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PAPER

Peptidomimetic bond formation by DNA-templated acyl transfer†

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The efficiencies of DNA-templated acyl transfer reactions between a thioester modified oligonucleotide and a series of amine and thiol based nucleophiles are directly compared. The reactivity of the nucleophile, reaction conditions (solvent, buffer, pH) and linker length all play important roles in determining the efficiency of the transfer reaction. Careful optimisation of the system enables the use of DNA-templated synthesis to form stable peptide-like bonds under mild aqueous conditions close to neutral pH.

Introduction

The synthesis of novel, biologically active peptidomimetic molecules normally requires sequential reactions of building blocks to build up a specific sequence. Building blocks can be based on natural and non-natural amino acids, or on molecules that can be connected using specific ligation chemistries such as amide formation, Wittig chemistry, Diels–Alder reaction or click chemistry. A key goal of synthetic chemistry is to maintain a high level of control of the sequence of reactions, in particular at low reagent concentrations. Many advances have been made towards this goal using the concept of DNA-templated synthesis (DTS),[1–9] using the unique sequence-directed interactions of DNA to control the relative positions of small-molecule reactants. DTS has been used in nucleic acid sensing, sequence-specific DNA modifications, the creation and assessment of DNA-encoded molecular libraries, and the directed evolution of molecules linked to DNA.[10–13] DTS permits incorporation of natural and unnatural building blocks into synthetic biomimetic oligomers, adding to its potential value for drug discovery.

Acyl transfer reactions are of particular interest in peptide chemistry and in the creation of peptidomimetic molecules for use in biology and medicine. Acyl transfer reactions require an acyl donor group, such as an ester or activated ester, which can be attacked by a nucleophile which is commonly a nitrogen based reagent. Liu and co-workers[14,15] have used DTS to carry out acyl transfer reactions between DNA-linked amino acids to generate tripeptides. Native chemical ligation[16] has been used by Grossmann and Seitz[17] to perform a DNA-catalyzed transfer of a reporter group using *iso*-cysteine and thioester-modified peptide nucleic acids, and by Stetsenko and Gait[18] to conjugate peptides to oligonucleotides. However, current synthetic systems lag behind the natural systems for the synthesis of polypeptides by ribosomal and non-ribosomal synthesis (NRPS).[19,20] These natural systems achieve efficient peptide bond formation with precise sequence control by templated acyl transfer chemistries. The NRPS reaction involves the nucleophilic attack of a primary amine on a thioester within a controlled hydrophobic environment under physiological conditions. Using DNA and peptide scaffolds, Joyce and co-workers[21] and Ghadiri and co-workers,[22] respectively, have shown that peptide-forming acyl transfer reactions can be performed, without enzymes, in aqueous solvents at neutral or slightly basic pH. This highlights the potential application of the acyl transfer reaction as a tool for peptide synthesis in a synthetic NRPS scheme.

Despite the many successful DNA templated acyl transfer reactions achieved to date, there is no published study that systematically compares alternative nucleophiles to the amine using the same templates. The use of nucleophiles with tunable reactivity could enable implementation of an adaptable system in which a library of components could be linked using a single set of reaction conditions. We hypothesized that stronger nucleophiles would react more quickly and at lower pH than regular amines and might therefore be suitable building blocks for acyl transfer under physiological conditions. We have evaluated a range of nucleophiles with different reactivities (amine, hydrazine, hydrazone-nicotinate, benzylamine, aminooxy, hydrazide) under the same reaction conditions and have varied temperature, pH

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† Electronic supplementary information (ESI) available: DNA sequences used in this work, synthesis procedures and characterisation of activated esters, HPLC chromatograms and ESI spectra of oligonucleotide adapters and products, additional experimental results. See DOI: 10.1039/c0ob00753f

