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Convenient RNA Synthesis Using a Phosphoramidite Possessing a Biotinylated Photocleavable Group

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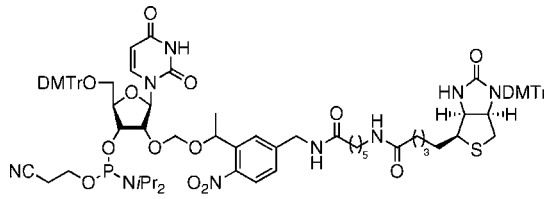
ABSTRACT



structure of phosphoramidite for a rapid RNA synthesis

A convenient RNA synthesis using a biotin−streptavidin interaction and a photocleavable protecting group is described. The biotinylated photocleavable group was introduced at the 2′-position of the uridine derivative. Using the phosphoramidite 12, we attempted the synthesis of a 21mer RNA, which is pure enough to show potent RNAi activity compared with a conventionally prepared and HPLC-purified 21mer RNA with the same sequence.

After the discovery of RNA interference (RNAi),[1] much effort has been dedicated to the application of short interfering RNAs (siRNAs), not only as biological tools but also as therapeutic agents.[2] In addition, investigation has also focused on the discovery of potentially new functions of RNAs, especially noncoding RNAs such as microRNAs (miRNAs).[3] This trend translates into a high demand for development of a more rapid and efficient synthetic protocol for solid-phase RNA synthesis. However, because of the existence of the 2′-hydroxyl group in its structure, the RNA synthesis is still comparatively difficult versus the optimized solid-phase DNA synthesis. Thus, selective protection of the 2′-hydroxyl group is first required. This protecting group must be stable and less bulky for a coupling reaction with a phosphoramidite unit throughout the solid-phase synthesis; yet it must be readily removable under mild conditions. In addition, after removal of the protecting groups at the 2′-hydroxyl position, the resulting free 2′-hydroxyl group may cause cleavage and/or migration of the RNA strand, especially under basic conditions. Therefore, a more rapid method of purification after removal of the protecting group at the 2′-position is also required.

For the protecting group at the 2′-position, a *tert*-butyldimethylsilyl (TBDMS) group is a popular choice, the phosphoramidite units of which are widely used for RNA synthesis. However, since this is not a robust method, extensive effort is underway to develop a more suitable protecting group at the 2′-position, such as triisopropylsilyloxymethyl (TOM) and bis(2-acetoxyethyloxy)methyl (ACE) protecting groups.[4−9] Ohgi et al. have recently developed one of the most successful examples with a 2-cyanoet-

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hoxymethyl (CEM) protecting group and have succeeded in preparing a 110mer precursor miRNA using this method.[10,11] Apart from these efforts, there have been few reports of novel technology for rapid purification, which would avoid cleavage and/or migration of the resulting RNA strand caused by the tedious purification procedure. To this end, Olejnik et al. developed a rapid purification of DNA using a nonnucleosidic photocleavable biotin phosphoramidite.[12] In their approach, the phosphoramidite is incorporated at the 5′-end of the DNA strand, so that the resulting 5′-end biotin-labeled DNA can be selectively isolated from the undesired shorter DNA fragments by incubation with immobilized streptavidin. After irradiation with UV light, the photocleavable biotin moiety at the 5′-end is cleaved to afford the desired DNA strand. This approach is ingenious for a rapid purification; however, the resulting DNA strand is inevitably 5′-phosphorylated. Fang and Bergstrom reported an RNA synthesis using a similar strategy based on biotin−avidin interaction.[13] In their method, the biotin group is introduced at the 5′-end of the resulting oligoRNA via a silyl acetal linkage, necessitating the ACE-protected phosphoramidite units,[6] and thus requires unusual reaction conditions in solid-phase RNA synthesis.

In view of the apparent limitations of existing methods, we report herein a new approach toward a convenient RNA synthesis. Our approach consists of the synthesis of a phosphoramidite possessing a biotinylated photocleavable group at the 2′-position, its incorporation into the 5′-end of the RNA strand under the usual reaction conditions in solid-phase RNA synthesis, and selective isolation by means of the biotin−streptavidin interaction, followed by photocleavage to afford the desired RNA strand pure enough for RNAi experiments.

In order to simplify our proposed approach, the type and introduction of a biotinylated photocleavable group in the phosphoramidite unit initially had to be considered. We first planned to introduce the photocleavable group at the nucleobase moiety, according to the report by Höbartner and Silverman.[14] The uridine derivative **1** was prepared, as shown in Figure 1. Prior to conversion into the corresponding

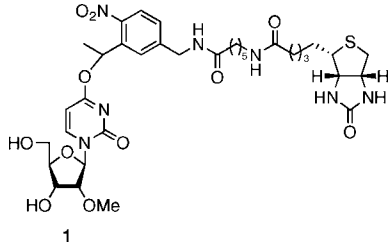
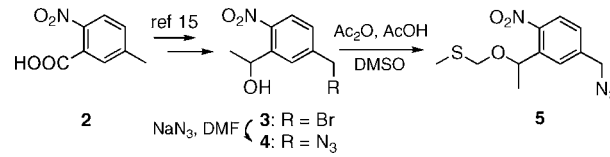


