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Reversible 5′-end biotinylation and affinity purification of synthetic RNA

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Abstract—A reversible biotinylation phosphoramidite was synthesized and incorporated onto the 5′-end of an oligoribonucleotide on a solid phase synthesizer. After cleavage and deprotection, the crude synthetic oligomer mixture was incubated with NeutrAvidin® coated microspheres, and the failure sequences removed by washing with a buffer followed by treating the microspheres with tetrabutylammonium fluoride to give a high quality unmodified full-length oligoribonucleotide.
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Besides wide application in biology and medicine for nonradioacitve labeling of biopolymers,[1–3] the strong noncovalent interaction (association constant $10^{15}\,M^{-1}$) between biotin and streptavidin or avidin has been proved to be useful for efficient isolation of biotinylated DNA from complex mixtures.[4–6] Typically, the mixture containing the biotinylated target is incubated with streptavidin or avidin coated microspheres, then, nonbiotinylated materials are removed by washing with buffer followed by recovering purified target molecules from the microspheres. In order to obtain unmodified DNA after affinity isolation, a special reversible linker between biotin and target is required. The reported linkers suitable for such a purpose include the acid-cleavable triphenylmethyl alkyl ether linkage reported by Gildea et al.[4] and the photocleavable *o*-nitrobenzyl alkyl phosphate diester linkage reported by Rothschild and co-workers.[5] Although successful in certain applications, these linkers have shortcomings such as inconvenient preparation, long cleavage time under acidic conditions and potential damage of biomolecules caused by exposure to UV irradiation.[7–9] Recently, we reported the preparation of two phosphoramidites containing a diisopropylsilyl acetal linkage, and their use for reversible biotinylation, phosphorylation and affinity purification of synthetic DNA.[10,11] We demonstrated that the phosphoramidites could be efficiently coupled onto the 5′-end of an oligodeoxyribonucleotide on a solid phase synthesizer, and that the linkage was completely stable under synthetic and cleavage/deprotection conditions. The biotinylated full-length DNA could be efficiently attached to NeutrAvidin® coated microspheres, failure sequences could be completely removed by simple washing with buffers, and high quality unmodified DNA could be recovered by treating with fluoride ion. As it is well known, oligoribonucleotide synthesis is generally less efficient than oligodeoxyribonucleotide synthesis, and thus, generates more failure sequences. Moreover, extreme caution is required to handle RNA because of ubiquitous RNAse. These factors coupled with the higher hydrophilicity of RNA make it more difficult to purify. Consequently, an efficient, simple RNA purification method should be valuable to the scientific community. Here we report the synthesis of a reversible biotinylation ribonucleotide phosphoramidite, its incorporation onto the 5′-end of an oligoribonucleotide on a solid phase synthesizer, and the application of this biotinylation methodology in affinity purification of synthetic RNA.

As shown in Scheme 1, the biotinylation phosphoramidite **1** was conveniently prepared from the known biotinyl alcohol **2**[10] in two steps. Thus, **2** (1 equiv),