Table 1 Characterisation data of modified oligonucleotides

DNA adapter	Modification	Expected Mass [Da]	Calculated Mass [Da]	%Yield[a]
T1-TAM	Thioester	7961.1	7959.4	95.1
T2-TAM	Thioester	9520.3	9519.1	92.6
T1-PEG-TAM	Thioester	8173.5	8171.9	35.2
T1-HIS-TAM	Histamine/thioester	8322.8	8320.9	55.2
N3	Benzylamine	9653.4	9652.1	83.9 (>95)
N4	Aminooxy	9593.3	9590.1	31.5 (>95)
N5	Hydrazide	9682.4	9681.2	49.9
N6	Hydrazine	9893.4[b]	9892.0	35.9 ([c])
N7	Hydrazino-nicotinate	9695.4[b]	9693.3	*ca.* 60 ([c])
N8	Thiol	9608.3	9607.8	67.2 (>99)
N9	Hydrazide	9894.5	9893.8	41.0

[a] Yields shown refer to activated ester couplings; data in parentheses refer to deprotection steps. [b] Mass of modified oligonucleotide with protecting group measured before UV_{363} deprotection (N5) or at pH 11.0 (N6). [c] Oligonucleotide conjugates degraded over time and could not be isolated after deprotection.

and solvent composition. In addition, both the linker to the DNA template and the distance between the reacting groups on the template have been varied, as the distance between reactive groups has been shown to be a key factor in DTS chemistries.[23] Reaction yields were measured using PAGE and HPLC, and the products were confirmed by mass spectrometry. As expected, lower pK_a values and α-effects favor acylation. However, we have found that a careful balance between all the reaction parameters is required to optimize acyl transfer from one DNA strand (acyl donor) to another (acyl acceptor). The more nucleophilic modified oligonucleotides are reactive at near-neutral pH and are promising reagents for DNA-templated acyl transfer chemistry. We propose that these transfer systems could serve as a platform for biomimetic DTS based on the NRPS system.

Results and discussion

pH stability of thioester-modified oligonucleotides

To investigate the use of thioesters as acyl donors in DTS, the fluorescent dye TAMRA (TAM) was attached to thiol-modified oligonucleotides, using its succinimide activated ester form, to give the fluorescent thioesters **T1-TAM**, **T1-PEG-TAM**, **T1-HIS-TAM**, and **T2-TAM** (Fig. 1) in 35–95% yields (Table 1). The first three thioester-modified adapter oligonucleotides T1 have the same nucleobase sequence (see ESI† for synthesis and sequences); T2 is complementary to the nucleophile-modified adapters (see below).

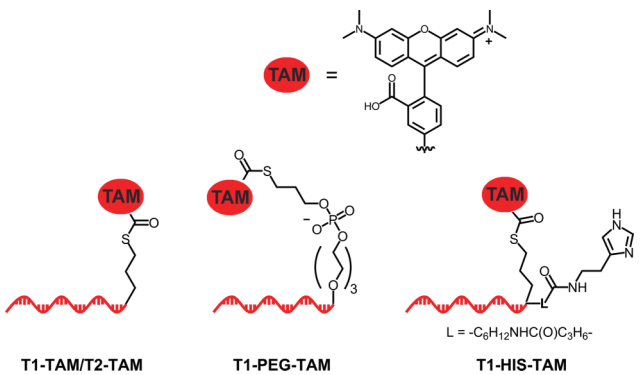


Fig. 1 Structures of thioester-modified oligonucleotides (acyl donor).

The hydrolytic stability of thioester-modified oligonucleotide **T1-TAM** was analyzed by PAGE and HPLC. The attached fluorophore permits easy monitoring of the thioester bond because the fluorescent tag is removed upon hydrolysis. After 48 h incubation at 38 °C, the TAM-thioester oligonucleotide, the thiolated oligonucleotide, and free TAMRA were separated by gel electrophoresis and reverse-phase HPLC (Fig. 2). The results show that **T1-TAM** is stable over a wide pH range in aqueous solutions. The formation of free TAMRA and thiolated DNA indicated that thioester hydrolysis occurs above pH 9.5. This confirms that thioester-modified oligonucleotides may be used as activated donors for DNA-templated acyl transfer reactions in aqueous solutions,[21] although high pH conditions should be avoided.

