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Versatile Site-Specific Conjugation of Small Molecules to siRNA Using Click Chemistry

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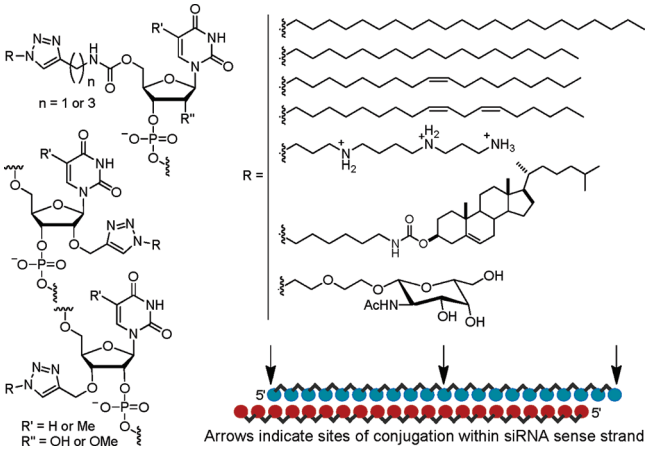

We have previously demonstrated that conjugation of small molecule ligands to small interfering RNAs (siRNAs) and anti-microRNAs results in functional siRNAs and antagomirs *in vivo*. Here we report on the development of an efficient chemical strategy to make oligoribonucleotide−ligand conjugates using the copper-catalyzed azide−alkyne cycloaddition (CuAAC) or click reaction. Three click reaction approaches were evaluated for their feasibility and suitability for high-throughput synthesis: the CuAAC reaction at the monomer level prior to oligonucleotide synthesis, the solution-phase postsynthetic "click conjugation", and the "click conjugation" on an immobilized and completely protected alkyne−oligonucleotide scaffold. Nucleosides bearing 5′-alkyne moieties were used for conjugation to the 5′-end of the oligonucleotide. Previously described 2′- and 3′-*O*-propargylated nucleosides were prepared to introduce the alkyne moiety to the 3′ and 5′ termini and to the internal positions of the scaffold. Azido-functionalized ligands bearing lipophilic long chain alkyls, cholesterol, oligoamine, and carbohydrate were utilized to study the effect of physicochemical characteristics of the incoming azide on click conjugation to the alkyne−oligonucleotide scaffold in solution and on immobilized solid support. We found that microwave-assisted click conjugation of azido-functionalized ligands to a fully protected solid-support bound alkyne−oligonucleotide prior to deprotection was the most efficient "click conjugation" strategy for site-specific, high-throughput oligonucleotide conjugate synthesis tested. The siRNA conjugates synthesized using this approach effectively silenced expression of a luciferase gene in a stably transformed HeLa cell line.

Introduction

The so-called click chemistry strategy allows efficient coupling of modular building blocks bearing azide and alkyne.[1] Due to improvements independently made to the Huisgen 1,3-dipolar azide−alkyne cycloaddition reaction[2] by the laboratories of Meldal[3] and Sharpless,[1,4] the click reaction

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can be performed in aqueous and nonaqueous solvent and on solid-support.[5] The inertness of azide and alkyne toward other functional groups such as amines, carboxylates, alcohols, thiols, and esters makes this strategy advantageous for selective postsynthetic chemical ligation of ligands to proteins, peptides, nucleic acids, carbohydrates, and polymers.

Click chemistry has proven very useful to the oligonucleotide chemistry field. The copper-catalyzed azide−alkyne cycloaddition (CuAAC) reaction has been successfully used for ligation and cyclization of DNA using appropriately azido- and alkyne-functionalized DNA strands.[6−8] The CuAAC reaction has also been extensively used for synthesis of modified nucleosides,[9−13] carbohydrate,[14−16] fluorophore,[17−21] and lipophilic[22] oligonucleotide conjugates; for synthesis of chimeras of peptide−DNA/PNA[23] and oligonucleotides;[12] and to explore G-quadruplex solution structures.[24] A recent report from Morvan's laboratory demonstrated a very efficient click chemistry approach for the synthesis of a dendrimeric oligonucleotide−glycoconjugate bearing 16 galactose moieties on its periphery.[25] In addition, Best has reviewed the usefulness of azide−alkyne cycloaddition in bioorthogonal reactions for labeling of biomolecules, including proteins, viruses, sugars, nucleic acids, and lipids, and its impact on chemical biology.[26]