Figure 1. 2′-*O*-Methyluridine possessing a photocleavable group in its nucleobase moiety.

phosphoramidite unit, the stability of **1** against conditions used in solid-phase RNA synthesis had to be addressed. As a result, **1** was converted into 2′-*O*-methylcytidine when it was treated with concentrated NH_4OH/EtOH (3:1) at room temperature ($t_{1/2}$ estimated to be 12 h), whereas under 0.05 M K_2CO_3 in MeOH, it was converted into a 4-methoxypyrimidine nucleoside ($t_{1/2}$ estimated to be 1.3 h). Thus, it was concluded that incorporation of the functional group at the uracil moiety was not suitable for our approach (data not shown).

We next planed to introduce the photocleavable group at the 2′-position via an acetal linkage, which can be introduced selectively into the desired position and will not migrate to the 3′-position during further chemical conversion, and which works as a protecting group. The synthesis of the phosphoramidite unit possessing a biotinylated photocleavable group is shown in Schemes 1 and 2. Starting with 5-methyl-

Scheme 1



2-nitrobenzoic acid (**2**), we prepared compound **3** in four steps.[15] The bromo group of **3** was then converted to an azido group to afford **4**. Methylthiomethylation of **4** was achieved by treatment with acetic anhydride in a mixture of dimethyl sulfoxide and acetic acid to afford **5**,[16] which was introduced into the 2′-position of the uridine derivative **6** (Scheme 2). Thus, a mixture of **5**, **6**, and *N*-iodosuccinimide (NIS) in THF was treated with trifluoromethanesulfonic acid (TfOH) at −40 °C, and the 1,1,3,3-tetraisopropyldisiloxane-1,3-diyl (TIPDS) group of the crude product was removed by ammonium fluoride to give **7** in 81% yield in two steps. The 5′-hydroxyl group was then protected by the dimethoxytrityl (DMTr) group, and the azido group of the resulting **8** was reduced by Ph_3P in the presence of H_2O under reflux to give **9**. In order to introduce a biotin unit, compound **9** was treated with **10**[17] in the presence of *O*-(benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HBTU), 1-hy-

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Scheme 2

droxybenzotriazole (HOBt), and Et_3N to give **11**. Finally, compound **11** was converted into the corresponding phosphoramidite **12**. Since this conversion was slow and the starting material was not consumed, 4-dimethylaminopyridine (DMAP) was added as a catalyst to promote the reaction.

With the desired phosphoramidite **12** in hand, we next examined the solid-phase RNA synthesis. The sequence prepared in this study was a 21mer RNA with two consecutive 2′-deoxythymidine (**t**) units at its 3′-end (**RNA1**), which can directly be used for gene silencing experiments by RNAi if it does not require further purification. The schematic representation of our approach is shown in Figure 2. First, a 20mer sequence was prepared on a DNA/RNA synthesizer using combination of 2′-deoxythymidine and 2′-*O*-TBDMS phosphoramidite units following the standard procedure (step 1). After deprotection of the DMTr group at the 5′-end, **12** (represented as U possessing an asterisk in Figure 2) was coupled with the resulting 5′-hydroxyl group to give the 21mer sequence on the CPG support. In the usual synthesis, oligoDNA or RNA is synthesized as DMTr-On at its 5′-end in order to ease its purification, whereas in our approach, the DMTr group at the 5′-end was removed (step 2). After completion of the solid-phase synthesis, the CPG support was treated with concentrated NH_4OH/EtOH (3:1) for 16 h at room temperature to remove the protecting groups and detach the RNA strand from the CPG support. The CPG support was filtered off, and the filtrate was successively treated with $Et_3N{\cdot}3HF$[18] for 14 h at room temperature to remove the 2′-*O*-TBDMS groups (step 3). In this stage, an aliquot of the reaction mixture was analyzed by HPLC. As can be seen in Figure 3a, a full-length sequence was observed at around 17 min, which was observed as two peaks arising from the existence of diastereomers at the photocleavable moiety, and undesired shorter sequences were observed between 11 and 13 min. The solution containing the full-length and failure sequences and other impurities (50% of the 0.2 μmol synthesis) was then incubated with magnetic beads having streptavidin (Dynabeads MyOne Streptavidin C1) in a buffer for 30 min at room temperature (step 4). Since the full-length RNA strand has a biotin functional group, only the desired RNA will be immobilized to the beads through the high-affinity biotin−streptavidin interac-