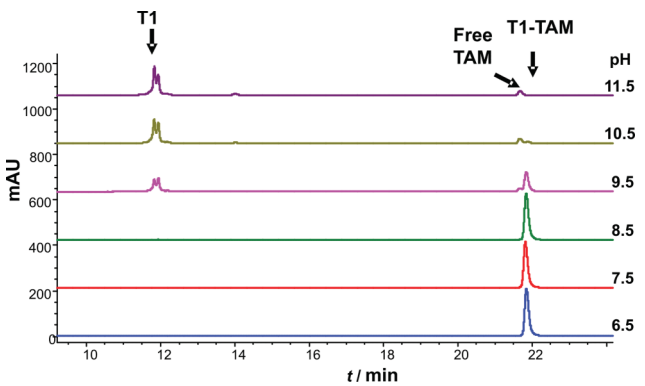


Fig. 2 Hydrolytic stability of the fluorescent TAMRA thioester, **T1-TAM**: HPLC chromatograms of **T1-TAM** after 48 h incubation at 38 °C in universal buffer at indicated pH.

Nucleophile-modified oligonucleotides

The most commonly used nucleophiles in acyl transfer reactions are primary amines, which upon acyl transfer form stable amide bonds. However, without enzymatic catalysis, these reactions are problematic under physiological conditions as amine protonation greatly decreases reaction rates. It is therefore important to investigate the use of nucleophiles with lower pK_a values that could potentially yield higher transfer efficiencies while still generating stable linkages. Benzyl amine, aminooxy, hydrazide and hydrazine moieties were chosen because they are more nucleophilic

than simple amines at pH 7.0 as a result of either an α-effect (neighbouring heteroatom) or the benzylic substitution, and all form stable peptidomimetic bonds when reacting with carbonyl compounds.[24,25] The reactivities of the nucleophiles, based on their pK_a value, are estimated to be in the order; amine < benzylamine < hydrazine < aminooxy < hydrazide < hydrazino-nicotinate.[26] Acyl transfer reactions between these enhanced nucleophiles and thioesters should generate benzylamides, alkoxyamides, and hydrazones. Several research groups have already modified oligonucleotides with these moieties using solid phase synthesis[27,28] or post-synthetic modifications.[29,30]

A series of nucleophile-modified oligonucleotides were prepared post-synthetically by coupling activated esters to amino-modified oligonucleotides **N1** and **N2** (Fig. 3). Thiol oligonucleotide **N8** was included as a positive control as it should effect partial transfer of the fluorescent marker by *trans*-thioesterification. In some cases, the nucleophilic groups prepared contained a photocleavable protecting group, nitro-veratryloxycarbonyl (NVOC),[31] to allow facile unmasking of the nucleophile by exposure to UV_{363}. The hydrazine-modified oligonucleotides **N6** and **N7** partially decomposed during HPLC purification after deprotection (see ESI†). Decomposition of hydrazine conjugates has been reported previously.[32,33] Deprotection was therefore carried out immediately before or immediately after the oligonucleotides were combined to initiate an acylation reaction. Synthesis yields of nucleophilic and thioester-modified oligonucleotides are shown in Table 1.

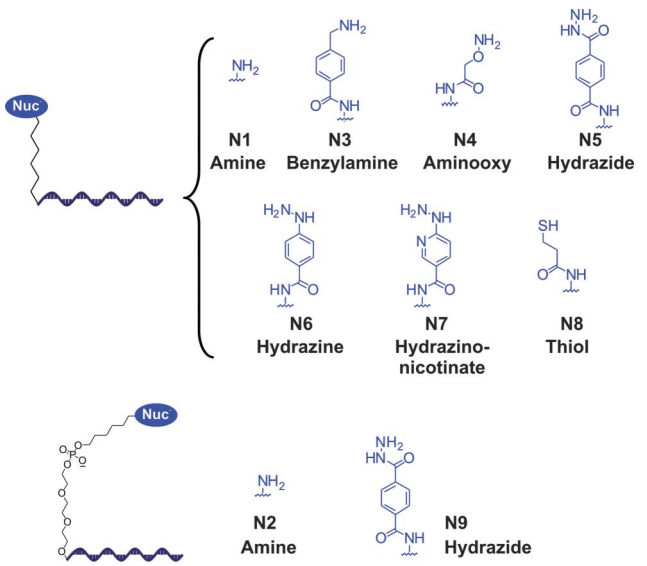
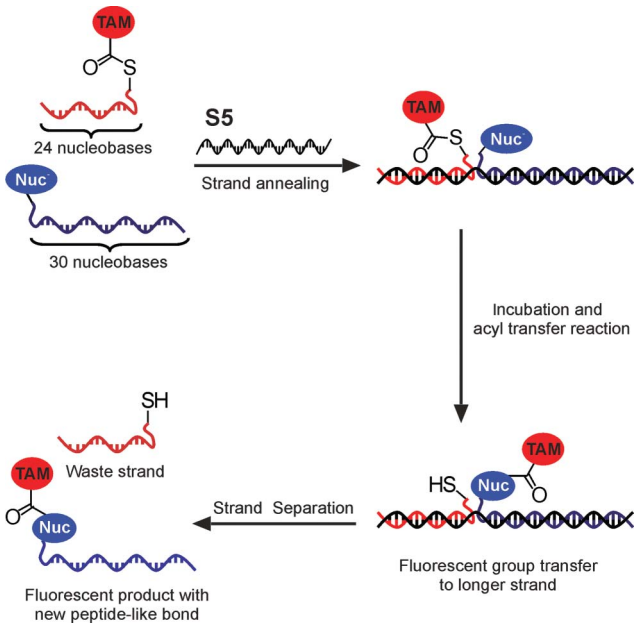