In order to make small interfering RNAs (siRNAs) therapeutically active, the molecules must be delivered into the cytoplasm of the target cells, where the RNA-induced silencing complex (RISC) is located. Various approaches, such as chemical modifications, conjugation of small molecules, liposomes, nanoparticles, polymers, polyamines, and cell-penetrating peptides have been investigated to enhance cellular uptake.[27−34] Conjugation of small molecules such as cholesterol, fatty acids, vitamin E, polycationic compounds, and receptor specific ligand like folic acid and carbohydrates can also improve pharmacokinetic and pharmacodynamic properties of oligonucleotide-based therapeutic agents.[35] Oligonucleotide conjugates with lipophilic molecules have been shown to exhibit improved protein binding, nuclease resistance, and broader biodistribution and uptake in liver and jejunum *in vivo* relative to unconjugated controls.[29,30,35−38] Cholesterol-conjugated siRNAs,[29,30] antisense microRNAs (antagomirs),[39] and antisense oligonucleotides[40] enter cells in the absence of cationic lipids *in vivo*. Recently, a report demonstrated that siRNAs conjugated to polyspermine entered cells in the absence of transfection reagents *in vitro*.[31] Conjugation of receptor-specific *N*-acetylgalactosamine (GalNAc),[41−43] folic acid,[44] or α-tocopherol[45] to

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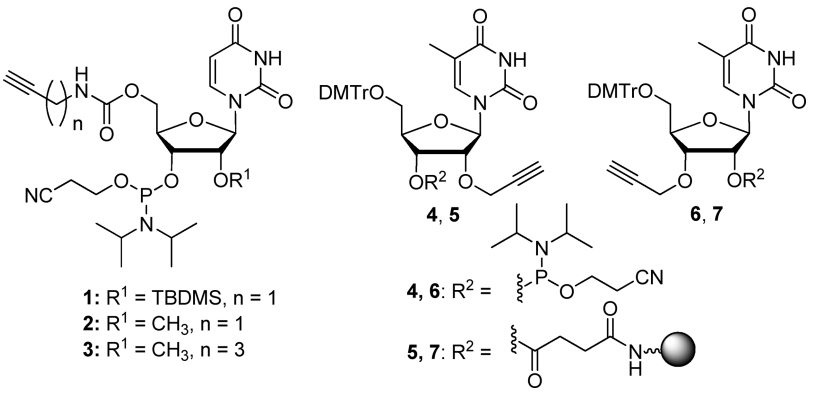

FIGURE 1. Alkyne building blocks for postsynthetic CuAAC reaction on RNA.

antisense oligonucleotides, peptide nucleic acid, or siRNAs elicited receptor-mediated cellular uptake of the conjugated oligonucleotides *in vitro* or *in vivo*. However, synthesis of oligonucleotide conjugates is challenging due to lengthy synthetic procedures and frequent incompatibility with solid-phase oligonucleotide synthesis and deprotection conditions.

Considering the enormous potential of oligonucleotide−ligand conjugates for delivering nucleic acid therapeutics to organs or tissues of interest, we evaluated click chemistry for synthesis of oligoribonucleotide/ligand conjugates. Three strategies were evaluated. In the first one, the oligonucleotide conjugate was obtained by solid-phase synthesis using modified building blocks which were obtained by click reaction at the monomer stage. In the second approach, solution-phase postsynthetic click conjugation of azido functionalized ligands to an alkyne−oligonucleotide scaffold bearing one or more alkyne moieties was evaluated. In the third strategy, azido-functionalized ligands were conjugated to a fully protected solid-support bound alkyne−oligonucleotide. We evaluated the effect of the physicochemical characteristics of the incoming azide on click conjugation with azido-functionalized ligands derived from lipophilic long chain alkyls, cholesterol, oligoamine and carbohydrate. The siRNA conjugates synthesized using the CuAAC approach were evaluated for RNAi activity *in vitro* using a HeLa cell line stably transformed with the firefly and renilla luciferase gene.

