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Kinetic model studies on the chemical ligation of oligonucleotides via hydrazone formation

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Abstract—We report on the suitability of hydrazone formation for activator-free ligation of oligonucleotides. 5′-Acyl hydrazides were synthesized using a previously described phosphoramidite modifier, whereas 3′-hydrazides resulted from a hydrazinolysis of an ester group serving as a linker to the solid support. Aromatic aldehydes could be directly introduced on the 5′-terminus via the respective phosphoramidates. Aliphatic aldehydes were generated by periodate cleavage of the corresponding 3′- and 5′-modified diol precursors. Ligation of a 3′-hydrazide-modified oligonucleotide with oligonucleotides bearing an aromatic aldehyde in 5′-position showed a fast reaction kinetics (k_1 about 10^{-1}) and irreversible hydrazone formation. The ligation of a 5′-hydrazide-modified oligonucleotide and a 3′-ribobisaldehyde appeared to proceed reversibly at the beginning, but became irreversible with increasing reaction time. Hydrazide-modified oligonucleotides were found to be somewhat unstable in aqueous solutions.

1. Introduction

In the context of our search for oligonucleotide based chemical self-replicating systems we were interested in finding ligation methods that are not based on hydrolyzable condensing agents. Former approaches, which made use of the formation of phosphodiesters, pyrophosphates, and phosphoramidates to ligate oligonucleotides always necessitated the addition of activating reagents such as water soluble carbodiimide or cyanogen bromide.[1] Recent publications reported on a number of alternative chemical ligation reactions proceeding without activating reagents using so-called 'pre-activated' building blocks. Examples include the reaction of 3′-phosphorothioate- or 3′-phosphoroselenoate components with 5′-iodine-substituted compounds,[2] the ligation of 3′-phosphorothioates with 5′-mercapto oligonucleotides under formation of monophosphoryldisulfides,[3] the formation of metallosalen-DNA-complexes,[4] the photochemical ligation of oligonucleotides using 5-vinyldesoxyuridine[5] or the template-directed oligomerization of a 5′-amino-3′-aldehyde-substituted nucleoside under reductive amination conditions.[6]

However, the pre-activated building blocks described so far are either unstable, difficult to synthesize, or lack broad applicability. A very promising ligation reaction in this context is the formation of hydrazones using aldehyde-modified and hydrazine or hydrazide-modified oligonucleotides. This chemistry is claimed to be stable, irreversible, easy to use, highly selective, and not susceptible to non-specific binding.[7] It is widely used for the immobilization of oligonucleotides on surfaces or for the conjugation of oligonucleotides with, for example, proteins or small organic compounds.[8] During the course of studies on chemical copying of trisoligonucleotides the formation of hydrazones proved to be particularly suitable.[9] Herein we report on the evaluation of suitable hydrazide- and aldehyde-modified oligonucleotides and on kinetic studies of their ligation reaction.

2. Results and discussion

It is highly desirable to introduce any new functionality at the 3′- and 5′-ends of oligonucleotides as modular building blocks compatible with automated oligonucleotide synthesis. This also holds for aldehyde and hydrazide modifiers needed for hydrazone-based ligation. For both, the 3′-modification of oligonucleotides with