Fig. 3 Structures of the nucleophile-modified oligonucleotides.

Design of DNA-templated system to monitor transfer of fluorescent tag

A simple DTS system was designed to allow the progress of DNA-templated acyl transfer reactions to be monitored by PAGE (Scheme 1).[34] Thioester- and nucleophile-modified oligonucleotides were designed with different lengths, which could easily be separated by PAGE; the progress of the reaction could therefore be monitored by observing the transfer of fluorescence between gel bands. The reaction was triggered by annealing stoichiometric quantities of both adapter oligonucleotides to a complementary



Scheme 1 DTS system for monitoring acyl transfer reactions.

template **S5**. Various experimental conditions were investigated to determine factors that affect acyl transfer efficiency, as described below.

Length of linker and adjacent residues affect acyl transfer efficiency

It is possible that additional adapter length and flexibility could favour interaction between nucleophile and thioester. To test this hypothesis, a PEG spacer was incorporated in oligonucleotides **T1-PEG-TAM**, **N2** and **N9**. Virtually no fluorescent group transfer was observed from PEG-containing thioesters (see ESI†). The effects of incorporating a PEG spacer in the nucleophile adapter were much smaller, but it reduced reaction yield with both amine- and hydrazide-modified oligonucleotides. We infer that the advantage of increased conformational freedom imparted by the flexible linker is outweighed by the increased distance between tethered reactants.

Functional groups adjacent to the reaction site may also influence reactivity. For example, NRPS makes use of catalytic residues, particularly histidines, in close proximity to the transfer site. In addition, it has been shown that histidines on peptide scaffolds can catalyze acylation reactions.[35,36] To explore this effect in our DTS system, we designed strand **T1-HIS-TAM** (Fig. 1) and explored its effectiveness in acyl transfer reactions. Contrary to what was expected, when a histamine residue is added to the system, the reaction yields were reduced significantly. The imidazole residue promoted thioester hydrolysis rather than acylation, particularly at higher pH values. Ester hydrolysis has also been reported in synthetic peptide esterases, which use histidine residues.[37,38]

Effects of temperature, co-solvents and added base on acyl transfer efficiency

Fig. 4 shows the results of a comparison between reactions performed at room temperature and at 38 °C. The yields of acyl transfer were significantly higher at 38 °C, but a further

