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Systematic Evaluation and Optimization of Modification Reactions of Oligonucleotides with Amines and Carboxylic Acids for the Synthesis of DNA-Encoded Chemical Libraries

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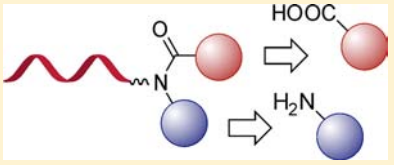
Supporting Information

ABSTRACT: DNA-encoded chemical libraries are collections of small molecules, attached to DNA fragments serving as identification barcodes, which can be screened against multiple protein targets, thus facilitating the drug discovery process. The preparation of large DNA-encoded chemical libraries crucially depends on the availability of robust synthetic methods, which enable the efficient conjugation to oligonucleotides of structurally diverse building blocks, sharing a common reactive group. Reactions of DNA derivatives with amines and/or carboxylic acids are particularly attractive for the synthesis of encoded libraries, in view of the very large number of building blocks that are commercially available. However, systematic studies on these reactions in the presence of DNA have not been reported so far. We first investigated conditions for the coupling of primary amines to oligonucleotides, using either a nucleophilic attack on chloroacetamide derivatives or a reductive amination on aldehyde-modified DNA. While both methods could be used for the production of secondary amines, the reductive amination approach was generally associated with higher yields and better purity. In a second endeavor, we optimized conditions for the coupling of a diverse set of 501 carboxylic acids to DNA derivatives, carrying primary and secondary amine functions. The coupling efficiency was generally higher for primary amines, compared to secondary amine substituents, but varied considerably depending on the structure of the acids and on the synthetic methods used. Optimal reaction conditions could be found for certain sets of compounds (with conversions >80%), but multiple reaction schemes are needed when assembling large libraries with highly diverse building blocks. The reactions and experimental conditions presented in this article should facilitate the synthesis of future DNA-encoded chemical libraries, while outlining the synthetic challenges that remain to be overcome.



INTRODUCTION

DNA-encoded chemical libraries (DECLs) are increasingly being used as a tool for drug-discovery applications.[1−4] In DECLs, each small organic molecule is individually attached to a unique DNA fragment, which serves as an amplifiable identification barcode. As a result, very large mixtures of compounds can be used simultaneously and screened for their ability to interact with target proteins of interest. The affinity-selection of DECLs for immobilized proteins, followed by high-throughput DNA sequencing of the enriched conjugates, facilitates the identification of small-molecule binders.[5] Compared to standard drug screening methodologies (e.g., high-throughput screening, fragment-based screening), DECL technology may allow the construction and screening of larger libraries, in a less expensive procedure and under multiple experimental conditions.[1] As a result, this emerging technology is increasingly generating lead structures for chemical, biological, and pharmaceutical applications.[6−14]

The preparation of DECLs relies on the availability of robust and efficient DNA-conjugation reactions, which can be performed in parallel on large series of functionally related compounds. One approach for the preparation of DECLs is to use DNA-templated reactions, which rely on hybridization of a reactant DNA to accelerate synthesis.[9,15] However, in most cases, split-and-pool synthetic strategies are used for library construction,[8,10,12,16] since these procedures can be performed by a direct reaction of oligonucleotide with "off-the-shelf" reagents. In these strategies, the first step of library construction consists of the coupling of compounds to cognate oligonucleotides, followed by HPLC purification of the individual conjugates. All successive steps, however, are performed with pools of compounds. Thus, heterogeneous reaction yields for different library members may lower the purity of the library and may compromise the evaluation of selection results. The availability of robust and general synthetic methods crucially contributes to the performance of DECL technology. Thus, there is considerable interest to reinvestigate organic reactions in the context of DNA modification with structurally diverse sets of building blocks.

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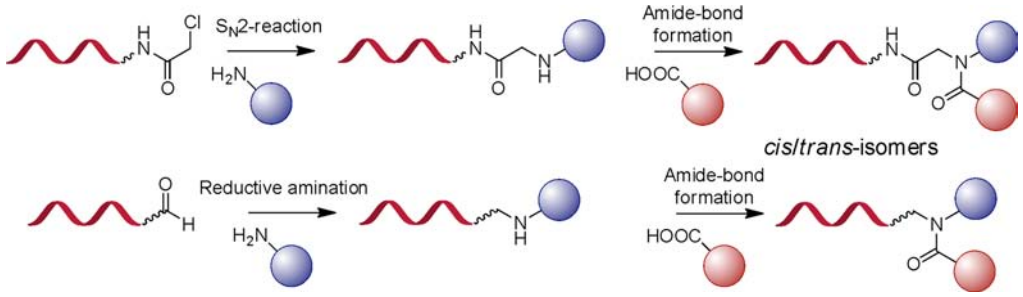

Figure 1. Preparation of DNA-small molecule conjugates via a two-step procedure. In a first step, DNA containing an electrophilic (chloroacetamide or aldehyde) group reacts with primary amines, generating a secondary amine linked to the DNA. In the second step, amide bond formation of a carboxylic acid with the secondary amine forms a DNA-conjugate of small molecules with compact structure. Two reactions are applicable for the attachment of the amine to the DNA: S_N2-displacement of a chloroacetamide (upper scheme) and reductive amination (lower scheme).

