of DNA detection by these methods has been reported not to exceed 0.5 pmol of DNA, which is less than the sensitivity of MALDI-TOF mass spectrometry at optimal conditions.[9] This can probably be explained by the analyte loss during its purification before analysis. The analysis method reported here combines high sensitivity of MALDI-TOF mass spectrometry in PNA detection and signal amplification due to catalytic turnover. PNA is analyzed directly from reaction mixtures without prepurification to minimize PNA losses, which is an important advantage, since PNA tends to bind to surfaces of reaction vials, HPLC columns, etc. This is the first assay for DNA detection in which metal complex catalyzed reaction is used.

One can envision that application of other more efficient metal catalyst−substrate pairs may shorten analysis time and, potentially, increase catalysis turnover.

Conclusions

The DNA-templated hydrolysis of an ester in a substrate PNA catalyzed by a Cu^{2+} complex of a catalyst PNA has been optimized. This reaction provides >100-fold kinetic discrimination between DNAs that are different from each other at a single nucleotide position. Natural enzyme T4 DNA ligase is slightly less selective. On the basis of this reaction a fully homogeneous, sensitive assay for sequence-specific DNA detection has been developed (10 fmol DNA). Identification of one of four DNAs (variation at one position) can be done in a single experiment. Since the Cu^{2+} ion is tightly bound in an associate containing the substrate PNA, the catalyst PNA, and the template DNA, application of this method in buffers containing other Cu^{2+}-binding ligands, e.g., PCR buffer and physiological buffer, is possible.

Experimental Section

The best commercially available chemicals from Acros (Geel, Belgium), Aldrich/Sigma/Fluka (Deisenhofen, Germany), Advanced Chemtech (Louisville, KY), and Novabiochem (Läufeltingen, Switzerland) were obtained and used without purification. HPLC-purified DNA was obtained from Metabion. Buffers used for templated reactions: buffer A, MOPS 10 mM, pH 7 and NaCl 50 mM; buffer B, ATP 200 μM, $MgCl_2$ 1.5 mM, KCl 50 mM; buffer C, phosphate buffer 10 mM, pH 7, NaCl 150 mM.

The reagents for PNA synthesis were obtained from PerSeptive Biosystems (Hamburg, Germany). MALDI-TOF mass spectra were recorded on a Bruker BIFLEX III spectrometer. The solution of 3,5-dimethoxy-4-hydroxycinnamic acid (27 mM in TFA 1%/CH_3CN 33.9%/MeOH 33%/H_2O 33%) was used as a matrix for MALDI-TOF analysis of the PNA conjugates. Samples for mass spectrometry were prepared on a Bruker MAP II probe preparation station using a dried droplet method with 1:2 probe/matrix ratio for water and water/CH_3CN solutions (HPLC fractions) and a 1:20 ratio for TFA/*m*-cresol (4:1) solutions. Mass accuracy with external calibration was 0.1% of the peak mass, i.e., ±3.0 at *m*/*z* 3000. Preparative and analytical HPLC was performed at 49 °C on a Shimadzu liquid chromatograph equipped with UV−vis detector, column oven, and Advantec SF-2100W fraction collector. A Macherey-Nagel Nucleosil C4 250 × 4.6 mm column with gradients of CH_3CN (0.1% TFA, solvent B) in water (0.1% TFA, solvent A) was used: 49 °C, 0% B for 5 min, in 30 min to 35% B, in 10 min to 90% B, 90% B for 10 min.

Synthesis of PNA Conjugates. The PNA part of conjugates (Scheme 1, PNA **2** and PNA **7**) was synthesized on an Expedite 8909 PNA/DNA synthesizer according to the manufacturer's recommendations for 2 μM scale synthesis. A lysine residue was attached to the PNA C-terminus using Fmoc-Lys(Boc)-OH to increase PNA solubility in aqueous buffers.