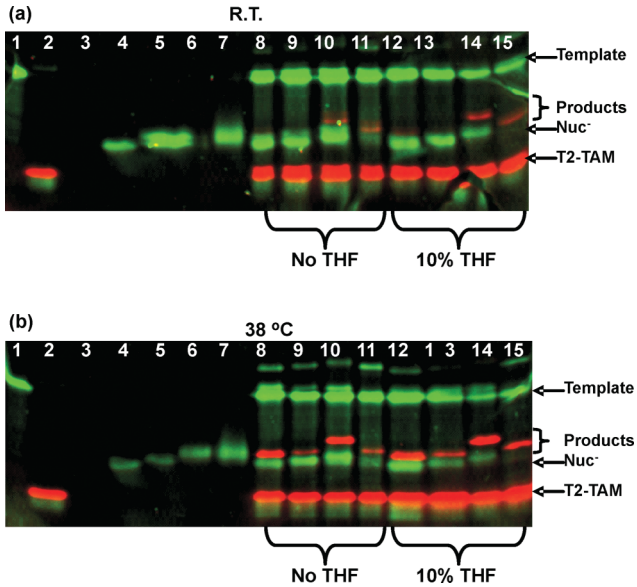


Fig. 4 Comparison between acyl transfer reactions at room temperature (R.T.) and at 38 °C. Acyl transfer reactions were carried out for 24 h in 100 mM sodium phosphate buffer, pH 8.5, with (lanes 12–15) and without (lanes 8–11) 10% THF at R.T. (a) and at 38 °C (b). Lane 1: Template oligonucleotide **S5**; Lane 2: **T1-TAM**; Lane 3: blank; Lane 4: **N1**; Lane 5: **N2**; Lane 6: blank (a)/**N5** (b) ; Lane 7: **N9**; lanes 8–15: acyl transfer reactions with the nucleophiles as in lanes 4–7. Red channel: TAMRA (Ex532/Em605 nm); green channel: SybrGold DNA stain (Ex488/Em530 nm).

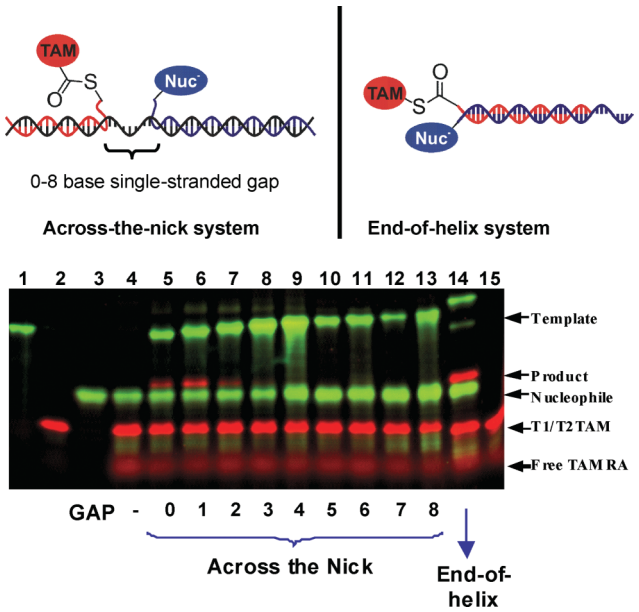


Fig. 5 DNA architectures and acyl transfer efficiency. Lane 1: **S5** (template oligonucleotide); lane 2: **T1-TAM**; lane 3: **N1**; lane 4: **T1-TAM** + **N1** control (no template); lanes 5-13: **T1-TAM** + **N1** in the presence of a template oligonucleotide incorporating a 0–8 nucleotide single-stranded gap (oligonucleotides 5–13, ESI†); lane 14: **T2-TAM** + **N1** (end-of-helix architecture); lane 15: **T2-TAM**. Reactions were carried out in 100 mM sodium phosphate buffer, 100 mM NaCl, pH 9.0, 10% THF at 38 °C for 24 h.

increase to 45 °C or 55 °C promoted hydrolysis rather than higher acylation yields. Co-solvents THF (10%), and DMF (10%, not shown) were also added to the reaction mixtures to test their effects on acylation. Thioester-activated acyl transfer experiments using model small molecules showed an improvement in both efficiency and purity when 10% THF was added as a co-solvent (see ESI†), and improvements in rate and purity were also observed for the DNA-templated acyl transfer system studied here. However, no significant changes in conversion were observed using 10% DMF as a co-solvent. Addition of bases such as 10% (v/v) DIPEA or 10 mM Mg^{2+}-imidazole[39] resulted in thioester hydrolysis rather than enhanced acyl transfer, as observed with the histamine-modified thioester **T1-HIS-TAM**. As a result of these observations, all subsequent experiments were performed under optimized conditions in the presence of 10% THF and at 38 °C without additional base.

