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Multifunctional dinucleotide analogs for the generation of complex RNA conjugates

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Abstract—Oligonucleotide conjugates are needed for in vitro selection schemes aiming at reactions between small, organic reactants. A general strategy is provided for the generation of the required RNA reactant conjugates based on multifunctional dinucleotide analogs. These modified dinucleotides allow for enzymatic ligation to native or enzymatically transcribed RNAs. They further contain a flexible polyethylene glycol spacer for correct spatial positioning and a photolabile cleavage site for selective release. The dinucleotides can be derivatized with the desired organic compounds by activated ester chemistry as was demonstrated by coupling to several nucleobases and nucleotides.

1. Introduction

The past decade has seen an increasing interest in functionalized oligonucleotides.[1] The conjugation to chromophores and fluorescent probes has facilitated the detection of nucleic acids significantly and nearly eliminated the use of radioactively labeled oligonucleotides in standard techniques such as Sanger sequencing or Northern/Southern blotting.[2] Metal chelators attached to nucleic acids serve as artificial nucleases[3] and conjugated markers such as acridine or psoralene are used to study DNA–protein or RNA–protein interactions. The advent of antisense or antigene therapy has set new demands on the in vivo stability and membrane permeability which were met by nuclease-resistant or lipophilic modifications.[4] The site-specific immobilization of nucleic acids is essential for the fabrication of high-density DNA chips which has revolutionized whole genome analysis.[5]

Recently, complex RNA conjugates have been shown to be a major improvement for in vitro selection experiments aiming at the isolation of new catalytic oligonucleotides.[6,7] In this technique, combinatorial nucleic acid libraries are successively screened for catalytic activity followed by enzymatic amplification of the selected sequences. Since an intramolecular catalysis event is exploited for the selection, the basic protocol using unmodified DNA/RNA is strictly restricted to oligonucleotide-modifying reactions. Catalysts for reactions between two small, organic reactants cannot be investigated using this approach. As an additional drawback, several classical selection experiments have also been prone to side reactions at internal positions of the nucleic acid.[8,9]

To expand the in vitro selection technique to previously inaccessible reactions, conjugates of enzymatically generated nucleic acids are needed.[10] In this approach, potential (deoxy-) ribozymes can intramolecularily act on their coupled reactant instead of on themselves. Through the covalent linkage to their catalytically generated products they become tagged, thus allowing for partitioning from the unmodified bulk of inactive sequences in the selection step. The use of such RNA conjugates ultimately led to the isolation of some remarkable new ribozymes catalyzing fundamental organic reactions like amide or *N*-glycosidic bond formation[11,12] and Diels–Alder cycloaddition.[13,14]

The key step in such expanded selection protocols based on oligonucleotide conjugates is the introduction of potential reactants into enzymatically generated nucleic acids. While several methods exist for the site-specific modification of oligonucleotides during their automated chemical synthesis (e.g. by incorporating suitably functionalized phosphoramidites),[15,16] labeling of native or enzymatically generated DNA/RNA has been described only for a few selected reporter groups with limited specificity and efficiency.

We recently proposed photocleavable dinucleotide analogs as versatile tools to tackle the inherent limitations of the in vitro selection.[17] They allow for efficient and site-specific derivatization of randomized RNA transcripts and are compatible with the overall selection scheme. Here we present a general conjugation strategy based on these dinucleotide analogs which vastly expands the repertoire of potential reactants to be investigated and significantly

Keywords: oligonucleotide derivatives; RNA conjugates; in vitro selection; ribozymes; photocleavage.

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reduces the preparative manipulations needed for their conjugation.

2. Results and discussion

2.1. Design and synthesis of the dinucleotide analogs

Previous experiments have shown that photocleavable linkers are compatible with the direct selection protocol.[17] Therefore they can be used to expand the scope of the method to reactions between two small molecules. To access new classes of linker conjugates and thereby apply the concept of photocleavable linkers to a broader range of reactants, the dinucleotides **1**–**3** were designed containing the following features in one molecule (Fig. 1).

- Two cytidine residues and a 5′-terminal phosphate group for the T4 RNA ligase-mediated attachment to RNA transcripts or other oligonucleotides.[18]
- Two to five hexaethylene glycol (HEG) units to ensure optimal positioning of RNA and coupled reactant. For example, ligating linker **3a** containing 18 ethylene glycol units to the acceptor stem of a typical tRNA should allow the attached reagent to interact with the anticodon loop, whereas the five hexaethylene glycol units of **2b** should be sufficient to span the length of an extended 30 base pair DNA duplex.[19] Polyethylene glycol (PEG) was chosen as a spacer because of its high flexibility, chemical inertness and structural neutrality, thereby minimizing a participation of the linker in the catalysis.[20]
- An *o*-nitrobenzyl moiety[21] which allows light-induced cleavage of the linker under a variety of conditions. Because photocleavage is independent of pH, redox potential, solvent polarity or ion strength, it offers a highly stringent selection criterion. The regioselective release of RNAs immobilized via their attached reactants should be useful to suppress side reactions which occur during the selection process and have been shown to complicate the selection process.[8,9]
- A 3′-terminal functional group to allow the attachment of potential reactants or other small molecules of interest. With biotin as a versatile affinity tag, this was realized during the automated synthesis of the dinucleotide analogs **1a** and **1b** by using a biotinylated solid support. As such appropriately pre-derivatized solid supports are usually not commercially available, we introduced a primary amine or a carboxyl group into the dinucleotide analogs **2** and **3**. These functional groups allow for the post-synthetic