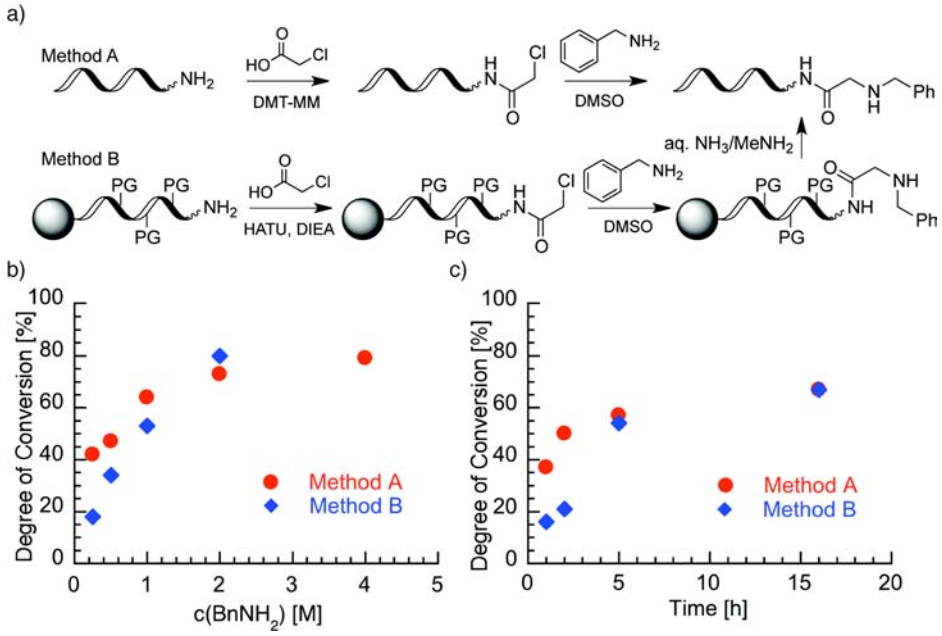

Figure 2. Formation of DNA-conjugates of secondary amines by the reaction of ClAcNH-DNA with primary amines. (a) Schematic representation of reaction modes on pseudo-solid phase with deprotected DNA (Method A) and on solid-phase with protected DNA (Method B). Dependence of the yield of conversion on (b) $BnNH_2$-concentration (t = 2 h) and (c) the reaction time [$c(BnNH_2)$ = 0.25 M]. For the associated HPLC traces see Figures S2 and S3 in the Supporting Information.

Amines and carboxylic acids represent the most widely available building blocks. Reactions that involve these substrates are thus ideal for DECL synthesis. Preferably, encoded compounds should be compatible with medicinal chemistry applications and should preferentially adhere to Lipinski's Rule of 5.[17] Structural compactness of encoded compounds is an additional attractive feature. In an optimal design, diverse building blocks would be connected by only a few chemical bonds, in order to maximize library purity and decrease molecular weight of library members. Despite the evident need for robust bioconjugation strategies, few systematic studies[18−20] have been dedicated to the examination of chemical reactions for DECL synthesis (the reaction scope for DECLs prepared by DNA-templated synthesis[15] has been studied more thoroughly[21,22]).

We first investigated the reaction of primary amines in nucleophilic substitutions and in reductive amination procedures, yielding secondary amine DNA-conjugates. In a second set of reactions, we studied the coupling of carboxylic acids to primary and secondary amines. The two steps can be combined conveniently in a sequential fashion, thus generating large combinatorial libraries (Figure 1).

Reactions yielding secondary amines represent well-established bioconjugation strategies, which however have rarely been used for the preparation of DNA-conjugates. Reductive amination has been applied to the attachment of peptides and proteins to DNA[23−25] but rarely to the formation of small-molecule conjugates. Reports of nucleophilic substitution reactions on oligonucleotides modified with leaving groups include the reaction of 5′-iodinated[26] or tosyl-containing DNAs[27] with thiols and other nucleophiles. Furthermore, chloroacetamide (ClAcNH−) phosphoramidites have been described and preliminary investigations of their reactivity with amines have been reported.[28,29] In one study, Wrenn et al. performed the modification of DNA with chloroacetamide groups and subsequent nucleophilic displacement with amines for the synthesis of a library of β-peptoids on DNA.[7]

DNA-attached primary and secondary amines are particularly suited for an efficient chemical modification with electrophiles. In this context, carboxylic acids are especially attractive, as a large number of reagents are currently available from