Effect of DNA adapter design on acyl transfer efficiency

The architecture of the template has been shown to significantly affect the rates of DNA-templated reactions.[40,41] We have compared acyl transfer efficiencies with the "across-the-nick" system, with variable gap length, and the end-of-helix geometry (Fig. 5). As expected, no product was observed in a control reaction using the across-the-nick system from which the template oligonucleotide was omitted (lane 4). A single-stranded DNA spacer of 0–8 nucleotides was introduced into the template oligonucleotide to separate the reactive groups attached to the 5′ and 3′ ends of the hybridized oligonucleotide adapters (lanes 5–13). The optimum spacer was found to be a single unpaired nucleotide (lane 6). The end-of-helix geometry, in which reactive groups are conjugated to the 5′ and 3′ ends of complementary oligonucleotides, produced the highest product yield, as recently observed with the Wittig-based DTS synthesis of longer oligomers.[42] The end-of-helix configuration was therefore used in reactions presented below.

Effect of pH on acyl transfer efficiency

pH has an important effect on nucleophile reactivity, which is reduced by protonation. The reactivities of amine (**N1**) and hydrazide (**N5**) nucleophilic oligonucleotides over the pH range 7.0–9.5 were compared using the end-of-helix configuration. Peptide bond formation was observed at the higher end of the pH range for both nucleophiles, but thioester hydrolysis also increased with pH as expected (see ESI†). Product formation near neutral pH, with negligible hydrolysis, occurred with the hydrazide oligonucleotide only.

The difference in reactivity between the amine and hydrazide nucleophiles was assessed by measuring product formation as a function of time. The reactions were quenched by adding a displacer oligonucleotide complementary to the **T2-TAM** adapter (see ESI†) at specific time points; products were analyzed by HPLC. The results, which are summarized in Fig. 6, show that hydrazide oligonucleotide **N5** has a 3- to 10-fold faster acylation rate at pH 7.5 than amine **N1**, while at pH 9.5 the rate of acylation for both nucleophiles is similar. It should be noted that even for the most reactive nucleophile **N5** the acyl transfer yields are surprisingly low.

Acyl transfer reactions between nucleophiles **N1**–**N6** and thioester **T2-TAM**, using the end-of-helix configuration, were

Table 2 Characterization data of acyl transfer products with **N1**–**N9**

DNA adapter	Expected Mass [Da]	Calculated Mass [Da]
N1	9933.5	9932.4
N2	10145.8	10144.4
N3	10066.5	10065.4
N4	10006.5	10003.2
N5	10095.5	10093.3
N6	10067.5	17450.5[a]
N7	10068.5	*n.d.*[b]
N8	10021.5	10019.2
N9	10307.4	10306.1

[a] Mass of acylation product was not observed; detected mass could correspond to crosslinked product between the two reactive oligonucleotides. [b] Not determined.

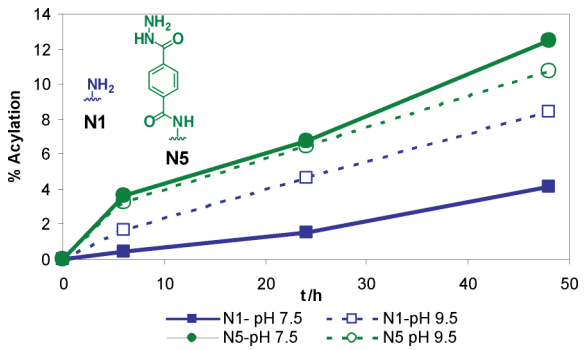


Fig. 6 Comparison between reactivities of amine- and hydrazide-modified oligonucleotides as functions of pH. Time courses of acylation reactions, monitored by HPLC, using **N1** and **N5** nucleophiles in 100 mM sodium phosphate buffer at the indicated pH.

carried out by incubating the modified oligonucleotides for 48 h at 38 °C (Fig. 7, Table 2). Thioester **N8** was used as a positive control (the *trans*-thioesterification product equilibrated to approx 40–50% with **T2-TAM** under all conditions studied). The reactions were quenched by adding a displacer oligonucleotide **S14**. New peaks corresponding to the products were identified from the chromatograms (see ESI†) and were characterized by mass spectrometry. HPLC peak areas were used to compare