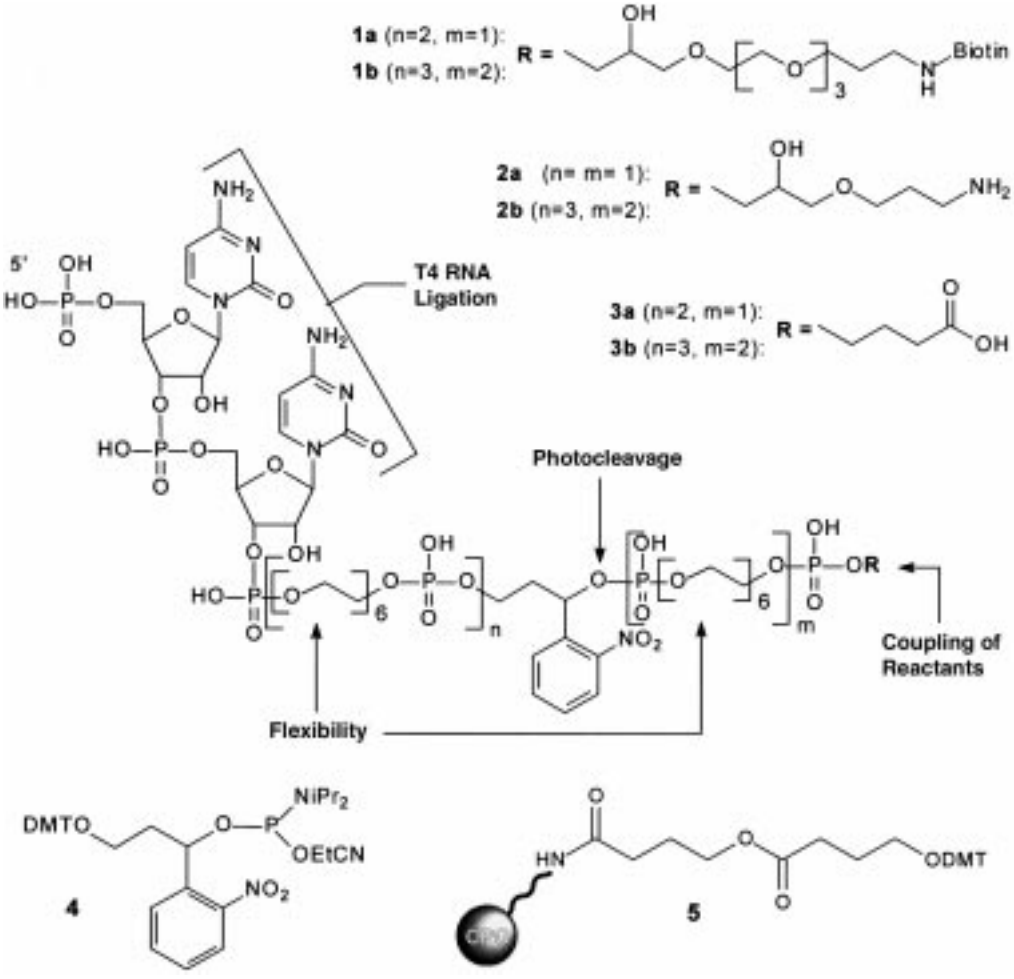

Figure 1. Design of the multifunctional dinucleotide analogs **1**–**3** containing the 5′-pCC residues for T4 RNA ligation, the photocleavable *o*-nitrobenzyl group, up to five hexaethylene glycol units and either a biotin, amino or carboxyl moiety at the 3′-terminus.

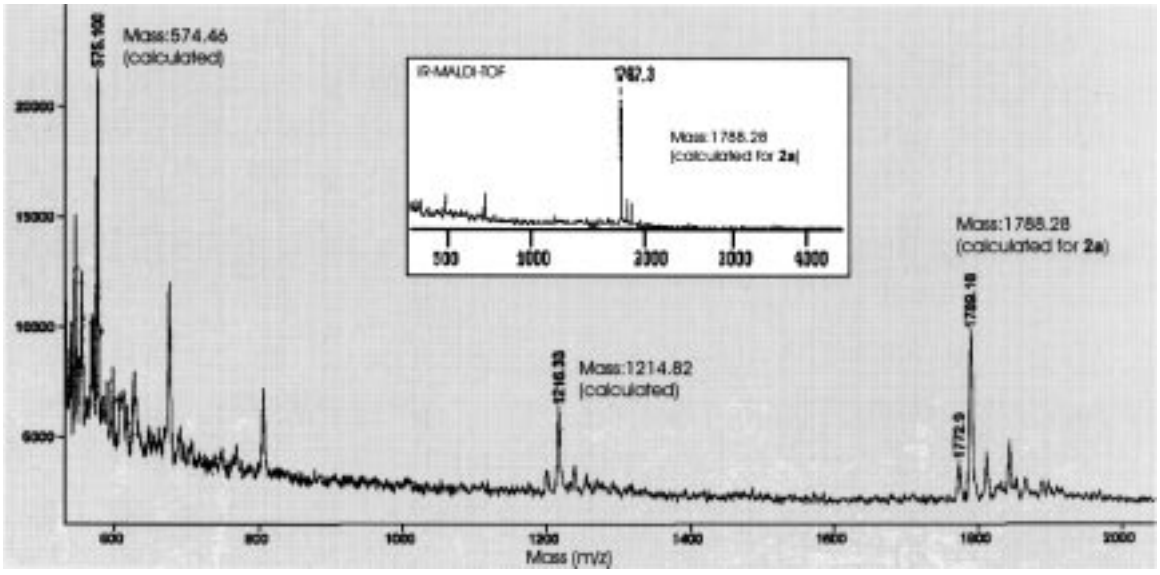

Figure 2. MALDI-TOF spectrum of the dinucleotide **2a** in the positive detection mode using hydroxypicolinic acid as a matrix with added NH_4^+-ion exchanger beads and excitation at 355 nm. The inset shows the IR-MALDI-TOF using succinic acid as matrix with excitation at 2940 nm.

attachment of potential reactants as carboxylic acids or primary amines, respectively.