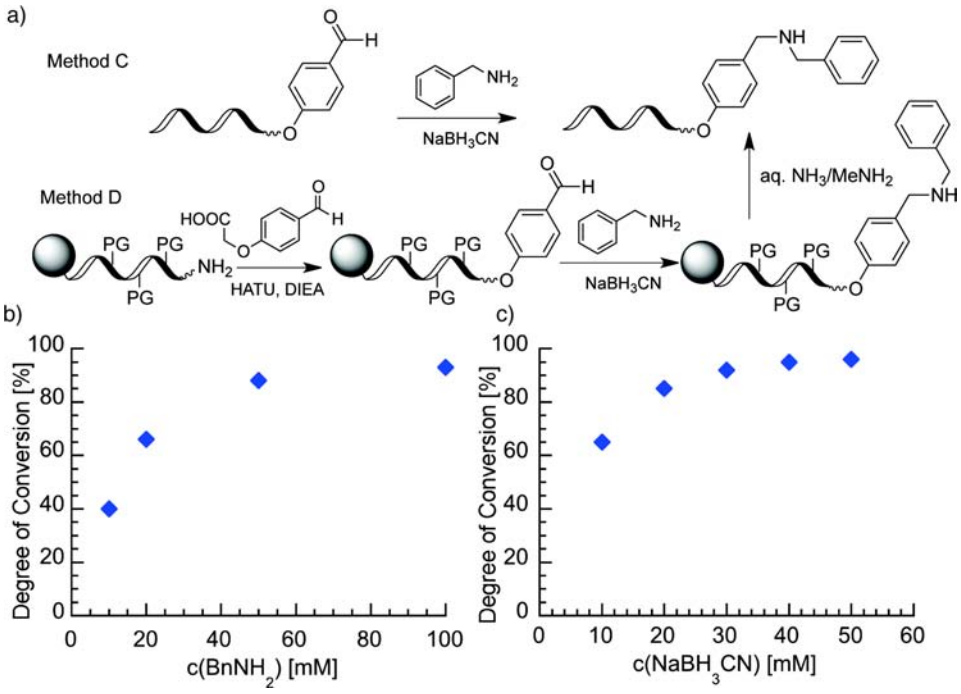

Figure 3. Formation of DNA-conjugates containing secondary amines by reductive amination of DNA-CHO with primary amines. (a) Schematic representation of two possible reaction modes on liquid phase (Method A) and solid phase (Method B). (b) Dependence of the rate of conversion by Method A on the $BnNH_2$-concentration [$c(NaCNBH_3)$ = 15 mM, t = 16 h]. (c) Dependence of the rate of conversion by Method A on the concentration of $NaBH_3CN$ [$c(BnNH_2)$ = 50 mM, t = 16 h] . For the associated HPLC traces see Figures S5 and S6 in the Supporting Information.

commercial sources. Other reagents (e.g., sulfonyl chlorides, isocyanates, isothiocyanates) could also be considered. Modification of DNA derivatives carrying primary or secondary amine groups (amino-DNAs) with activated carboxylic acids is a standard reaction in DNA-conjugation chemistry. This conversion typically proceeds with good yields and is frequently used for the preparation of DECLs.[8,12,16,30] However, little information is available concerning the substrate scope, conversion yields, and product purity for the coupling of structurally diverse carboxylic acids to DNA, using amide bond formation.

Here we report a systematic study of reactions and experimental conditions for the coupling of amines and of carboxylic acids to DNA derivatives. In particular, we tested several reaction conditions and substrates, performing bioconjugation reactions in solution, on solid phase, and on DNA immobilized on anion exchange resin[20] in a "pseudo-solid phase" protocol. A variety of reaction protocols were identified for each of these transformations and their efficiency was assessed for a representative group of reagents, thus allowing a direct comparison of reaction protocols. While coupling efficiency was sometimes strongly dependent on the structure of the amine and carboxylic acid building blocks, methods were identified which allow the efficient incorporation of certain sets of compounds (with conversions >80%), thus facilitating the future construction of DECLs.

RESULTS

Reaction of Chloroacetamide-Modified DNAs with Amines. The reaction of oligonucleotides bearing terminal leaving groups with primary amines represents a general avenue for the preparation of DNA-conjugates containing secondary amines. We investigated two modalities for this reaction (Figure 2a) using benzylamine ($BnNH_2$, **AM-1**) as the test substrate and DNA modified with a 5′-terminal chloroacetamide electrophile (ClAcNH-DNA). In Method A, the reaction was performed using unprotected DNA immobilized on an anion exchange resin according to a modified protocol initially reported by Harbury and co-workers.[7] Alternatively, the reaction was performed on nucleobase-protected DNA prior to cleavage from the solid support (Method B).

ClAcNH-DNA was prepared by the reaction of chloroacetic acid with amine-modified DNA, using the peptide-coupling reagent DMT-MM for the pseudo-solid phase method and HATU/DIEA for DNA on solid support. Attempts to introduce bromo- or iodoacetic acid groups were unsuccessful even for protected DNA, likely because of alkylation of the DNA bases (data not shown). Incubation of ClAcNH-DNA with $BnNH_2$ provided benzylaminoacetamide-DNA (BnNHAcNH-DNA) from both methods. The reaction exhibited a distinct solvent preference and proceeded most efficiently in DMSO from the tested solvents (Supporting Information Figure S1). The degree of conversion of the reaction strongly depended on the $BnNH_2$ concentration (Figures 2c, Supporting Information Figures S2 and S3). On pseudo-solid phase, the product fraction increased from 42% for 0.25 M $BnNH_2$ to 79% at 4.0 M $BnNH_2$ and on solid phase from 18% for 0.25 M to 80% at 2.0 M $BnNH_2$ (t = 2 h). Prolonged reaction times also enhanced the rate of conversion. For Method A, 1 h incubation time yielded 37% of the desired conjugate, whereas for 16 h it increased to 67% (16% to 67% for Method B). However, analysis of HPLC chromatograms showed that the degree of conversion plateaued prior to maximum amine concentrations and long reaction times because of the formation of side products. Concerns that prolonged incubation of the DNAs on solid support with high concentrations of amines may prematurely cleave DNA from the solid support were not substantiated and the recovered