Results and Discussion

Preparation of Nucleoside−Alkyne Building Blocks for Postsynthetic Oligonucleotide Conjugation. The nucleoside−alkyne building blocks shown in Figure 1 were selected for evaluating site-specific conjugation of small molecule ligands to siRNA under CuAAC reaction conditions. Alkynylation at the 2′-, 3′-, or 5′-hydroxyl of ribonucleosides enables incorporation of one or more alkyne moiety to the desired position of an oligoribonucleotide for small molecule conjugation under CuAAC reaction conditions. The phosphoramidites **1**−**3** were designed to introduce alkyne functionality to the 5′-terminus of the oligonucleotide. The phosphoramidites **4** and **6** were chosen to incorporate the alkynes containing nucleosides at the 5′-terminal or internal positions of the oligonucleotide, whereas the solid supports **5** and **7** were selected for insertion of the alkyne containing nucleoside moiety at the 3′-terminus.

The alkyne group was introduced via a carbamate linkage at the 5′ position of uridine and 2′-*O*-methyluridine as shown in Scheme 1. Treatment of 2′,3′-*O*-isopropylideneuridine (**8**) with phenyl chloroformate in anhydrous pyridine and subsequent treatment of the intermediate formed with propargylamine afforded compound **9** in >90% yield. The isopropylidene protection was removed by refluxing compound **9** in 80% acetic acid for 18 h to afford compound **10** in quantitative yield. Silylation of the hydroxyl group of compound **10** with *tert*-butyldimethylsilyl chloride (TBDMS-Cl) in anhydrous THF in the presence of pyridine and $AgNO_3$ yielded a mixture of 2′- and 3′-*O*-TBDMS derivatives **11** and **11a** (structure not shown in the Scheme), respectively. The isomeric ratio was approximately 7:2 (2′-*O*-TBDMS/3′-*O*-TBDMS) as determined by ^{1}H NMR analysis of the crude compounds. Chromatography on neutral alumina using 2% MeOH in CH_2Cl_2 as eluent produced good separation of the two isomers **11** and **11a**.

Phosphitylation of 2′-*O*-silylated nucleoside **11** afforded the phosphoramidite **1** in 71% isolated yield. The synthesis of phosphoramidite **2** was achieved with 45% overall yield from 3′-*O*-acetyl-2′-*O*-methyluridine (**12**)[46] (Scheme 1). The precursor **14** for the synthesis of phosphoramidite **3** was obtained from compound **12** by Curtius rearrangement. Addition of diphenylphosphoryl azide to a mixture of compound **12** and 5-hexynoic acid in the presence of triethylamine in anhydrous DMF at ambient temperature and subsequent stirring at 100 °C for 18 h afforded compound **14** in 71% yield. Deacetylation of compound **14** using NaOMe in methanol at ambient temperature followed by phosphitylation yielded the desired phosphoramidite **3** in 31% overall yield.