The dinucleotide analogs were assembled by automated synthesis using commercially available building blocks. The photocleavable phosphoramidite **4** used to generate the photocleavage site was synthesized in four steps with 40% overall yield starting from *o*-nitrobenzaldehyde.[21] The carboxyl-generating solid support **5** was synthesized by immobilizing DMT-protected 4-hydroxy butyric acid on an amino-functionalized CPG matrix. After capping and detritylation, the 4-hydroxyl group was esterified with another 4-DMTO butyric acid via the mixed anhydride procedure.[22]

After the automated synthesis of the dinucleotide analogs, the precursors of **3a** and **3b** were first cleaved from the solid support with aqueous TEA prior to the standard deprotection procedure to avoid amide formation. After desilylation, the dinucleotides **1**–**3** were purified by preparative HPLC (shown for **3a** in Fig. 4b) with 5–20% isolated yield and characterized by MALDI-TOF mass spectroscopy. A typical spectrum of **2a** is shown in Fig. 2 where the predicted molecular mass peak at *m*/*z* 1789.18 g/mol can clearly be detected. Since the UV excitation of the matrix during MALDI-TOF measurements is nearly identical to the photocleavage conditions of the *o*-nitrobenzyl moiety, two additional peaks corresponding to the two expected photofragments were observed, thereby confirming the anticipated cleavage of the photoactive *o*-nitrobenzyl unit. Representatively, the integrity of dinucleotide **2a** was checked by IR-MALDI-TOF[23] and the detection of the correct molecular mass of [MH^+] of 1787.3 g/mol clearly validated the UV-MALDI-TOF measurements. As shown in the inset of Fig. 2 no photofragmentation or other decomposition was observed upon IR excitation.

2.2. Coupling of the dinucleotide analogs

The most straightforward way to generate derivatized dinucleotide conjugates is the coupling of suitably activated esters to the amino group of the dinucleotides **2a** or **2b**. This was exemplified by the biotinylation of **2a** with commercially available sulfosuccinimidyl (biotinamido) hexanoate. The reaction proceeded to near completion as detected by HPLC analysis (data not shown).

While for some commonly used reagents preactivated derivatives are commercially available (e.g. biotin, fluorescein, protected amino acids), for most target compounds the appropriate NHS esters have to be prepared from the corresponding carboxylic acids prior to conjugation with the dinucleotide. In situ activation approaches[24] with TSTU, HBTU or EDC were not satisfying and led to complex reaction mixtures without significant amounts of the desired products.

A general procedure for the derivatization of the dinucleotide therefore consists in a separate activation of carboxylic acids followed by coupling to the amino-functionalized dinucleotide analogs. This was demonstrated by the conjugation of the nucleobases adenine, nicotinic acid and orotic acid to **2b** (Fig. 3). With these conjugates in hand, RNA catalyzed nucleobase/nucleotide metabolism reactions could be investigated which are fundamental to a hypothetical RNA world.[25] For the coupling of adenine, the carboxyl moiety first had to be introduced by reacting 6-chloro purine **6** with caproic acid **7** to yield **8a**[26]. The three nucleobases **8a–c** were then activated with DCC in DMF (HMPTA for **8a** due to superior solubility) followed by a conversion to the corresponding NHS esters after the addition of *N*-hydroxy succinimide. The dicyclohexyl urea byproduct was precipitated and the activated esters **9a–c** were purified by crystallization from petroleum ether:isopropanol (6:1). All products were characterized by mass spectroscopy and NMR which revealed significant traces of unreacted orotic acid (35%) in **9c**, possibly due to hydrolysis during purification. Since this did not inhibit the following derivatization experiments, further purification was not necessary. The reactivity of the activated esters towards hydrolysis