PNAs 6a. Bromoacetyl bromide (8.6 μL, 100 μmol) was dissolved in DMF (1 mL) and mixed with DIEA (38 μL, 200 μmol). The resulting dark solution was added to Rink resin bound PNA **2** (~2 μmol of terminal NH_2 groups). The suspension was mixed at 22 °C for 30 min using an automatic mixer with temperature-controlling capability, filtered, washed with 2 × DMF (2 mL) and 2 × CH_3CN (2 mL), and dried at 0.01 mbar. A portion of dry resin (PNA **3**) was treated with TFA/*m*-cresol (4:1, v/v, 20 μL) for 90 min at 22 °C, and the solution obtained was analyzed by MALDI-TOF mass spectrometry. Starting material was not detected, while bromoacetylated PNA **3** appeared at 2655.0, calcd for $C_{104}H_{136}N_{54}BrO_{27}$ $[M + H]^+$ 2655.5.

Aminoethanol (22 μL, 350 μmol) and DIEA (61.3 μL, 350 μmol) were dissolved in DMF (1 mL) and added to PNA **3** resin. After 24 h mixing at 22 °C the resin was worked up and analyzed analogously to the protocol used for PNA **3** synthesis. Starting material was not detected, while aminated PNA **4** appeared at 2631.7, calcd for $C_{106}H_{142}N_{55}O_{28}$ $[M + H]^+$ 2634.6.

1-Thymineacetic acid (37 mg, 200 μmol), HBTU (68 mg, 180 μmol), and HOBT (28 mg, 200 μmol) were dried at 0.01 mbar for 30 min, then dissolved in DMF (1 mL), and mixed with DIEA (76 μL, 400 μmol). A slightly darkened solution was added to the PNA **4** resin, and the resulting suspension was mixed for 60 min at 22 °C. After this the resin was worked up and analyzed analogously to the protocol used for PNA **3** synthesis. Starting material was not detected, while O,N-diacylated PNA product appeared at 2964.2, calcd for $C_{120}H_{154}N_{59}O_{34}$ $[M + H]^+$ 2967.9. The product obtained was treated with aqueous ammonia (25%) for 24 h, worked up, and analyzed in a standard way. Starting material was not detected, while deprotected PNA **5** appeared at 2800.3, calcd for $C_{113}H_{148}N_{57}O_{34}$ $[M + H]^+$ 2801.8. Picolinic acid (25 mg, 200 μmol) and HOBT (28 mg, 200 μmol) were dried for 30 min at 0.01 mbar, dissolved in DMF (1 mL), and mixed first with DIC (31 μL, 200 μmol) and then with DMAP (1.2 mg, 10 μmol). After complete DMAP dissolution the resulting solution was added to PNA **5** resin, which was dried for 24 h at 0.01 mbar. The suspension was mixed for 3 h at 22 °C and worked up in a standard way. The PNA **6** obtained was cleaved from the solid support and deprotected using a TFA/*m*-cresol mixture (4:1, v/v, 150 μL), precipitated by addition of 10 volumes of diethyl ether, filtered, washed with diethyl ether (2 × 2 mL), dried, dissolved in water, and purified by HPLC. PNA **6a**: HPLC t_R = 26.0 min. Yield: 2.9%. MALDI-TOF MS: calcd for $C_{119}H_{150}N_{58}O_{32}$ $[M + H]^+$ 2905.9, found 2903.7. PNA **6b** was synthesized analogously. HPLC t_R = 29.7 min. Yield: 3.5%. MALDI-TOF MS: calcd for $C_{172}H_{276}N_{86}O_{46}$ $[M + H]^+$ 4225.1, found 4225.0.