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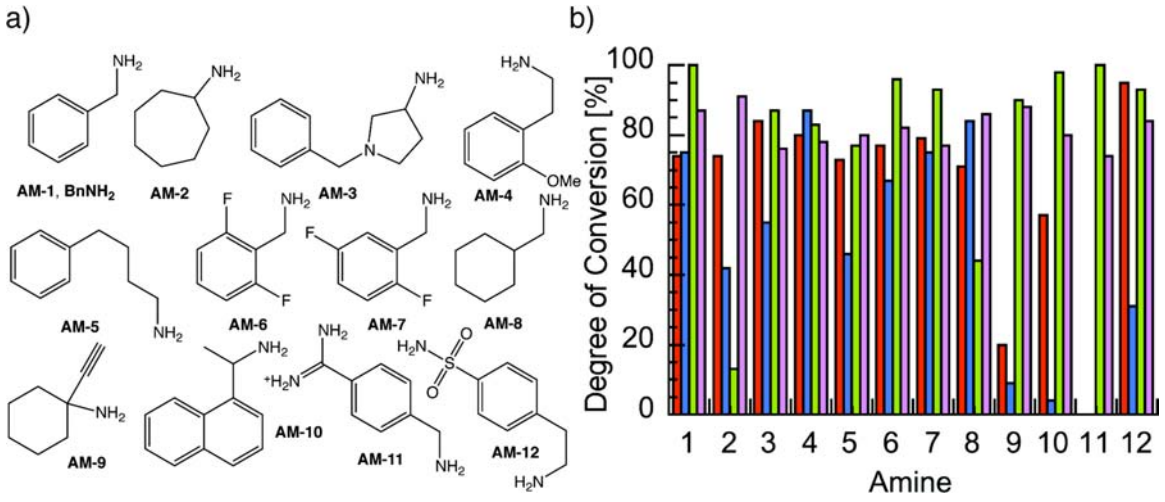

Figure 4. (a) Structures of amines selected to evaluate the scope of the bioconjugation protocols. (b) Results of the analysis of Methods A–D for the preparation of DNA-conjugates containing secondary amines. Red columns: nucleophilic substitution on pseudo-solid phase (Method A); blue columns: nucleophilic substitution on solid support (Method B); green columns: reductive amination in solution (Method C); pink columns: reductive amination on solid support (Method D). For **AM-11**, determination of product yields was prevented for Methods A and B by overlapping impurities; the product obtained for Method D may be the hydrolysis product (carboxamide).

yields of DNAs were largely independent of the amine concentrations and the reaction time (Supporting Information Figure S3).

We performed a test reaction between $BnNH_2$ and ClAcNH-DNA on solid support at 50 °C and observed a marked enhancement of the product formation by HPLC analysis even for relatively low $BnNH_2$ concentrations (100 mM). However, mass spectrometric analysis revealed that multiple additions of the amine to the DNA had occurred under these conditions (Supporting Information Figure S4a), possibly by trans-amination of protected cytosine bases.[31] On the other hand, microwave irradiation (10 pulses of 15 s at 900 W followed by cooling at room temperature for 5 min) enhanced the yield of the expected product and a single mass was seen in the mass spectrum corresponding to BnNHAcNH-DNA (Supporting Information Figure S4b). Therefore, whereas elevated temperatures are unfavorable because of side reactions, microwave irradiation is a viable means for enhancing the reaction.

Reductive Amination of Aldehyde-Modified DNAs. We tested reductive amination as a route for the synthesis of DNA-conjugates with secondary amines, prepared from aldehyde-modified DNA (DNA-CHO) and primary amines (Figure 3a). In one approach (Method C), the reductive amination was performed in aqueous solution. The aldehyde modification was introduced by incorporation of a commercially available aldehyde modifier during solid-phase DNA synthesis.[32] Incubating DNA-CHO in buffer (300 mM MOPS, pH 7.4) with $BnNH_2$ as the test substrate and sodium cyanoborohydride ($NaBH_3CN$) as the reducing agent provided the anticipated BnNH–DNA conjugate. The concentration of $BnNH_2$ strongly affected conversion yields ($c(NaBH_3CN)$ = 15 mM, t = 16 h, T = 37 °C) and at 100 mM near-complete conversion was achieved (Figure 3b and Supporting Information Figure S5). Similarly, increasing the concentration of $NaBH_3CN$ enhanced product yield (Figures 3c and Supporting Information Figure S6). In contrast, the presence of organic solvent had a negative effect on the reaction; addition of DMF gradually decreased the formation of the BnNH–DNA conjugate from 100% for without DMF to 60% for 1:1 DMF/buffer ($c(BnNH_2)$ = 50 mM, $c(NaBH_3CN)$ = 50 mM, t = 16 h, T = 37 °C; Supporting Information Figure S7). Importantly, the reaction was clean and almost no side products were observed. Only in acidic solutions (acetate buffer, pH 5.5) and at elevated $NaBH_3CN$ concentrations was reduction of the aldehyde to the benzylhydroxy-conjugate a competing side reaction (not shown). Using the same reaction conditions, reductive amination could also be efficiently performed on solid support (Figure 3a, Method D).

Comparative Analysis of Bioconjugation Methods. We examined the scope of the four bioconjugation methods by testing a set of 12 representative primary amines (Figure 4). The tested molecules encompassed amines with varying substituents at the α-position and several functional groups (Figure 4a).

For the nucleophilic substitution on pseudo-solid phase (Method A; $c(R\text{-}NH_2)$ = 1.0 M, t = 16 h), most amines provided the expected conjugates in good yields (>70%). Similar results were obtained for the nucleophilic substitution on solid support (Method B; $c(R\text{-}NH_2)$ = 1.0 M, t = 16 h) although the reaction yields were generally lower, mostly because of the formation of side products and the presence of impurities associated with DNA-solid phase synthesis. The α-substituted amines **AM-9** and **AM-10** provided lower yields for nucleophilic displacement (Methods A and B), indicating that DNA conjugation by substitution may be sensitive to steric hindrance at this position, although the effect was less pronounced for **AM-2** and **AM-3**. The product peak of **AM-11** overlaps with those of impurities preventing the determination of the yield by HPLC. In general, nucleophilic substitution provides the conjugates in good yields; however, the reaction is accompanied by side products, which limit the yield largely to <85% under these conditions.