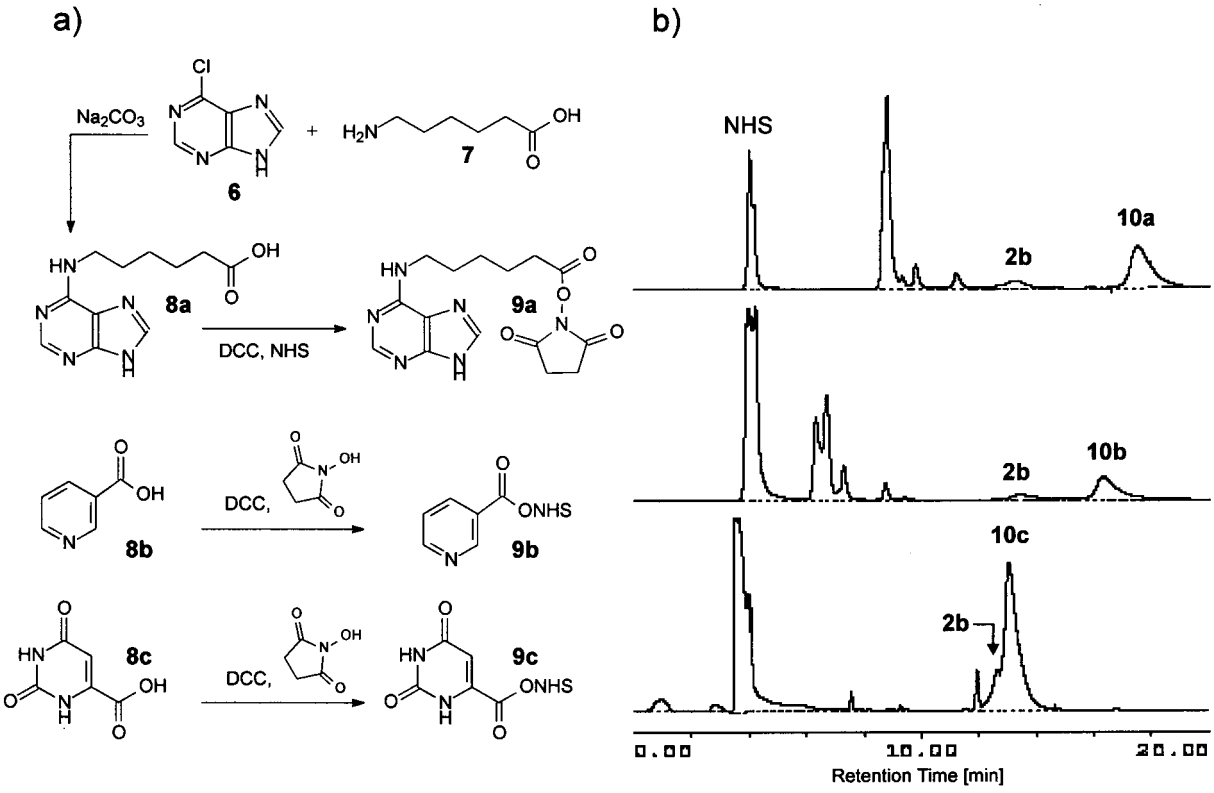

Figure 3. (a) Synthesis and *N*-hydroxysuccinimide (NHS) activation of the nucleobases *N*-(6-purinyl)-caproic acid (**8a**), nicotinic acid (**8b**) and orotic acid (**8c**); (b) HPLC purification of the coupling reactions of the nucleobase–NHS esters **9a–c** to the amino-functionalized dinucleotide **2b** (gradient B).

increased in the order **9a**<**9b**<**9c**. Whereas **9a** was moderately stable in aqueous neutral solutions (e.g. HPLC buffers), rapid precipitation was observed during the coupling of **9c** indicating very fast hydrolysis. When stored at −20°C under argon, no decomposition was detected for any of the NHS esters over several months.

During the coupling reactions an approximately 40-fold excess of activated ester ensured over 80% conversion of the dinucleotide **2b**. As shown in Fig. 3b, conjugation with adenine or nicotinic acid resulted in significantly higher retention times during HPLC purification. Besides the desired products and traces of unreacted dinucleotides, the main byproducts were identified as hydrolyzed or quenched esters of **9a–c** and NHS. Because the orotic acid conjugate and the underivatized dinucleotide analog **2b** have similar retention times, traces of the unreacted starting material could not be resolved. Additional HPLC analysis of isolated **10c** and co-injection with unmodified dinucleotide **2b**, however, verified a conjugation to orotic acid. Preparative HPLC yielded ca. 50% of isolated product under various reaction conditions and scales. The correct identities of the isolated products were confirmed by MALDI-TOF mass spectroscopy. Due to the specific photofragmentation pattern side reactions at the cytidine residues could be excluded.

2.3. Conjugation with complex molecules

Many reagents of interest are neither amenable to a separate activation as NHS-esters nor are they suited for in situ activation. Especially natural products may contain additional functional groups interfering with the coupling chemistry or they might lack the carboxyl group necessary for conjugation. Inherent instability might also preclude a coupling procedure as in Fig. 3. To address these classes of compounds, the dinucleotide analogs **3a** and **3b** were designed and synthesized carrying a 3′-terminal carboxyl group. Preliminary experiments[27] have shown that this functionality can be used for conjugation to primary amines. Since in this approach the amine component is now in large excess, the limiting carboxyl group is amenable to in situ activation with EDC at pH 6. This was demonstrated by coupling the dinucleotide **3a** to N^6-(6-aminohexyl)-AMP **11b** and to 5-(biotinamido)-pentylamine **11c**. Fig. 4b depicts the HPLC chromatograms of the starting material **3a** and of the products after pre-purification by PAGE and desalting on a Sephadex G10 column. To our surprise, no side reactions occurred at the 5′-phoshate group of **3a** as suggested by the literature.[28] This was corroborated by identifying the correct photofragments of the products **12b** and **12c** by MALDI-TOF mass spectroscopy. For reactants susceptible to decomposition the reaction times can be shortened as was demonstrated by the coupling of the ATP analog **11a** to **3a** on an analytical scale (Fig. 4c).

To check the applicability of the multifunctional linkers **1**–**3** for the generation of novel RNA reactant conjugate libraries, the derivatized dinucleotides **10a–c** were ligated overnight to a randomized 157nt RNA pool with approximately 80% efficiency as determined by a gel shift during PAGE purification. No influence of the length of the PEG spacer or the conjugated 3′-terminal reactant on the ligation efficiency was observed. A deoxy version of