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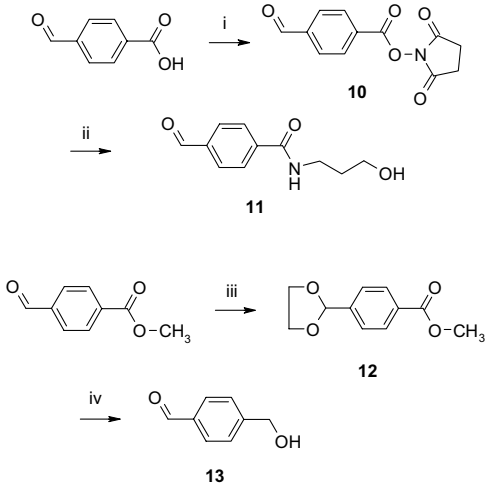
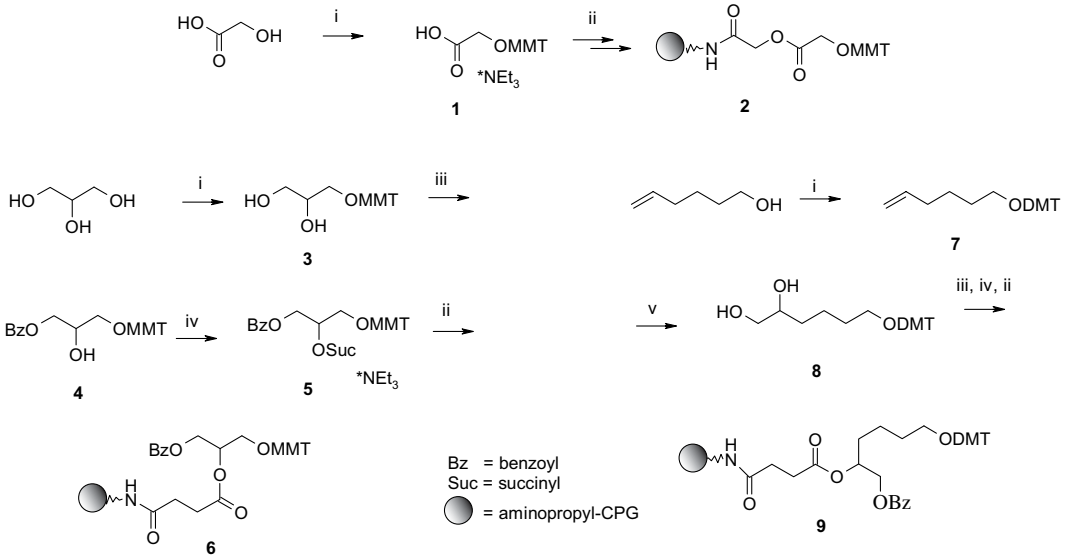
a hydrazide group and with an aldehyde function, the modification of the solid phase used for automated oligonucleotide synthesis seemed most suitable. Solid phase **2** was obtained over several steps: Starting from glycolic acid the hydroxyl group was protected with 4-methoxytrityl chloride (MMT-Cl)/pyridine and the carboxylic group was subsequently reacted with aminopropyl-controlled-pore-glass (CPG) using [2-(1*H*-benzotriazol-1-yl)-1,2,3,3-tetramethyluroniumhexafluorophosphate] (HBTU) as coupling reagent. Cleavage of the MMT-group with trifluoroacetic acid and repeated coupling of **1** gave **2** (Scheme 1). Automated oligonucleotide synthesis was carried out and deprotection of the oligonucleotide (1. $NHEt_2$, dichloromethane, 18 h, rt; 2. 24% NH_2NH_2, 18 h, 4 °C),[8a] removal of excess hydrazine via SepPak®-cartridges and HPLC-purification resulted in 5′-(4,4′-dimethoxytrityl) (DMT)-protected-3′-hydrazide-modified oligonucleotides. 3′-Diol-modified oligonucleotides were employed as precursors for 3′-aldehydes. Solid phases **6** and **9** were used for the synthesis of 3′-diol-modified oligonucleotides. Support **6** was synthesized starting from propane-1,2,3-triol (Scheme 1). Since the synthesis previously described did not result in good yields in our hands,[10] we made use of an alternative synthesis strategy. In the first step, one of the primary hydroxyl groups was protected with DMT-Cl/pyridine. In the second step, the remaining primary hydroxyl group was esterified with benzoyl chloride, and last, the secondary hydroxyl group was acylated with succinic acid anhydride. The fully protected compound was subsequently attached to aminopropyl-CPG using HBTU as coupling reagent. Starting compound for solid phase **9** was 5-hexen-1-ol, which was protected with DMT-Cl/pyridine (Scheme 1). Following oxidation with osmium tetroxide/*N*-methylmorpholine-*N*-oxide to give the vicinal diol, the introduction of the other protective groups and the attachment of the protected compound to the solid phase was performed as described for **2**. 5′-DMT-protected-3′-(vicinal-dihydroxyalkyl)phosphate-modified oligonucleotides were obtained via automated oligonucleotide synthesis on supports **6** and **9**, deprotection (25% ammonia, 6–8 h, 65 °C), and HPLC-purification. These compounds were converted to the respective aldehyde-modified oligonucleotides **20** and **21** (Scheme 4) using sodium periodate (1. 4-fold excess, 75 min, rt; 2. 20-fold excess of sodium thiosulfate, lyophilization). 3′-Ribobisaldehyde **22** was obtained starting from a solid support preloaded with the corresponding ribonucleoside, automated synthesis, cleavage (1. 25% ammonia, 16 h, 55 °C; 2. HF*triethylamine/DMF (3/1), 1.5 h, 55 °C), and glycol oxidation using