High degrees of conversion were generally obtained for DNA-conjugation by reductive amination in solution phase (Figure 4b; Method C; $c(R\text{-}NH_2)$ = 50 mM, $c(NaBH_3CN)$ = 50 mM, 300 mM MOPS Buffer, pH 7.4, T = 37 °C, t = 16 h). For most amines the reaction reached near-completion (>90%) even for the aforementioned problematic amines **AM-9** and **AM-10** although for a few amines (i.e., **AM-2** and **AM-8**) significantly lower yields were isolated for unknown reasons

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(Figure 4b). Good conversions (>75%) were also obtained for Method D (Figure 4b; $c(BnNH_2)$ = 50 mM, $c(NaBH_3CN)$ = 50 mM, 300 mM MOPS Buffer, pH 7.4, T = 37 °C, t = 16 h) even for the amines that were problematic for Method C. Impurities formed during solid-phase synthesis of DNA somewhat decreased the fraction of product for Method D. In conclusion, this analysis has shown that for the four approaches tested reaction yields appear to be most consistent for Method D.

Efficiency of Amide-Bond Formation on Unprotected DNA. Having established robust methods for the introduction of secondary amines at the terminus of DNAs, we turned next to their subsequent reaction with activated carboxylic acids. In a first step, we tested the coupling efficiency of a structurally diverse set of 355 carboxylic acids to a DNA modified with a 5′-terminal primary amine (DNA-NH_2) using a pseudo-solid-phase reaction protocol (c(R-COOH) = 50 mM, c(EDC) = 50 mM, c(HOAt) = 5 mM, 3 × 2 h in DMSO).[12,20] The reaction yields varied considerably across the carboxylic acids (Figure 5a). A subset of the acids coupled efficiently to DNA-NH_2 (128 with >80% yield; 220 with >50% yield; product yields estimated by analytical HPLC) whereas for other acids no product was observed. No clear trends between coupling efficiency and structure were apparent. For example, similar coupling yields were observed for different types of carboxylic acids (i.e., primary, secondary, and tertiary carboxylic acids; benzoic acids, carboxylic acids of aromatic heterocycles). In a previous report, it was described that several Fmoc-protected amino acids coupled near-quantitatively to amine-modified DNAs contrasting with our observations.[20] We therefore tested a set of 146 Fmoc-protected amino acids and found that these react generally with good yields under our standard conditions (Figure 5b). The obtained data illustrates the necessity to thoroughly test the building blocks prior to DECL synthesis and the risk of relying on a small set of test substrates.

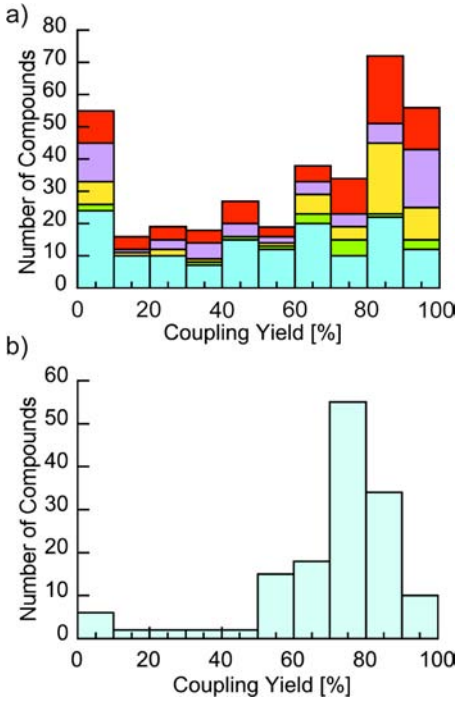


Figure 5. Analysis of amide bond formation of a set of structurally diverse carboxylic acids to DNA-NH_2 on pseudo-solid phase. (a) Histogram analysis of the coupling yields of 355 carboxylic acids. Thirteen additional carboxylic acids were not included in the analysis because of limited solubility in DMSO and 5 for problems associated with the recovery of the DNA (red: Ar-COOH; purple: R-CH_2-COOH; yellow: R,R′-CH-COOH; green: R,R′,R″C-COOH; blue: Heterocycle-COOH). (b) Histogram analysis of the coupling yields of 146 Fmoc-protected amino acids.

In a few cases, mass spectrometric analysis of the obtained conjugates exhibited additional peaks with masses corresponding to the expected product plus one or several EDC adducts (Supporting Information Figures S9 and S10). The adduct formation of EDC with G- and T-nucleobases is known[33,34] but has so far remained unrecognized as a potential difficulty in the context of DECL synthesis.

Assessing Peptide Bond Formation on DNA-Conjugates of Secondary Amines. We further tested the coupling of carboxylic acids to DNA-conjugates bearing aliphatic secondary amines (RNHAcNH-DNA; R: aliphatic substituent). Using the standardized EDC-coupling protocol, yields of amide bond forming reactions were distinctly lower for RNHAcNH-DNA than for DNA-NH_2 (Table 1, Supporting Information Table S2).

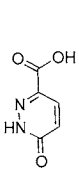
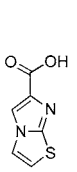
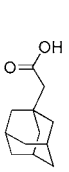
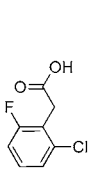
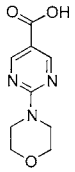
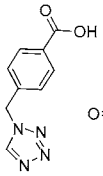
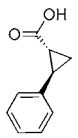
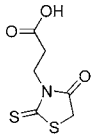
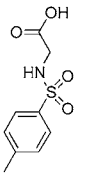
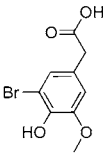
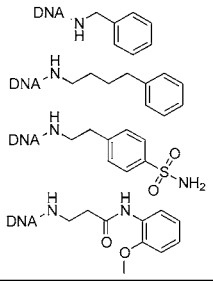
Steric bulk at the α-carbon (e.g., substitutents or branching points) further reduced the coupling efficiency substantially. For example, carboxylic acids reacted inefficiently with a DNA-conjugate of 1-benzylpyrrolidine-3-amine (**AM-3**), and with the exception of CA-2, all tested acids had coupling yields of <30% under these conditions (Table 2).