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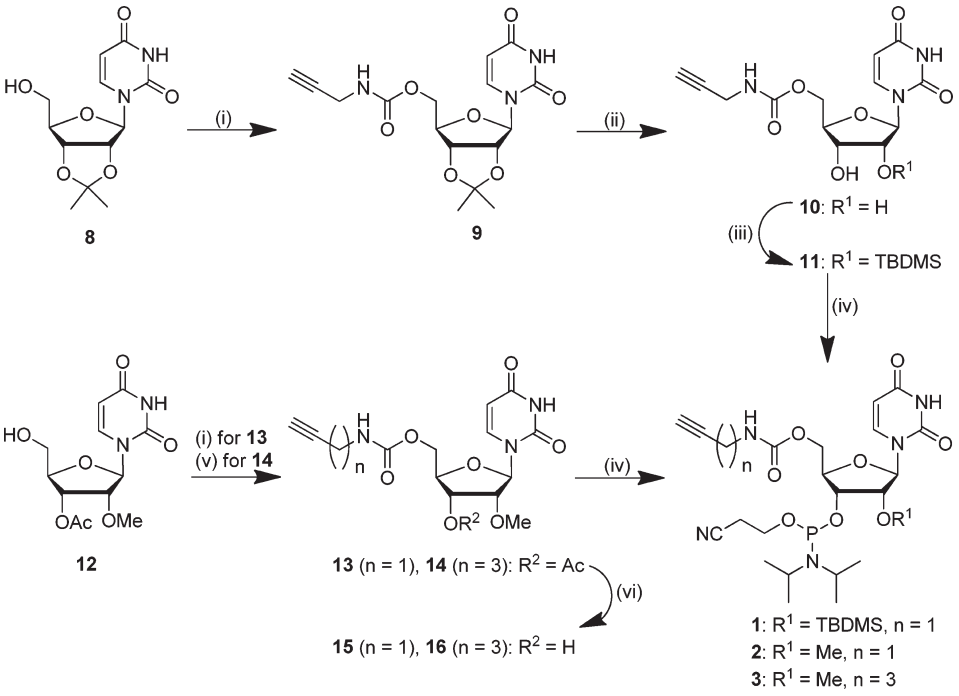
SCHEME 1. Syntheses of 5′ Alkyne-Functionalized Uridine Phosphoramidites[a]



[a]Key: (i) (a) phenyl chloroformate, pyridine, rt, 3 h, (b) propargylamine, rt, 18 h, yield **9** (91%) and **13** (>95%); (ii) 80% AcOH in MeOH, reflux, 18 h, quant; (iii) $AgNO_3$, pyridine, TBDMS-Cl, THF, rt, 18 h, 20%; (iv) 2-cyanoethyl *N*,*N*-diisopropylchlorophosphoramidite, *N*,*N*-diisopropylethylamine (DIEA), CH_2Cl_2, rt, 4 h, yields **1** (71%), **2** (70%) and **3** (70%); (v) diphenylphosphoryl azide, 5-hexynoic acid, Et_3N, DMF, rt to 100 °C, 18 h, 71%; (vi) NaOMe, MeOH, rt, 2 h, yields **15** (67%) and **16** (65%).

The 2′- and 3′-alkyne-derived nucleosides were synthesized following modifications of the known procedures. 5′-*O*-[Bis(4-methoxyphenyl)phenylmethyl] (5′-*O*-DMTr) derivatives **18** and **19** of 2′- and 3′-*O*-propargyl-5-methyluridine were directly synthesized from 5′-*O*-DMTr-5-methyluridine (**17**) by microwave-assisted alkylation (Scheme 2). The tin-mediated alkylation of uridine with propargyl chloride in the presence of TBAI[47] was optimized by replacing uridine with 5′-*O*-DMTr-uridine in a mixture of benzene and CH_3CN. The microwave-assisted reaction was performed in a sealed bottle, and the reaction was complete within 4 h with a total isolated yield of about 70% (40% for 2′- and 32% for 3′-*O*-propargyl derivatives). The 5′-*O*-DMTr protection of the nucleoside remained intact and provided a good handle for purification after the tin-mediated alkylation. An attempt to alkylate **17** with propargyl chloride under non-microwave conditions resulted in partial deprotection of the 5′-*O*-DMTr group due to the extended alkylation time, and this made the isolation of the product difficult. The microwave-assisted propargylation of 5′-*O*-DMTr-uridine mediated by dibutyltin-(IV) oxide was successful and is a promising approach for small-scale synthesis of 2′- and 3′-*O*-propargylated pyrimidines. The method was successfully extended to other ribonucleosides with protection of exocyclic amino groups (rA^{Bz}, rC^{Bz}, and rG^{iBu}) to give the corresponding 2′- and 3′-*O*-propargylated nucleosides in good yields (Table S1, Supporting Information). The phosphoramidite **6** was prepared as described above. Succinylation of **19** followed by coupling to long chain alkyl amine controlled pore glass support (lcaa-CPG) under amide coupling conditions afforded the solid support **7** with 80 μM/g loading.