PNA 9. *N*-Methyl-2-imidazolylcarboxylic acid (25 mg, 200 μmol) and HOBT (28 mg, 200 μmol) were dried for 30 min at 0.01 mbar, dissolved in DMF (1 mL), and mixed first with DIC (31 μL, 200 μmol) and then with DMAP (1.2 mg, 10 μmol). After complete DMAP dissolution the resulting solution was added to PNA **8** resin obtained in accordance with the reported protocol.[5] The suspension was mixed for 3 h at 22 °C and worked up in a standard way. The PNA **9** obtained was cleaved from the solid support and deprotected using TFA/*m*-cresol (4:1, v/v, 150 μL), precipitated by addition of 10 volumes of diethyl ether, filtered, washed with diethyl ether (2 × 2 mL), dried, redissolved in water, and purified by HPLC. PNAs **11a**,**b** were obtained analogously, except that in the synthesis of the latter compounds PNA **10** [4] bound to the resin was used in place of PNA **8**. PNA **9**: HPLC t_R = 22 min. Yield: 4.5%. MALDI-TOF MS: calcd for $C_{110}H_{143}N_{56}O_{30}$ $[M + H]^+$ 2728.1, found 2730.4. PNA **11a-C**: HPLC t_R = 21.0 min. Yield: 12.3%. MALDI-TOF MS: calcd for $C_{109}H_{141}N_{56}O_{29}$ $[M + H]^+$ 2700.0, found 2699.4. PNA **11a-T**: HPLC t_R = 21.0 min. MALDI-TOF MS: calcd for $C_{110}H_{142}N_{55}O_{30}$ $[M + H]^+$ 2714.0, found 2714.4. PNA **11a-A**: HPLC t_R = 21.0 min. MALDI-TOF MS: calcd for $C_{110}H_{141}N_{58}O_{28}$ $[M + H]^+$ 2722.9, found 2723.6. PNA **11a-G**: HPLC t_R = 21.0 min. MALDI-TOF MS: calcd for $C_{110}H_{141}N_{58}O_{29}$ $[M + H]^+$ 2738.8, found 2740.7. PNA **11b-C**: HPLC t_R = 23.4 min. Yield: 5.4%. MALDI-TOF MS: calcd for $C_{108}H_{141}N_{54}O_{32}$ $[M + H]^+$ 2708.6, found 2706.2. PNA **11b-T**: HPLC t_R = 23.4 min. MALDI-TOF MS: calcd for $C_{109}H_{142}N_{53}O_{33}$ $[M + H]^+$ 2723.6, found 2721.2. PNA **11b-A**: HPLC t_R = 23.4 min. MALDI-TOF MS: calcd for $C_{109}H_{140}N_{56}O_{31}$ $[M + H]^+$ 2731.7, found 2730.2. PNA **11b-G**: HPLC t_R = 23.4 min. MALDI-TOF MS: calcd for $C_{109}H_{141}N_{56}O_{32}$ $[M + H]^+$ 2748.7, found 2746.1.

PNAs **12**, **13**, and **14a** were obtained as reported,[5] and PNA **14b** was obtained analogously to PNA **14a**. PNA **14b**: HPLC t_R = 22.9 min. Yield: 3.1%. MALDI-TOF MS: calcd for $C_{125}H_{167}N_{54}O_{37}$ $[M + H]^+$ 3019.0, found 3018.2.

UV-Melting Experiments. UV melting experiments were performed on a Varian Cary 100 Bio UV−vis spectrophotometer measuring absorbance at 260 nm in 1 cm optical path black wall semimicrocuvettes with a sample volume of 0.7 mL, using 2 μM strand concentration in MOPS buffer (10 mM, pH 7), NaCl (50 mM). Cooling and heating rates were 0.5 °C/min. Melting points were averages of the extrema of the first derivative of the 61-point smoothed curves from at least two cooling and two heating curves.

MALDI-TOF Mass Spectrometric Assays. In a typical DNA-templated ester hydrolysis reaction, a solution of PNA **14a** (1 μM) and $CuSO_4$ (1 μM) in buffer (MOPS 10 mM, NaCl 50 mM, pH 7) was prepared. This mixture was allowed to stand at 22 °C for 24 h for equilibration, then an equimolar mixture of 4 PNAs **11b** (each 5 μM final concentration) was added. The reaction was initiated by addition of template DNA **14** (1 μM final concentration). Probes (1 μL) were taken at definite times and mixed with 3,5-dimethoxy-4-hydroxy-cinnamic acid matrix (2 μL). The resulting mixture was spotted on a MALDI plate and allowed to dry. Prepared in such a way probes were analyzed by MALDI-TOF mass spectrometry. Each reaction was repeated at least three times.

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