Alternative peptide coupling reagents were tested to enhance the yield of amide bond formation for secondary amines. Several coupling reagents (DIC, DCC, DMT-MM) provided consistently lower yields in test reactions and were not further investigated. *N*-Ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ; similar results were obtained for IIDQ) in contrast afforded encouraging results, providing higher coupling efficiencies for RHNAcNH-DNA with branched amines than the EDC/HOAt system (Table 2). However, for some of the EEDQ-mediated reactions the formation of a side product was observed by HPLC analysis (Supporting Information Figure S11), which was identified as the ethyl carbamate adduct in agreement with reports in the literature.[35] The ratio between the two products depended on the structure of the carboxylic acid and was relatively independent of the amino DNA conjugate. PyBrOP was tested as an example of a phosphonium salt coupling reagent and provided better coupling efficiencies for some acids but the results were inconsistent and the coupling efficiency was low for other acids (Table 2, Supporting Information Table S3).

■ DISCUSSION

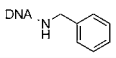
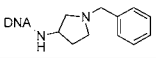
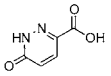
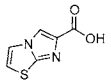
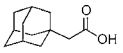
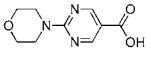
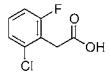
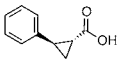
The construction of good-quality DNA-encoded chemical libraries crucially relies on the possibility to perform reactions at high yields and good purity with a large number of functionally related compounds. Amines and carboxylic acids represent two of the classes of building blocks, which can be purchased from commercial suppliers with a wide range of substituents. In this report, we systematically investigated synthetic strategies for the incorporation of amines and carboxylic acids into DNA-derivatives, with procedures suitable for library construction. We used a two-step protocol for the preparation of these DNA-conjugates. In principle, it would be possible to couple amines and carboxylic acids in a first step and conjugate them to the DNA. However, such an approach would

Table 1. Summary of Test-Coupling Reactions of Carboxylic Acids with DNA-Conjugates Containing a Secondary Amine[a]

	 CA-1	 CA-2	 CA-3	 CA-4	 CA-5	 CA-6	 CA-7	 CA-8	 CA-9	 CA-10	 CA-11
DNA-NH_2	47%	95%	96%	86%[b]	84%	85%	91%	99%	91%	73%[b]	94%[b]
	25%	83%	19%	52%	66%	51%	56%	80%	47%	20%	63%
	36%	88%	30%	31%	65%	57%	54%	57%	13%	20%	70%
	29%	67%	65%	66%	71%	59%	54%	95%	20%	15%	78%
	30%	75%	41%	58%	69%	69%	40%	82%	21%	15%	82%

[a]Conditions: CA-1 − CA-11: c(CA) = 50 mM, c(EDC) = 50 mM, c(HOAt) = 5 mM, 16h at RT in DMSO. Conjugates were confirmed by ESI for reactions with >30% yield. [b]Coupling values were taken from ref 11.

Table 2. Summary of Coupling Yields Using Different Activating Agents on a Range of Acids with Branched and Non-Branched Amines[a]

		DNA−NH_2						
		EDC (1 eq.)/ HOAt (0.1eq.)	EDC (1 eq.)/ HOAt (0.1eq.)	EEDQ (4 eq.)	PyBroP (2 eq.)	EDC (1 eq.)/ HOAt (0.1eq.)	EEDQ (4 eq.)	PyBroP (2 eq.)
	CA-1	47%	30%	90%	96%	<5%	76%	15%
	CA-2	95%	80%	77%	83%	81%	83%	70%
	CA-3	96%	13%	13%	8%	<5%	10%	<5%
	CA-4	84%	66%	<5%	36%	25%	<5%	22%
	CA-5	86%	31%	20%	61%	<5%	29%	<5%
	CA-6	99%	80%	37%	59%	<5%	67%	52%

[a]Conjugates were confirmed by ESI for reactions with >30% yield.

be uneconomical for larger libraries, and split-and-pool strategies are most efficient for the generation of DECLs.

Two reactions have been studied for the formation of the DNA-conjugates modified with secondary amines: nucleophilic attack of primary amines to a chloroacetamide group on DNA and reductive amination reaction between small-molecule amines and aldehyde-bearing DNA (Figure 1). The two reactions have been tested both on nucleobase-protected DNAs attached to the DNA controlled pore glass support (S_N2-reaction, Method B; reductive amination, Method D) and on deprotected and purified DNA in solution (reductive amination, Method C) and on DNA immobilized on anion exchange resin (S_N2-reaction, Method A). We found that each of the studied bioconjugation approaches was effective and appropriate for library synthesis. Nevertheless, each of the different reactions and experimental setups has advantages and disadvantages. Nucleophilic substitution of ClAcNH-DNA—both on anion-exchange resin (Method A) and on solid support (Method B)—may be sensitive to substitution at the α-carbon. The reaction proceeds effectively for α-methylene amines, but yields were lower for some amines with substituents or branching points at the α-carbon. The yield of the nucleophilic substitution reaction strongly depends on the amine concentration and generally high amine concentrations (typically >0.5 M) are necessary for efficient DNA-conjugation, which can be problematic for expensive and poorly soluble amines. Microwave irradiation may further enhance the reactivity and decrease the required amine concentration but is slightly cumbersome from a practical perspective.