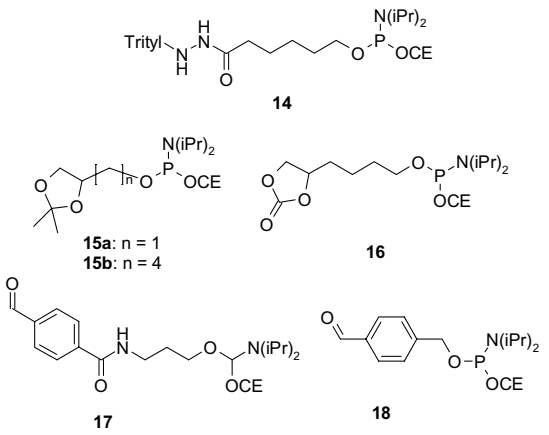
Scheme 2. Synthesis of aromatic alcohols **11** and **13**. Reagents and conditions: (i) *N*-hydroxysuccimide, dicyclohexyl carbodiimide, dioxane/THF, 0 °C; (ii) 3-aminopropane-1-ol, dioxane/water; (iii) ethyleneglycol, toluol, 4-toluenesulfonic acid (cat.); (iv) lithiumaluminium hydride, THF, acidic work-up.



Scheme 1. Synthesis of solid phases **2**, **6**, and **9** for 3′-modification of oligonucleotides. Reagents and conditions: (i) 1.1 equiv MMT-Cl or DMT-Cl, pyridine, 0 °C, 16 h; (ii) HBTU, diisopropylamine, DMF, 1 h; (iii) benzoylchloride, pyridine, 0 °C, 16 h; (iv) succinic acid anhydride, pyridine, 16 h; (v) OsO_4 (cat.), *N*-methylmorpholine-*N*-oxide, acetone/water (8/1), 24 h, 0 °C.