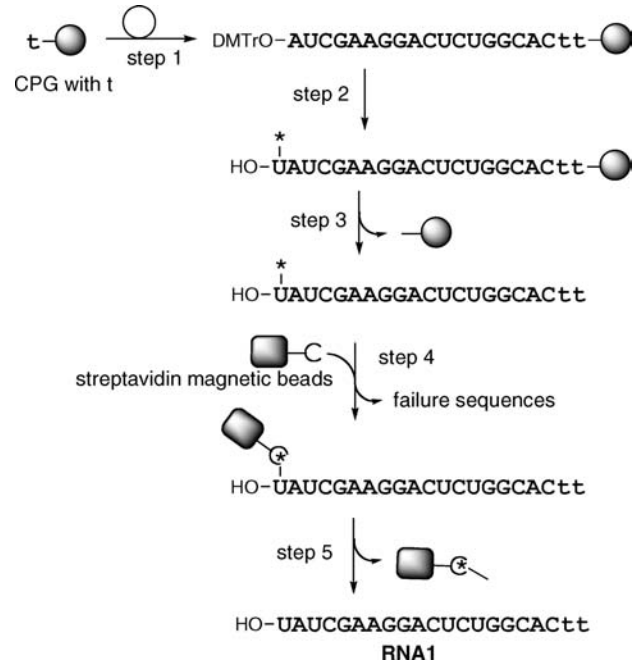

Figure 2. Schematic representation of our approach.

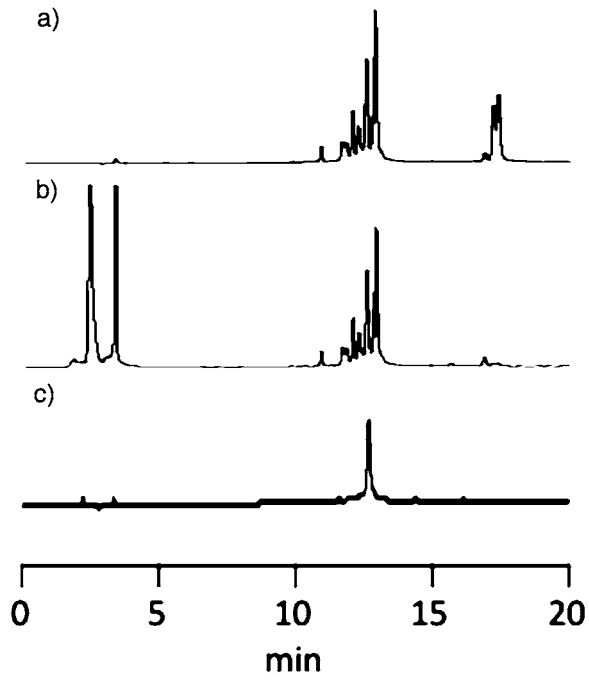

Figure 3. HPLC profiles of (a) reaction mixture after step 3 (see Figure 2); (b) supernatant after step 4; (c) resulting RNA after step 5.

tion. Figure 3b shows an HPLC chart of the supernatant after the procedure of step 4.[19] As expected, the peaks corresponding to the full-length completely disappeared, whereas the undesired shorter RNAs were observed. As the last step of our approach, a 0.2 M phosphate buffer of the magnetic beads loaded with the full-length RNA was irradiated with a high-pressure Hg lamp (400 W) using a Pyrex filter (>300 nm) for 30 min (step 5). After filtration of the magnetic beads, the filtrate was analyzed by HPLC. Accordingly, a single peak was observed at 13 min (Figure 3c), which was identical with that of **RNA1** prepared by the usual protocol (i.e., purification by C-18 silica gel, followed by HPLC). Additionally, the structure of **RNA1** prepared by this method was confirmed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF/MS).[20]

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(19) The peaks appearing at about 3 min came from the washing buffer solution. This was confirmed by injection of this solution alone.

(20) **RNA1** calculated mass $C_{201}H_{250}N_{77}O_{144}P_{20}$ 6664.9 (M − H), observed mass 6666.0.

In order to evaluate the quality of the oligoRNA prepared in our approach, the RNAi activity of **RNA1** was compared with the 21mer RNA having the same sequence using a conventional method and HPLC purification. The sequence of **RNA1** is complementary to firefly luciferase mRNA (pGL3). Thus, **RNA1** and its complementary strand (5′-GUGCCAGAGUCCUUCGAUAtt-3′) prepared separately were annealed to form siRNA (duplex), and its RNAi activity was measured by transfection into HeLa cells constitutively expressing the luciferase gene at 5, 25, and 50 nM concentrations. As a result, the siRNA consisting of **RNA1** showed almost equal RNAi activity compared with the siRNA prepared by the usual method (the data are given in the Supporting Information).

In conclusion, we have developed a rapid and efficient RNA synthesis using a phosphoramidite unit possessing a biotinylated photocleavable group. The biotinylated photocleavable group was introduced at the 2′-position of uridine. Since this functional group was bonded via an acetal linkage, no migration to the 3′-hydroxyl group occurred and it was stable toward conditions used in solid-phase RNA synthesis. After incorporation of the phosphoramidite **12** at the 5′-terminal of the RNA strand, followed by appropriate procedures, the desired full-length RNA was selectively "caught" by magnetic beads possessing streptavidin and then "released" by photoirradiation to give the desired RNA. Since the resulting RNA is pure enough to show potent RNAi activity compared with a conventionally prepared and HPLC-purified RNA, the "catch and release" approach presented in this work would be applicable to high-throughput screening to determine the best sequence of siRNAs. Application of this approach for the synthesis of longer RNA sequences, such as pre-miRNA, is now in progress.

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Supporting Information Available: Experimental procedure for new compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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