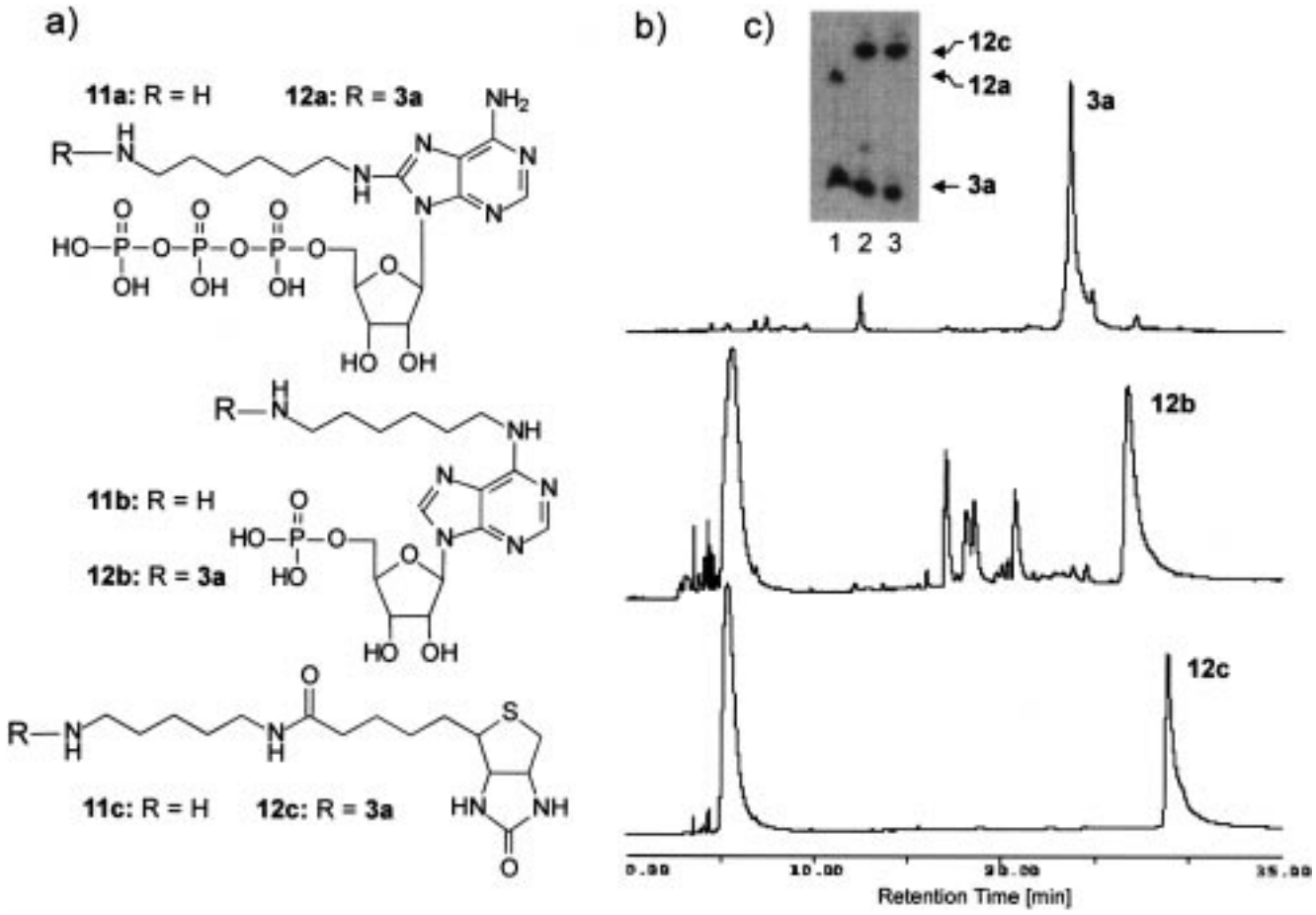

Figure 4. (a) Primary amines **11a–c** used for in situ coupling to the carboxy-terminated dinucleotide **3a**; (b) HPLC purification of the crude dinucleotide **3a** after automated synthesis (top) and its EDC-mediated conjugation products with the primary amines **11b** (middle) and **11c** (bottom) after PAGE and size exclusion chromatography; (c) Autoradiography of a denaturing 20% polyacrylamide gel analyzing the EDC conjugation products of **3a**. Lane 1 with **12a** for 2 h, lane 2 with **12c** for 25 min and lane 3 with **12c** for 2 h.

the dinucleotide analogs containing two deoxycytidines (dC) instead of ribocytidines (rC) at the 5′-end could also be ligated to RNA pools, albeit with only half the efficiency compared to the RNA linker (data not shown).

With the RNA libraries conjugated to the dinucleotides **10a–c** in hand, RNA-catalyzed nucleobase/nucleotide metabolism reactions can be investigated which are fundamental to a hypothetical RNA world. These nucleobase–RNA conjugates are currently being used in selection experiments for ribozymes synthesizing their own building blocks by catalyzing *N*-glycosidic bond formations. Along similar lines, AMP/ATP–RNA conjugates like **12a** and **12b** might allow selection experiments for ATP-generating ribozymes which are crucial for activating these building blocks, e.g. for subsequent RNA-catalyzed polymerization.[25]

Recently, the strategy of RNA–reactant conjugates coupled via photocleavable linkers was also applied to the selection of acyl-transfer catalysts. The isolated ribozymes accelerated the aminoacylation of small, external oligonucleotides. These findings demonstrated the regioselective selection pressure exerted by the photocleavable linker since the aminoacylation of the single 3′-terminal hydroxyl group was favored in the presence of 160 competing internal 2′-OH of the riboses or exocyclic amines of the nucleobases.[29]

Suitably derivatized dinucleotides (e.g. with puromycin) may also improve stringency in peptide selection experiments based on RNA–peptide fusions.[30] In combination with recently described multifunctional primers, the presented coupling chemistry can be applied to the generation of various DNA conjugates using appropriate PCR protocols.[31]

3. Conclusion

The dinucleotide analogs described here provide a straightforward and generally applicable way for site-specific derivatization of larger RNA transcripts. This allows for the expansion of the in vitro selection protocol to new, previously inaccessible reactions. The desired reactants can be coupled to the dinucleotides either via NHS-activated carboxyl groups or with lower yields as primary amines by in situ EDC-mediated condensation. The dinucleotide analogs presented in this work should serve as valuable tools in a variety of in vitro selection experiments as well as in RNA interaction studies.