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Reductive amination of DNA-CHO generates the product conjugates in excellent yields for the majority of amines, especially when performed on solid support. Furthermore, the amine concentration required for reductive amination is substantially lower than for nucleophilic substitution (50 mM instead of >1 M) and formation of side products is negligible. Among the tested conditions, reductive amination proceeded most efficiently in aqueous buffer and addition of organic cosolvents decreased product formation substantially, which can be problematic for amines with limited solubility in water. Moreover, certain amines provide low yields with reductive amination (at least in solution phase) for reasons that remain unclear. Furthermore, for some amines HPLC separation from the starting DNA-CHO is challenging.

The reactions investigated in this study could be successfully performed on unprotected DNA (S_N2 reaction, Method A; reductive amination, Method C) and on protected DNA on solid support (S_N2 reaction, Method B; reductive amination, Method D) with comparable reactivity. Solid support reactions do not require an HPLC purification step, which is necessary for deprotected DNAs, thus reducing time and work associated with library synthesis. However, the amine attachment on deprotected DNAs generally produces cleaner products, because the starting DNA has already been purified. Furthermore, DNAs attached to solid support require cleavage from the CPG under harsh conditions, which is incompatible with the chemical integrity of some building blocks (e.g., esters, amidines).

We also systematically studied amide-bond forming reactions, using amine-modified DNAs and over 500 different activated carboxylic acids. Amide-bond formation is one of the most widely used synthetic strategies for DNA-encoded chemical library construction. The study of a large set of building blocks revealed that the reaction yield varies substantially for different carboxylic acids. Little correlation between the coupling yield and structural features of the building blocks emerged from the analysis. These results emphasize the importance of comprehensively testing substrates in model reactions prior to library synthesis and, at the same time, the limitation of relying on small sets of test reactions. Whereas for DNA-NH_2 the majority of carboxylic acids coupled with good to excellent yields, coupling efficiency significantly decreased for a series of DNA-conjugates containing secondary amines. This finding is particularly relevant in view of the facile accessibility of DNA-derivatives with secondary amines, for example, using the S_N2 and reductive amination strategies described above. Substituents at the amine α-position decreased the reactivity, and for some combinations of DNA−amine conjugates and carboxylic acids, no or very little product was obtained. Further optimization of the protocol proved challenging, because modifications in reaction parameters (e.g., solvent) increased the yield for some acids while decreasing it for others (data not shown). Also, attempts to enhance reactivity by varying peptide-coupling reagents were of limited success. There appears to be a narrow window of reactivity that ensures the formation of conjugates in good yield and without the formation of side products by competing reactions with DNA (i.e., EDC-adducts, formation of ethyl-carbamate with EEDQ). EDC generally is the preferred peptide coupling agent, typically providing the best yields with primary amines. PyBrOP represents a potential alternative for acids that react poorly with EDC. For α-substituted amines EEDQ was the most efficient coupling agent and, for certain acids, coupling was observed for this reagent exclusively. Nevertheless, the reaction between activated carboxylic acids and DNA-attached secondary amines remains a feasible approach for the synthesis of DECLs, particularly as the major side-product—the unreacted DNA-conjugate—should not result in false positives during selections. Alternative reactions with secondary amines may be studied in the future (e.g., reactions with sulfonyl chlorides or isocyanates or reductive alkylation with aldehydes).

In summary, we have systematically studied chemical reactions for the preparation of DNA-derivatives, for which large sets of building blocks are commercially available. We tested more than 500 compounds in various experimental conditions and in multiple combinations. Certain general findings could be identified, which will help DNA-encoded chemical library planning and construction in the future. The observation of different reactivities for many of the tested reagents underline the importance of performing systematic evaluations of building blocks in model reactions, prior to library synthesis. In general, the proposed synthetic strategy has several advantages relative to previous synthetic schemes used for DECL preparation. For instance, the choice of amines and carboxylic acids as building blocks provides a wide selection of structural elements enabling the economic preparation of chemically diverse compounds. This is in contrast to libraries that relied on bifunctional building blocks (e.g., Fmoc-protected amino acids) or require building blocks that needed to be synthesized specifically for this purpose.[12,16] Furthermore, compounds formed by direct attachment of a carboxylic acid to a DNA-attached secondary amines are structurally compact making it possible to design libraries with compounds that have low molecular masses, which is an important parameter for drug likeness. One current drawback is that the amide bond formation with secondary amines on DNA is low for certain carboxylic acids, in particular, if the amine is sterically hindered. The development of alternative reactions that provide amide-bonds more efficiently and with higher chemoselectivity than classical coupling chemistry would be of great value for DECL preparation.[36,37] Alternatively, methods could be devised that remove unreacted amine-DNAs, the major side-product, thus providing the desired conjugate in good purity.

EXPERIMENTAL SECTION

Materials and Instrumentation. Standard reagents and solvents were acquired from commercial sources and used without further purification. Amine and carboxylic acid building blocks were purchased from several commercial suppliers including ABCR (Karlsruhe, Germany), ChemBridge (San Diego, CA), Sigma-Aldrich (St. Louis, MO), TCI Europe (Zwijndrecht, Belgium), Alfa Aesar (Ward Hill, MA), Matrix Scientific (Columbia, SC), and Acros Organics (Geel, Belgium). DNA-CHO and amino-modified oligonucleotides were provided by IBA-lifesciences (Goettingen, Germany). DEAE sepharose was obtained from GE Healthcare Life Science (Piscataway, NJ).