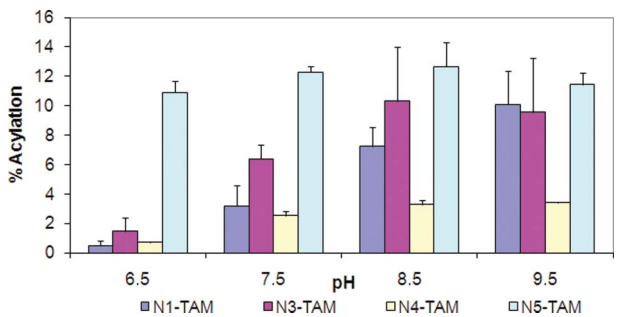


Fig. 7 Comparison of yields of acyl transfer products, using nucleophiles **N1**, **N3**–**N5**, determined from peak areas in HPLC chromatograms. Acylation reactions were carried out in 100 mM sodium phosphate buffer, 100 mM NaCl, at pH 6.5–9.5 for 48 h at 38 °C. Product peaks were identified by simultaneous measurement of TAMRA fluorescence and A_{260}.

product yields. Throughout the range of pH values studied, both the hydrazide- (**N5**) and hydrazine- (**N6**) modified oligonucleotides produced the greatest yields, as expected given the enhanced nucleophilicity conferred by the neighboring heteroatom α-effect. However, in the case of hydrazine **N6**, the fluorescent product isolated had a mass corresponding to the combined mass of modified oligonucleotides **T2-TAM** and **N6**, indicating that an undesired cross-linking reaction, rather than the expected acyl transfer, had occurred. Apparent yields corresponding to this reaction are therefore not included in Fig. 7. Hydrazide **N5** produced the stable fluorescent acyl transfer product **N5-TAM** as designed, with no crosslinked products observed. For nucleophiles **N1**–**N4**, the pattern of acylation varied significantly with pH, with yield initially increasing then saturating as pH was increased. The reaction yield with the amine (**N1**) saturates at higher pH than in the case of the benzylamine- (**N3**) and aminooxy- (**N4**) modified oligonucleotides, correlating with its higher pK_a, as expected. Unexpectedly, the aminooxy group **N4** was the least effective nucleophile studied under these conditions, and the amine gave higher yields than both aminooxy and benzylamine groups at pH 9.5. As discussed above, higher pH values increase thioester hydrolysis and are therefore undesirable. At all pHs studied, but especially at low pH, the hydrazide (**N5**) produced the highest yield of transfer product. The hydrazide-modified oligonucleotide, **N5-TAM** is therefore of particular interest for reactions to be carried out at near-neutral pH.

Conclusions

We have investigated the use of a range of alternative nucleophilic groups in acyl transfer reactions controlled by DNA-templated synthesis. A thioester group was chosen as the acyl donor due to its hydrolytic stability in aqueous buffers up to pH 9.5. The thioester-modified oligonucleotide included a fluorescent tag which allowed reaction progress to be readily monitored. Oligonucleotide adapters modified with amine, benzylamine, aminooxy, hydrazide, hydrazine, hydrazino-nicotinate, and thiol groups were synthesized and used as nucleophiles in acyl transfer reactions in aqueous buffers. The reactions yielded products with stable peptide-like linkages, including amide, alkoxyamide, hydrazide and thioester bonds.

Several parameters were found to affect acyl transfer efficiency, including pH and the architecture of the DNA adapters that enforce proximity between nucleophile and thioester. Nucleophiles with lower pK_a values are particularly effective in buffers close to neutral pH, where the hydrazide group displayed at least one order of magnitude enhanced reactivity relative to simple amines. Acyl transfer reactions at near-neutral pH values have the significant advantage that the thioester is more stable to hydrolysis than at higher pH values. The more reactive nucleophiles can also be expected to react at higher rates with other activated esters. This chemistry has the potential to enable biologically-relevant reactions such as the formation of protein- or peptide-oligonucleotide conjugates or peptidomimetic molecules, which may require neutral conditions to be stable or active. We propose that the DTS of peptidomimetic bonds in aqueous buffers could be used to create macromolecule libraries and to select and evolve novel bioactive compounds.[43,44]

Acknowledgements

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