4. Experimental

4.1. General

All organic reagents were purchased from Aldrich unless otherwise noted. Reversed phase HPLC was performed on a C-18 column (Beckman) with a 0.1 M triethylammonium acetate pH 7 (TEAAc) buffer and increasing percentage of acetonitrile (gradient A: 0.8%–16% in 15 min and 16%–24% in 20 min or gradient B: 0.8%–18% in 5 min and 18%–21.2% in 20 min, both followed by an 80% acetonitrile wash). Elution profiles were detected by measuring the absorbance at 270 nm.

MALDI-TOF measurements were performed on a Bruker Reflex spectrometer using hydroxypicolinic acid as a matrix containing additional NH_4^+-ion exchanger beads. After excitation with a Nd–YAG laser at 355 nm, cations were detected by time-of-flight measurement. Alternatively, 2,6-dihydroxybenzoic acid was used as a matrix in the negative detection mode with excitation at 377 nm. *o*-Nitrobenzyl containing samples were prone to photodegradation resulting in a characteristic loss of 16 mass units thereby obscuring the exact determination of these peaks.

4.1.1. Orotidyl-*N*-oxy-succinimide (9c). 1.20 g (10.4 mmol) *N*-hydroxy-succinimide (NHS) and 1.53 g (10.0 mmol) orotic acid **8c** (Fluka) were dissolved in 25 ml of dry DMF in an argon atmosphere. After addition of 2.26 g (11.0 mmol) dicyclohexyl carbodiimide (DCC) the solution was stirred overnight at room temperature. The precipitated solid was filtered off, the filter was rinsed with 5 ml DMF, and the filtrate was diluted with 180 ml of a mixture of petroleum ether (Merck, 40–60°C) and isopropanol (6:1). After 2 h at room temperature the crude product was recovered by filtration, redissolved in 5 ml DMF and recrystallized from 31 ml petroleum ether: isopropanol (6:1) to yield 0.71 g of **9c** (2.8 mmol, 28%) as a white solid after drying in vacuo. ^{1}H NMR (250 MHz, DMSO-d_6): δ=2.84 (s, 4H, NHS); 6.3 (s, 1H, CH); 11.58 (s, 2H, NH); traces of orotic acid and NHS at δ=2.58; 6.0; 10.82; 11.3. ^{13}C NMR (62.9 MHz, DMSO-d_6): δ=25.64; 106.54; 137.14; 150.74; 157.09; 163.29; 169.73, traces of orotic acid: 25.31; 103.27; 142.67; 150.95; 161.85; 164.18; 172.90.

4.1.2. Nicotinyl-*N*-oxy-succinimide (9b). 1.45 g (12.6 mmol) *N*-hydroxy succinimide and 1.48 g (12.0 mmol) nicotinic acid **8b** (Sigma) were dissolved in 25 ml dry DMF under argon and treated with 2.7 g (13.0 mmol) DCC overnight at room temperature. The precipitate was filtered off, the filtrate was concentrated by rotary evaporation and crystallized from petroleum ether:isopropanol (6:1) at room temperature. The white solid was recovered by filtration and dried under high vacuum to yield 1.67 g (63%) of the desired product **9b**. ^{1}H NMR (250 MHz, DMSO-d_6): δ=2.89 (s, 4H, NHS); 7.64–7.72 (m, 1H); 8.42–8.49 (m, 1H); 8.92–8.98 (m, 1H); 9.28–9.3 (m, 1H). ^{13}C NMR (62.9 MHz, DEPT=+/0/−): δ=25.60 (−); 121.08 (0); 124.49 (+); 137.77 (+); 150.54 (+); 155.65 (+); 161.01 (0); 170.21 (0). MS (EI): *m*/*z*=220 (2.3%, $M^{+\cdot}$); 106 (100%, M–NHS^{+}); 78 (50%, M–NHS–$CO^{+\cdot}$).

4.1.3. *N*-(6-Purinyl)-caproic acid (8a). A suspension of 1.67 g (12.7 mmol) caproic acid **7**, 0.77 g (7.0 mmol) Na_2CO_3 and 1.00 g (7.0 mmol) 6-chloro purine **6** in 10 ml water was adjusted to pH 9.5–10 by addition of 1 ml HCl (18%). The mixture dissolved upon refluxing and after 1 h the pH was readjusted with additional Na_2CO_3. After 3 h of refluxing the reaction mixture was cooled to 0°C, the product was precipitated by addition of 10 ml acetic acid and recovered by filtration. The crude product was purified twice by dissolving in 0.1 M NaOH and precipitating with acetic acid to yield 0.85 g (3.4 mmol, 50%) of HPLC-analyzed pure product **8a** after drying in vacuo. R_f (silica gel)=0.51 (CH_2Cl_2:MeOH:CF_3COOH=8:1.5:0.5). HPLC (gradient A)=16, 74 min. ^{1}H NMR (250 MHz, 4% NaOD/D_2O): δ=1.39–1.5 (quintet, 2H, *J*=7.5 Hz); 1.59–1.78 (m, 4H); 2.24–2.3 (t, 2H, *J*=7.5 Hz); 3.53–3.58 (t, 2H, *J*=7.5 Hz); 8.01 (s, 1H); 8.21 (s, 1H). ^{13}C NMR (62.9 MHz, DEPT=+/0/−): δ=28.40 (−); 28.88 (−); 31.54 (−); 40.32 (−); 43.44 (−); 122.69 (0); 152.24 (+); 154.20 (+); 156.43 (0); 160.70 (0); 186.34 (0). MS (EI): *m*/*z*=249 (24.5%, $M^{+\cdot}$); 190 (36%); 162 (35%); 148 (100%); 135 (45%).