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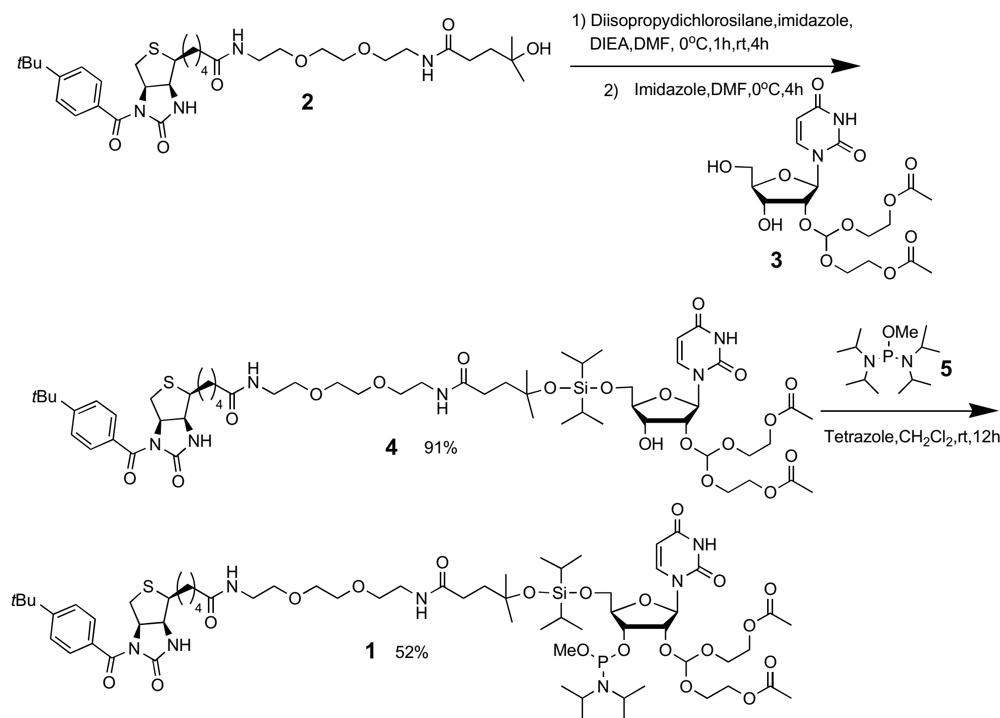
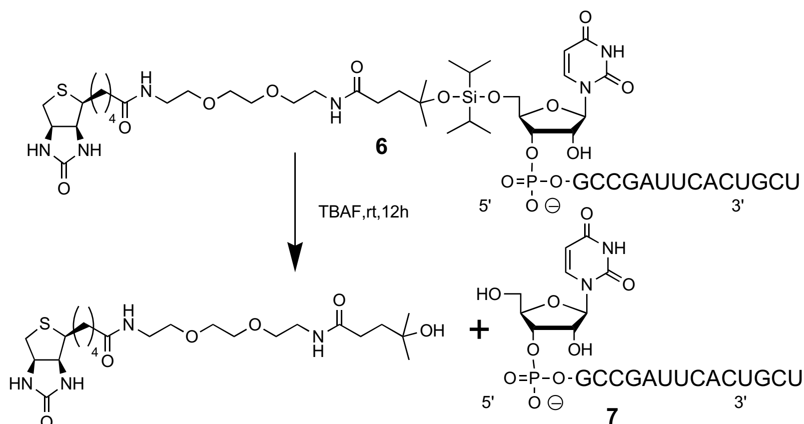


Scheme 1. Synthesis of the reversible biotinylation phosphoramidite **1**.

imidazole (1 equiv) and diisopropylethylamine (DIEA, 3 equiv) in dry DMF was cooled on an ice bath, diisopropyldichlorosilane was added, and the solution was stirred at 0 °C for 1 h and at room temperature for 4 h. The resulting solution was added slowly to a solution of 2′-O-ACE-uridine (**3**, 1 equiv)[12] and imidazole (1 equiv) in DMF via a cannula at 0 °C, and the mixture was stirred at the same temperature for 4 h. Partition between CH_2Cl_2 and 5% $NaHCO_3$ and flash chromatography (SiO_2, EtOAc/MeOH/Et_3N = 95:4:1) gave **4** as a white foam in 91% yield.[13] The vacuum dried **4** (1 equiv) was dissolved in CH_2Cl_2 and degassed by argon followed by the addition of phosphinylation agent **5** (1.3 equiv) via a syringe, and tetrazole (1.1 equiv) under positive argon pressure. After stirring at room temperature for 12 h, the mixture was partitioned between CH_2Cl_2 and 5% $NaHCO_3$. Purification by flash chromatography (SiO_2 pretreated with $CHCl_3$/THF/Et_3N = 3:2:0.5, then washed with $CHCl_3$/THF = 3:2; after loading the sample, eluted with $CHCl_3$/THF = 3:2) gave product **1** as a white foam in 52% yield.[14]

To test this biotinylation and affinity purification strategy, the short 5′-end biotinylated oligoribonucleotide **6** (Scheme 2) was next synthesized on a solid phase synthesizer at Dharmacon, Inc. on a 1 μmol scale.[15,16] The synthesis was carried out on a high cross-linked polystyrene support using the 2′-O-ACE protected phosphoramidites illustrated in Figure 1 and biotinylation phosphoramidite **1** as building blocks under standard conditions. After complete deprotection and cleavage,[15,16]



Scheme 2. Removal of biotin to generate unmodified RNA.