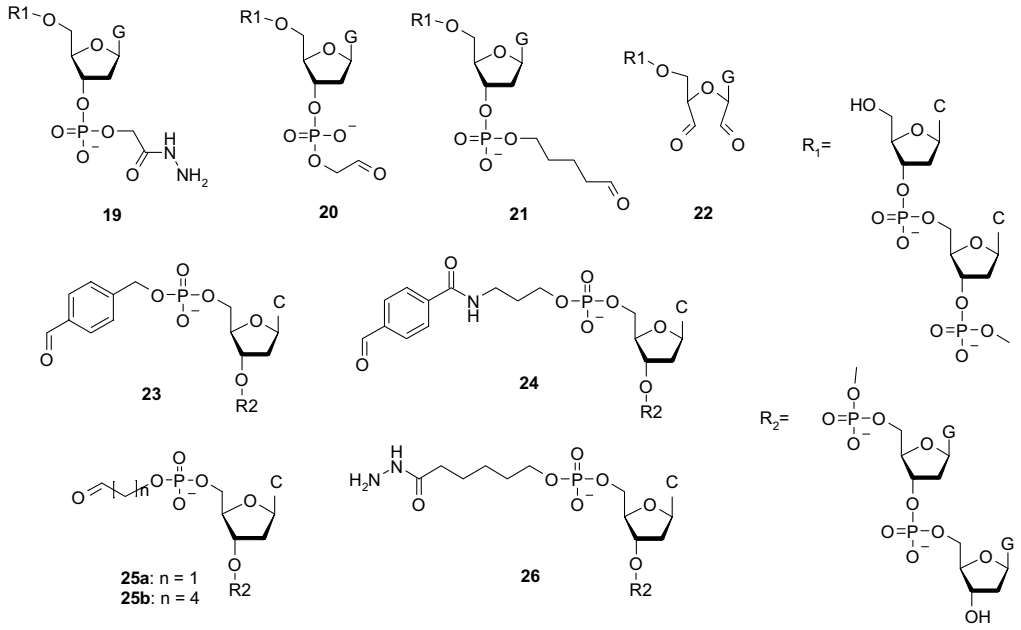
sodium periodate (1. 4-fold excess, 75 min, rt; 2. 20-fold excess sodium thiosulfate).[11]

For 5′-labeling, phosphoramidites derived from the reaction of the appropriate alcohol and *N*,*N*-diisopropyl-chloro-phosphoramidite as phosphitylating reagent were used.[12] 5′-Hydrazide-modified oligonucleotides were synthesized from the phosphoramidite **14** (Scheme 3) as previously described.[8a] For the synthesis of 5′-aldehyde-modified oligonucleotides two different approaches were taken: Amidite building blocks were either prepared from 5′-acetal or 5′-carbonyl-protected diols as precursors or in the case of aromatic and non-enolizable aliphatic aldehydes without protection. Quantitative activation of various 5′-acetal-modified oligonucleotides under strongly acidic conditions failed, presumably due to depurination. Phosphoramidites **15a**, **15b**, and **16** (Scheme 3) were used for 5′-diol modification, whereas the corresponding alcohols are commercially available and the required alcohol for the synthesis of **16** could be obtained by reacting hexan-1,2,6-triol with carbonyldiimidazole analogously to a previously described method.[13] Modified oligonucleotides derived from **15a** and **15b** could be cleaved under standard conditions. Following HPLC-purification, the ketal was cleaved with 80% acetic acid within 1 h.[14] The cyclic carbonate in the oligonucleotide derived from **16** was directly deprotected using lithium hydroxide/triethylamine in methanol as cleaving reagent.[15] The 5′-diol modified oligonucleotides obtained from **15b** and **16** were equivalent. One argument favoring the synthesis via the ketal is that the purification of the ketal by HPLC is improved compared to the less lipophilic diol. Diols could be converted to aldehydes following the above-mentioned conditions. Aromatic alcohol **11** (Scheme 2) needed for the synthesis of phosphoramidite **17** was synthesized analogously to literature.[16] Phosphoramidite **18** was obtained starting from methyl 4-formylbenzoate by protecting the aldehyde function with ethylene glycol and reducing the ester to the respective alcohol **13** using lithium aluminum hydride followed by acidic work-up (Scheme 2). All phosphoramidites applied for 5′-labeling are summarized in Scheme 3. Scheme 4 shows the 3′- and 5′-modified trinucleotides **19**–**26**, whose sequence in the case of 5′-modified oligonucleotides was d(CGG), whereas it was d(CCG) in the case of 3′-modified trimers. Oligonucleotides were stored as aqueous stock solutions (6.25 mM).



Scheme 3. Phosphoramidites **14**–**18** for 5′-modification.