4.1.4. *N*-(6-Purinyl) caproyl-*N*-oxysuccinimide (9a). 350 mg (1.4 mmol) *N*-(6-purinyl) caproic acid (**8a**) and 176 mg (1.5 mmol) *N*-hydroxy succinimide were dissolved in 1 ml of dry hexamethylphosphortriamide (HMPTA) under argon. The reaction was started by addition of 0.31 g (1.5 mmol) DCC at 0°C. After leaving the reaction overnight at room temperature the dicyclohexyl urea by-product was filtered off and rinsed with 0.1 ml HMPTA. The crude product was precipitated with 10 ml petroleum ether and 1.2 ml isopropanol for 2 h at room temperature and recovered by filtration. This residue was redissolved in 4 ml acetonitrile:acetone (1:1) and recrystallized with 7 ml petroleum ether:isopropanol (6:1) to yield 90 mg **9a** (0.26 mmol, 18%) as a white solid after drying in vacuo. ^{1}H NMR (250 MHz, DMSO-d_6): δ=1.34–1.46 (2H); 1.54–1.7 (m, 4H); 2.57 (s, 1H); 2.63–2.69 (t, 2H, *J*=7.5 Hz); 2.78 (s, 4H); 3.44 (br, 2H); 7.48 (s, 1H); 8.06 (s, 1H); 8.14 (s, 1H). ^{13}C NMR (62.9 MHz): δ=23.99; 25.43; 28.66; 30.14; 33.64; 36.45; 118.66; 138.54; 149.45; 152.36; 154.46; 168.97; 170.25. MS (FAB): *m*/*z*=347 ($M+H^+$).

4.2. Synthesis of the dinucleotide analogs

The dinucleotide analogs **1a** and **1b** were synthesized on a Pharmacia Gene Assembler on a 1.3 μmol DMT-on scale using phosphoramidites from Chemgenes, a biotinylated solid support from Glen Research and the photocleavable building block **4**. Coupling times were 90 s except for the cytidine residues (720 s) and the chemical phosphorylation (3×90 s).

The dinucleotide analogs were deprotected in 1 ml 33% NH_4OH/ethanol (3:1) overnight at 55°C. After lyophylization, the samples were treated with 300 μl 1 M nBu_4NF in THF for 48 h, quenched with 0.3 ml 2 M TEAAc (pH 7) and diluted to 10 ml with water. The crude product was passed over a 1.5 ml Sephadex A25 column (pre-equilibrated with 10 ml 0.05 M TEAAc), washed with 15 ml 0.1 M TEAAc and eluted with 2 M TEAAc. The UV active fractions were pooled, lyophylized and purified by HPLC. The lyophilized products were quantified by UV spectroscopy at 270 nm and characterized by UV-MALDI-TOF mass spectroscopy.

1a: 2.4 A_{270} (125 nmol, 10%); R_t=31.6 min (gradient A); mass (*m*/*z*)=2488.8 (calculated $[M-H]^-$=2487.6 g/mol); 3′-terminal photofragment (*m*/*z*)=931.8 (calculated=930.6 g/mol).

1b: 3.2 A_{270} (165 nmol, 13%); R_t=18.1 min (gradient B); mass (*m*/*z*)=3179.3, 3164.5 (calculated $[M+H]^+$=3178.6 g/mol); 5′-photofragment (*m*/*z*)=1904.2, 1889.2 (calculated=1903.4 g/mol); 3′-biotinylated photofragment (*m*/*z*)=1277.0 (calculated=1276.2 g/mol).

Dinucleotide **2a** was synthesized on an Applied Biosystems 391 synthesizer on a 1 μmol DMT-on scale using a 3′-aminomodifier-C6-CPG support from Chemgenes. Deprotection and purification were analogous to the dinucleotides **1** yielding 5.4 A_{270} (286 nmol, 22%) eluting at 21.5 min (gradient A). UV-MALDI-TOF: Mass found (*m*/*z*)=1789.18 (mass calculated $[M+H]^+$=1788.28 g/mol); cytidine-containing photofragment (*m*/*z*)=1216.33 (calculated=1214.82 g/mol); amine-containing photofragment (*m*/*z*)=575.1 (calculated=574.46 g/mol).

Dinucleotide **2b** was synthesized on a Milligen 8800 DNA Synthesizer on a 160 μmol DMT-on scale and deprotected with 12 ml 33% NH_4OH and 4 ml EtOH overnight at 55°C. The resulting lyophylized residue was desilylated in 1 ml triethylammonium trihydrofluoride at room temperature for 48 h, desalted on a Sephadex G10-column and purified by preparative HPLC on a C_{18}-Eurosphere column (Knauer) yielding 150 A_{270} (8 μmol, 5%) eluting at 13.3 min (gradient B). Mass found (*m*/*z*)=2821.0 (mass calculated $[M+H]^+$=2821.2 g/mol); cytidine-containing photofragment (*m*/*z*)=1904.2 (calculated=1903.4 g/mol); amine-containing photofragment (*m*/*z*)=918.4 (calculated=918.8 g/mol).

The dinucleotides **3a** and **3b** were synthesized as described for **1** starting with solid support **5**. Prior to deprotection the dinucleotide analogs **3** were first cleaved from the solid matrix in 0.75 ml H_2O/TEA (2:1) for 12 h at 55°C and then deprotected with an additional 0.5 ml 33% NH_4OH/EtOH (3:1) for 6 h at 55°C. Desilylation and purification were performed as for the analogs **1**.

3a: 4 A_{270} (200 nmol, 15%); R_t=23.6 min (gradient A); mass (*m*/*z*)=2084.8 (calculated $[M-H]^-$=2084.1 g/mol); 3′-terminal photofragment (*m*/*z*)=527.4 (calculated=527.1 g/mol).