Purification of DNA- and fluorescein-conjugates were performed by reverse phase HPLC (Waters, Milford, CT) using a C18-XTerra column (5 μm, 10 on 10 × 150 mm, Waters, Milford, CT) and analyzed by LC-MS on a tandem-quadrupole mass spectrometer (Quattro Micro API, Waters, Milford, CT) with electrospray ionization source.

General Protocol for Nucleophilic Substitution Reaction on Pseudo-Solid Phase (Method A). DEAE

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sepharose was washed with 10 mM aq. AcOH (2×) and water (2×). DNA-C_{12}-NH_2 (5 nmol) was immobilized on the anion exchange resin by incubation of an aqueous solution of the amino-oligonucleotide for 5 min, followed by washing with water (2×) and MeOH (2×). A solution of 4-(4,5-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (DMT-MM, 75 mM) and sodium chloroacetate (50 mM) in dry MeOH (1 mL) was preincubated for 5 min and added to the immobilized DNA. The reaction mixture was gently stirred for 40 min. The resin was rinsed with MeOH (2×), 1.0 M *n*-propylamine in MeOH (2×) and MeOH (2×) and dried briefly. The coupling and washing steps were repeated (2×).

A solution of the amine (1.0 M) in DMSO was added to the resin-immobilized DNA and the reaction mixture gently stirred for 16 h at room temperature. The resin was washed with DMSO (2×) and 10 mM aq. AcOH (2×). The DNA was eluted with AcOH buffer (3.0 M, pH 4.75) and precipitated with EtOH (4-fold volume of buffer, 0 °C overnight).

General Protocol for Nucleophilic Substitution Reaction on Solid Phase (Method B). DNA-C_{12}-NH_2 (nucleobase- and phosphodiester-protected, after removal of the MMT-protecting group) on the solid-support (controlled pore glass, CPG) used for DNA synthesis was rinsed with MeCN (2×) and DMF (2×). A solution of chloroacetic acid (50 mM), HATU (50 mM), and DIEA (150 mM) in DMF was added to the solid support. The mixture was gently shaken for 2 h at room temperature. The solid support was rinsed with DMF (2×) and MeCN (2×) and dried in a gentle stream of air. A solution of the amine (1.0 M) in DMSO was added to the solid-support and the mixture incubated at room temperature for 16 h (unless stated differently). The DNA was deprotected and cleaved from the solid support by incubation for 3 h in AMA solution (1:1 conc. aq. NH_3/conc. aq. $MeNH_2$) at room temperature. The AMA solution was evaporated and the residue was dissolved in water.

General Protocol for Reductive Amination in Solution (Method C). To a solution (total volume 100 μL) of DNA-CHO (5 nmol) and amine (50 μmol; added as the conjugate acid by addition of 1 equiv AcOH unless present as an ammonium salt) in MOPS buffer (300 mM, pH 7.4) was added an aqueous solution of $NaBH_3CN$ (50 μmol). The reaction mixture was incubated at 37 °C for 16 h. The reaction was quenched by addition of acetic acid buffer (200 μL, 3.0 M, pH 4.75) and the DNA precipitated with EtOH.

General Protocol for Reductive Amination on Solid Support (Method D). DNA-C_{12}-NH_2 (after removal of the MMT-protecting group) on the synthesis solid-support (CPG) was rinsed with MeCN (2×) and DMF (2×). A solution of 4-formylphenoxyacetic acid (50 mM), HATU (50 mM), and DIEA (150 mM) in DMF was added to the solid support. The mixture was gently shaken for 2 h at room temperature. The solid support was rinsed with DMF (2×) and MeCN (2×) and dried in a gentle stream of air. A solution of the primary amine (50 mM), acetic acid (50 mM; omitted for ammonium salts), $NaBH_3CN$ (50 mM) in MOPS buffer (300 mM, pH 7.4; 5−10% of DMF were added in some cases to aid solubilization of the amine) was added to the solid support and the mixture incubated at 37 °C for 16 h. The DNA was deprotected and cleaved from the solid support by incubation for 3 h in AMA solution (1:1 conc. aq. NH_3/conc. aq. $MeNH_2$) at room temperature. The AMA solution was evaporated and the residue dissolved in water.

Functionalization of Amine-Modified DNAs with Carboxylic Acids on Pseudo-Solid Support. Amine-modified DNAs (1−2 nmol) were immobilized on DEAE sepharose (0.1 mL of slurry) as described for Method A. The resin was washed with 10 mM aq. AcOH (2 × 0.5 mL), water (2 × 0.5 mL) and DMSO (2 × 0.5 mL). To the resin-immobilized DNA was added a solution of the corresponding carboxylic acid (50 mM), EDC (50 mM), and HOAt (5 mM) in DMSO (0.5 mL). The slurry was agitated for 2 h at room temperature. The solution was removed and the resin washed with DMSO (1 × 0.5 mL) and treated with freshly activated reaction solution. These steps were repeated to reach two coupling steps of 2 h and one coupling step of either 2 h or overnight. The reaction solution was removed and the resin washed with DMSO (2 × 0.5 mL) and 10 mM aq. AcOH (3 × 0.5 mL). The DNA was eluted from the resin by incubation with 3 M AcOH buffer (pH 4.75) for 5 min. The DNA-conjugates were isolated by ethanol precipitation and the pellets redissolved in deionized water for analysis by HPLC and LC-MS.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

HPLC and mass-spectrometry analysis of conjugation reactions. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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