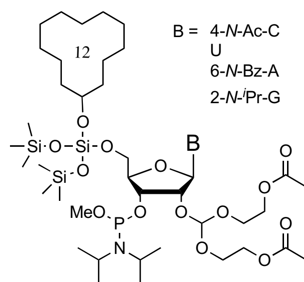


Figure 1. Phosphoramidite monomers used for RNA synthesis.

the crude oligoribonucleotide (10% of the 1 μmol synthesis), which contains the full-length oligomer, failure sequences, and other impurities, was incubated with NeutrAvidin® gel (600 μL) in PBS buffer (300 μL) at room temperature for 2 h.[17] Because the failure sequences generated in each synthetic cycle were capped with large excess of acetic anhydride, only the full-length oligoribonucleotide was biotinylated by the phosphoramidite **1**. As a result, only the desired RNA was attached to the microsphere through the noncovalent interaction between biotin and NeutrAvidin®, and the failure sequences and other impurities were removed by washing with PBS buffer (300 μL × 3) and water (500 μL × 3). After drying on a SpeedVac, the pure full-length unmodified RNA **7** (Scheme 2) was recovered from the gel by treating with tetrabutylammonium fluoride (TBAF, 1 M, THF) at room temperature for 12 h with gentle shaking followed by quenching by equal volume Tris–HCl buffer (100 mM, pH 7.4), and washing with water (300 μL × 5). In order to estimate the recovery yield of oligoribonucleotide from the microspheres, in addition to the full-length sequence recovered from gel, the failure sequences removed by washing were also collected, both were desalted by a NAP™-25 column,[18] and their quantities were determined by UV measurement at 260 nm, respectively. From these data, the recovery yield of RNA was estimated to be about 80%.

The efficacy of the affinity purification was next evaluated by 20% denatured (8 M urea) polyacrylamide gel electrophoresis (PAGE). In Figure 2, lane a is the crude RNA treated with TBAF (1 M, THF, 200 μL, *N*-methylpyrolidinone, 200 μL, rt, 12 h; 10% of the 1 μmol synthesis treated, 1% of which loaded).[19] Lane b is untreated

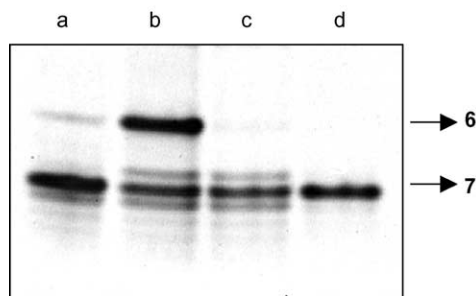


Figure 2. PAGE analysis of the results of NeutrAvidin® coated microspheres mediated affinity purification of synthetic RNA. See text for details.

crude RNA (same amount loaded). As can be seen, the biotinylated full-length RNA **6** runs much slower than the unmodified full-length RNA **7** and failure sequences, and the diisopropylsilylacetal linkage is not completely stable under deprotection/cleavage conditions. In order to estimate the degree of premature breakage, a separate preparative denatured PAGE was used to separate **6** and **7**,[20] and both were extracted from the gel with NH_4OAc buffer (0.1 M). UV measurement indicated that more than 70% **6** retained their biotin moiety during cleavage and deprotection. Lane c is recovered failure sequences removed from the NeutrAvidin® gel (concentrated on SpeedVac, desalted on a NAP™-25 column, 1% loaded) during the affinity purification. This lane demonstrated the high efficiency of the attachment of biotinyl RNA **6** to the avidin coated microsphere as **6** is not visible. Lane d is the affinity purified full-length unmodified RNA **7** (1% loaded), and shows that the failure sequences have been completely removed yielding pure RNA.

In summary, we prepared a novel reversible biotinylation phosphoramidite, and successfully incorporated it onto the 5′-end of oligoribonucleotide on a solid phase synthesizer. To the best of our knowledge, this represents the first chemical biotinylation of the 5′-end of RNA using a reversible linker, which, upon cleavage, affords unmodified RNA. We demonstrated that biotinylated full-length RNA could be efficiently attached to NeutrAvidin® coated microspheres by simple incubation in a buffer, failure sequences, and other impurities could be removed by simple washing, and high quality RNA could be conveniently obtained by cleavage from the microsphere.

Acknowledgements

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.tetlet.2004.09.019.