3b: 2.4 A_{273} (127 nmol, 10%); R_t=11.7 min (gradient B); mass found (3′-carboxyl-containing photofragment): *m*/*z*=874.1; (calculated $[M+H]^+$=873.7 g/mol).

4.2.1. Radioactive labeling of the dinucleotide 3a. 300 pmol of the dinucleotide **3a** were dephosphorylated by 2 U shrimp alkaline phosphatase (USB) in 10 μl buffer (20 mM Tris–HCl, pH 8.0, 20 mM $MgCl_2$) for 1 h at 37°C, then mixed with 1.5 μl 0.1 M EDTA and heated to 80°C for 10 min. After cooling, the reaction mixture was diluted with 2.5 μl T4 kinase buffer (10×, MBI Fermentas), 1.5 μl $MgCl_2$ 0.1 M, 2.5 μl dithiothreitol (DTT) 35 mM, 5 μl γ-^{32}P ATP (Amersham, 10 μCi/μl) and 2 μl T4 polynucleotide kinase (10 U/μl, MBI Fermentas) and incubated for 2 h at 37°C. The product was purified with 20% denaturing PAGE, visualized by autoradiography, eluted in water, concentrated by lyophylization and desalted on a 0.8 ml Sephadex G10-column.

4.2.2. Derivatization of the dinucleotide 2b. The conjugates **10a–c** were generally synthesized in 10 μl reactions containing 2.4 mM dinucleotide analog **2b**, 0.1 M of the respective *N*-hydroxysuccinimide-activated base **9a–c**, 0.4 M K_2HPO_4 (pH 8.0) and 20% DMF at room temperature. After 2–3 h the reactions were quenched with 2 μl ammonium acetate (1 M) for 30 min, diluted to 100 μl with water and purified by HPLC (gradient B).

10a: 0.27 OD_{270} (14 nmol, 60%); R_t=17.5 min; mass found (*m*/*z*)=3052.1 (calculated $[M+H]^+$=3051.1 g/mol); cytidine-containing photofragment (*m*/*z*)=1904.3 (calculated: 1903.4 g/mol); adenine-containing photofragment (*m*/*z*)=1149.6 (calculated: 1149.6 g/mol).

10b: 0.25 OD_{267} (13.2 nmol, 55%); R_t=16.4 min; mass found (*m*/*z*)=2925.7 (calculated $[M+H]^+$=2924.9 g/mol); cytidine-containing photofragment (*m*/*z*)=1904.1 (calculated: 1903.4 g/mol); nicotinyl-containing photofragment (*m*/*z*)=1023.9 (calculated: 1023.4 g/mol).

10c: 0.26 OD_{274} (13.4 nmol, 56%); R_t=12.8 min; mass found (*m*/*z*)=2959.0 (calculated $[M+H]^+$=2957.9 g/mol); cytidine-containing photofragment (*m*/*z*)=1904.2 (calculated: 1903.4 g/mol); orotidyl-containing photofragment (*m*/*z*)=1056.9 (calculated: 1056.4 g/mol).

4.2.3. Derivatization of the dinucleotide 3a. 20 nmol of partially 5′-^{32}P labeled dinucleotide **3a** were treated with 2 μmol of 5-(biotinylamido)-pentylamine **11c** or N^6-(6-aminohexyl)-AMP **11b** and 1 μmol EDC in 4 μl 2 M imidazole/HCl for 30 h and with 2 μl of fresh 0.25 M EDC for another 4 h. The reaction mixtures were then electrophoresed on a 20% denaturing polyacrylamide gel. The crude products were identified by autoradiography, eluted with water, desalted on a 4 ml Sephadex G10 column and purified by HPLC (gradient A).

12b: 68 mOD_{265} (2 nmol, 10%); R_t=26.8 min; mass found (AMP-containing photofragment): *m*/*z*=955.7 (calculated $[M-H]^-$=955.5 g/mol).

12c: 0.12 OD_{265} (6.25 nmol, 30%); R_t=28.9 min; mass found (biotinylated 3′-photofragment): *m*/*z*=837.5 (calculated $[M-H]^-$=837.6 g/mol).

4.3. Transcription of the RNA pool and ligation to the dinucleotide derivatives

The 157nt-RNA was synthesized by transcription of 10 pmol double-stranded DNA template in a 200 μl reaction containing 80 mM Hepes pH 7.5, 22 mM $MgCl_2$, 1 mM spermidine, 10 mM DDT, 0.2 mg/ml BSA, 4 mM of each nucleoside triphosphate (Roche), 1 U pyrophosphatase (Sigma), 100 μCi α-^{32}P CTP (Amersham) and 200 U T7 RNA polymerase. After 4 h at 37°C the DNA template was digested with 40 U DNase I (Roche) for 30 min at 37°C. The RNA was purified by denaturing PAGE (5%).

Full-length RNA (157 nt) was identified by autoradiography, eluted with 0.3 M NaOAc pH 5.5 and precipitated with ethanol, yielding 3.2 nmol. To suppress folding of the randomized RNA pool into undesired secondary structures interfering with ligation, the 3′-terminus was routinely annealed to 4 nmol of a complementary oligonucleotide at 95°C. For RNA substrates with accessible 3′-ends, this hybridization was not essential. Ligation of the hybridized RNA was performed in a 200 μl reaction with 50 mM Tris–HCl pH 7.8, 20 mM $MgCl_2$, 10 mM DTT, 20 μg/ml bovine serum albumin, 4 mM adenosine triphosphate (MBI), 10% DMSO, 100 μM of the respective dinucleotide derivative **10a–c** and 300 U T4 RNA ligase (MBI) with 70% conversion. Electrophoresis on a 5% denaturing polyacrylamide gel yielded 0.9 nmol (30%) of the isolated ligation product.

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