Hydrazone formation kinetics were performed in MES-buffer (pH 6.1, 0.1 M) at 30 °C, final concentration of oligonucleotides was about 1 mM, all experiments were run at least in duplicate. At various time-points samples were withdrawn and diluted hundredfold with water. The compositions of the reaction mixtures were analyzed by RP-HPLC (ammonium bicarbonate (0.1 M)/acetonitrile 95/5 → 80/20 in 15 min, detection: 254 nm,



Scheme 4. 3′- and 5′-Aldehyde and hydrazide-modified oligonucleotides **19**–**26** applied for investigations.

flow: 1 mL/min). The reaction between **20** and **26** yielded not only the expected hydrazone, but also a number of by-products. The analysis of the reaction mixture by MALDI-TOF-MS showed two main by-products, which could be explained by an oxidation of the 3′-phosphoglycaldehyde **20** to glyoxal under elimination of the 3′-phosphate oligonucleotide.[17] Glyoxal itself was reported to react with amino-group containing nucleobases under formation of a 1-*N*2-glyoxal-adducts and thus should exhibit this reaction also with **25a**. Due to this side reaction aldehyde **20** and its corresponding 5′-modified oligonucleotide **25a** are unsuitable for quantitative ligation experiments. To circumvent this side reaction, the next experiments were carried out with **21**, which differs from **20** by a longer spacer between the aldehyde function and the phosphate group. No quantitative product formation was observed in this case too, since **21** was consumed in a side reaction leaving 5′-hydrazide **26** unattached. The concentration of **21** also decreased when it was incubated with the unmodified trimer d(CGG). A MALDI-TOF analysis of the reaction mixture showed small amounts of the expected hydrazone and the remaining hydrazide—but no other products. Due to these results, experiments with compound **25b** were also not continued. The ligation reaction of the bisaldehyde **22** and hydrazide **26** appeared to be too fast under the above-mentioned conditions. The reaction was decelerated by increasing the pH and lowering the final concentration of oligonucleotides (HEPES-buffer, pH 7.5, oligonucleotide concentration: 0.79 mM). Three products having the same mass and close retention times were found and tentatively assigned to be the three major diastereomers of the dihydroxymorpholine unit (Fig. 1) as reported for similar cases.[18] Further experiments were carried out with the 3′-hydrazide **19** and the aromatic aldehydes **23** or **24**. The time course of product formation was monitored by HPLC and analyzed using our SimFit program (Figs. 2 and 3).[19] During fitting it became obvious that in all cases the concentration of the hydrazide-modified oligonucleotides decreased more rapidly than the product was formed. In independent studies we found evidence for the formation of dimeric and even

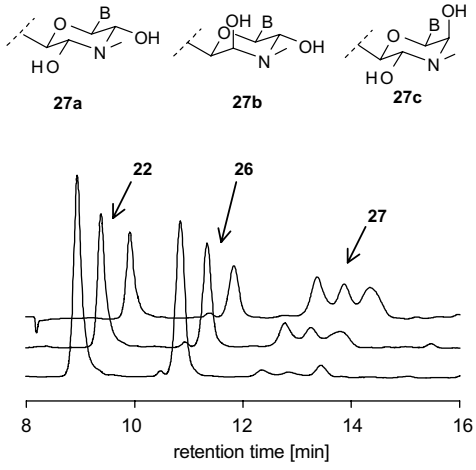


Figure 1. HPLC-chromatogram and possible isomers of the formed morpholine **27**.

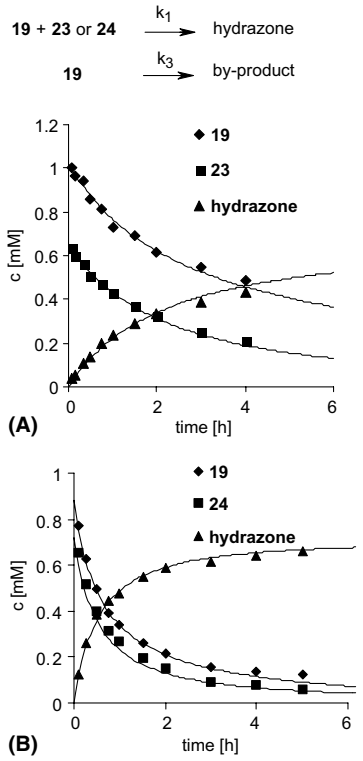


Figure 2. Reaction model and concentration versus time plot. (A) **19** and **23**; (B) **19** and **24**.