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12. 2′-O-ACE-uridine **3** was purchased from Dharmacon, Inc.
13. Biotinylurindine **4**: $R_f = 0.3$ (EtOAc/MeOH = 9:1); IR (thin film, cm^{-1}) ν 3309 (br), 2945, 2864, 1735, 1685, 1653, 1247, 1111, 1051; ^1H NMR ($CDCl_3$, 300 MHz) δ 0.97–1.11 (m, 14H), 1.28 (s, 6H), 1.32 (s, 9H), 1.46–1.87 (m, 8H), 2.05 (s, 3H), 2.06 (s, 3H), 2.19–2.30 (m, 4H), 3.02–3.09 (m, 2H), 3.22–3.26 (m, 1H), 3.34–3.43 (m, 4H), 3.45–3.68 (m, 8H), 3.75–4.34 (m, 14H), 5.20–5.24 (m, 1H), 5.59, (s, 1H), 5.66 (d, J = 8.4 Hz, 1H), 5.96 (d, J = 2.1 Hz, 1H), 7.39 (d, J = 8.4 Hz, 2H), 7.57 (d, J = 8.4 Hz, 2H), 7.94 (d, J = 8.1 Hz, 1H); ^{13}C NMR ($CDCl_3$, 300 MHz) δ 1.85, 13.26, 13.41, 17.69, 20.85, 25.40, 27.79, 27.95, 29.69, 29.78, 31.15, 31.66, 34.94, 35.62, 38.24, 39.09, 39.26, 40.01, 55.15, 57.44, 61.01, 62.49, 62.71, 62.79, 63.07, 68.16, 69.78, 70.04, 73.74, 84.17, 88.23, 101.97, 112.37, 116.52, 124.48, 128.97, 131.79, 140.15, 150.44, 154.91, 156.36, 163.67, 169.91, 170.92, 173.18, 173.61. MS (ESI, M−H) calcd for $C_{57}H_{89}N_6O_{19}SSi$ 1221.56, found 1221.35.
14. Phosphoramidite **1**: $R_f = 0.2$ ($CHCl_3$/THF = 1:1); ^1H NMR ($CDCl_3$, 300 MHz) δ 0.97–1.12 (m, 14H), 1.12–1.22 (m, 12H), 1.25–1.28 (m, 6H), 1.32 (s, 9H), 1.47–1.87 (m, 8H), 2.05–2.06 (m, 6H), 2.18–2.31 (m, 4H), 3.05–3.08 (m, 2H), 3.22–3.49 (m, 10H), 3.50–3.70 (m, 8H), 3.72–3.98 (m, 15H), 4.32–4.43 (m, 1H), 5.21–5.25 (m, 1H), 5.48 (s, 0.5H), 5.56 (s, 0.5H), 5.67 (d, J = 8.1 Hz, 1H), 5.99 (d, J = 2.7 Hz, 0.5 H), 6.02 (d, J = 3.9 Hz, 0.5 H), 7.39 (d, J = 8.4 Hz, 2H), 7.58 (d, J = 8.4 Hz, 2H), 7.85 (d, J = 7.5 Hz, 1H); ^{13}C NMR ($CDCl_3$, 300 MHz) δ 10.06, 13.10, 13.67, 17.64, 17.75, 17.88, 20.85, 21.19, 24.64, 24.75, 25.37, 25.60, 29.80, 30.31, 31.15, 31.61, 34.22, 34.94, 35.51, 38.25, 39.08, 39.24, 40.05, 42.82, 42.92, 43.08, 45.46, 55.08, 57.37, 61.23, 61.48, 61.65, 61.88, 62.47, 62.68, 63.17, 67.93, 69.79, 69.86, 70.01, 70.09, 73.68, 83.46, 84.26, 87.85, 88.97, 101.89, 111.79, 111.96, 124.48, 125.50, 128.23, 128.97, 131.75, 135.80, 140.70, 140.94, 150.21, 150.30, 151.50, 154.88, 156.29, 163.58, 169.95, 170.87, 173.06, 173.33; ^{31}P NMR ($CDCl_3$, 300 MHz) δ 168.30, 168.57.
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17. NeutrAvidin® gel was purchased from Pierce Biotechnology, Inc. PBS buffer: 136.9 mM NaCl, 2.7 mM KCl, 10.1 mM Na_2HPO_4, 1.8 mM KH_2PO_4, adjusted to pH 7.0 with HCl.
18. NAP™-25 column was purchased from Amersham Biosciences. Desalting was performed following manufacture-suggested procedure.
19. The gel was imaged on a Molecular Dynamics Storm (860) system after staining in a SYBR® Gold (purchased from Molecular Probes, Inc.) solution in 1× TBE buffer (1:10,000) at rt for 30 min.
20. RNAs **6** and **7** were identified with MALDI mass spectra. Biotinyl RNA **6**: calcd 5287, found 5289. Unmodified RNA **7**: calcd 4687, found 4687.