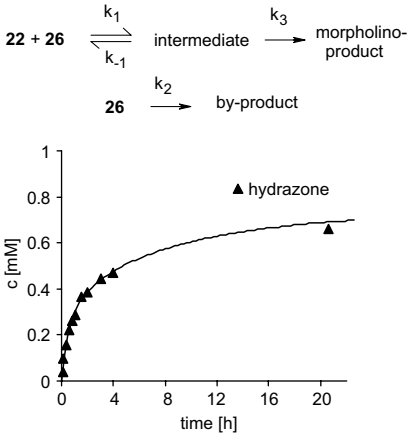


Figure 3. Reaction model and concentration versus time plot for the reaction of **22** and **26**.

oligomeric products of 5′- and 3′-hydrazide-modified oligonucleotides.[20] Reactions of the nucleobases with nucleophiles such as bisulfite, hydroxylamine, or hydrazine and *O*-alkyl or *N*-alkyl derivatives depending on pH are well known side reactions.[21] Up to now such side reactions have not been described for reactions with acyl hydrazides. Variation of the reaction model (Figs. 2 and 3) by taking into account a side reaction to an undetectable (possibly oligomeric) product, which lowers the hydrazide concentration during the reaction, resulted in acceptable fits. Rate constants are summarized in Table 1. In cases where the oligonucleotides were

Table 1. Kinetic parameters for the ligation of **19** and **23** or **24** and **22** and **26**

Rate constants	**19** and **23**	**19** and **24**	**22** and **26**
$k_1/M^{-1}s^{-1}$	$6.15\pm0.01\times10^{-1}$	$1.31\pm0.06\times10^{-1}$	$2.42\pm0.13\times10^{-1}$
k_{-1}/s^{-1}	—	—	$1.57\pm0.36\times10^{-4}$
k_2/s^{-1}	$2.85\pm0.02\times10^{-5}$	$1.08\pm0.12\times0^{-5}$	$5.73\pm3.34\times10^{-6}$
k_3/s^{-1}	—	—	$1.18\pm0.40\times10^{-4}$
RMS[a]/%	4.7	2.9	2.6

[a] RMS = root-mean square.

modified with aromatic aldehydes in the 5′-position having a 3′-hydrazide as the reaction partner, the reaction was fast and irreversible exhibiting comparable rate constants. The kinetic data from the reaction of the 3′-bisaldehyde **22** and the 5′-hydrazide **26** could be explained by the assumption of a consecutive mechanism: The reaction seems to proceed reversibly in the beginning, but becomes irreversible with increasing reaction time. As a reason for this finding we presume a two-step model: first the hydrazide reacts reversibly with one aldehyde function of the bisaldehyde, before the formation of the morpholine ring renders the reaction irreversible. The reversibility of morpholine formation from ribobisaldehydes and amino group containing compounds has been described, but for morpholines derived from acyl hydrazides reversibility could not implicitly be suspected.[8d,22]

We have described the synthesis and utilization of a number of versatile building blocks for the 3′- and 5′-labeling of oligonucleotides with aldehyde and hydrazide functionalities. The reactions of hydrazide- and aldehyde-modified oligonucleotides as a means to achieve activator-free ligation were found to be fast, efficient, and irreversible for the case of aromatic aldehydes. Hydrazone ligation using ribobisaldehydes appeared to be reversible at the stage of open-chained hydrazones or another intermediate and irreversible at the stage of morpholines. Side reactions of the hydrazide building blocks raised difficulties for quantitative ligation studies. The nature of this side reaction has to be ascertained in further studies. Nevertheless, the hydrazone formation fulfills most requirements to be employed as an efficient activator-free ligation chemistry.

Acknowledgements

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