Preparation of Small Molecules Carrying an Azido Group. The small molecules carrying azido groups shown in Figure 2 were chosen to evaluate the CuAAC reaction for high-throughput synthesis of oligonucleotide/ligand conjugates. The azides **20**−**23** are of fatty acid/alcohol origin to evaluate the CuAAC reaction between hydrophobic compounds and highly hydrophilic oligonucleotides in aqueous media. A more hydrophobic azide, **24**, derived from cholest-5-en-3-ol-(3β)-(6-hydroxyhexyl)carbamate[48] was also evaluated. Amino azide **25** and its corresponding trifluoroacetamido-protected derivative **25a** were synthesized from spermine (Scheme S2, Supporting Information) to evaluate the reaction of oligocationic small molecules with alkyne-functionalized oligonucleotides.[49] Lastly, GalNAc derivatives **26** and **27** were chosen to evaluate whether hydrophilic small molecules would serve as effective reactants. GalNAc serves to deliver payloads to hepatocytes.[42,50,51]

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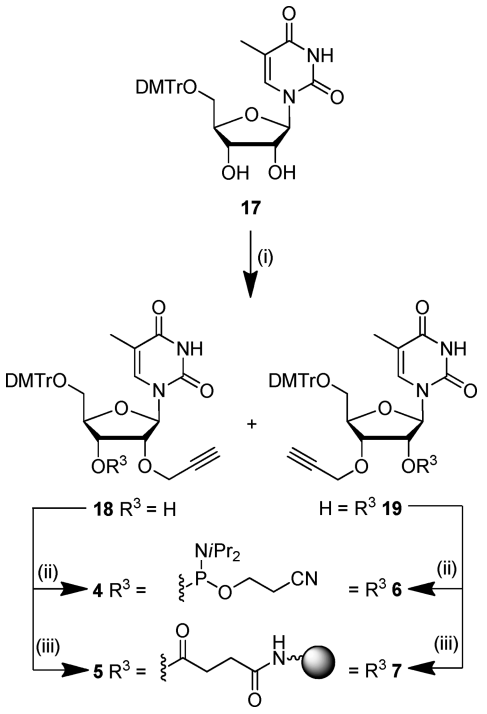
SCHEME 2. Syntheses of 2′- and 3′-*O*-Propargyl-5-methyluridine Phosphoramidites and CPG Supports[a]



[a]Key: (i) Bu_2SnO (1.1 equiv), propargyl chloride (2.0 equiv), TBAI (0.5 equiv), benzene/CH_3CN, microwave at 100 °C, yields **18** 40% and **19** 32%; (ii) 2-cyanoethyl *N*,*N*-diisopropylchlorophosphoramidite, DIEA, CH_2Cl_2, rt, 4−6 h, yield **6** 51%; (iii) (a) succinic anhydride, DMAP, CH_2Cl_2, rt, 18 h, (b) HBTU, DIEA, lcaa-CPG (500 Å, loading: 140 μM/g), DMF, 4 h.

Lipophilic azides[52−54] **20**−**23** (Figure 2) were synthesized from commercially available alcohols or bromides. Treatment of cholest-5-en-3-ol-(3β)-(6-hydroxyhexyl)carbamate[48] (**S17**, Scheme S1, Supporting Information) with sodium azide in DMF afforded the azide **24** in 84% yield. The carbohydrate derivatives **26** and **27** were synthesized according to reported procedures.[55]

Synthesis of Click-Conjugated Monomers for Solid-Phase Oligonucleotide Synthesis. To demonstrate the click reaction at the monomer stage, we synthesized 5′-modified uridine **28** and **29** bearing linoleyl and GalNAc moieties, respectively. The conditions reported by Jatsch *et al.*[56] were used (Scheme 3) for the synthesis. The reaction proceeded successfully with >85% isolated product yield. 2′ to 3′ TBDMS migration in MeOH/CH_2Cl_2 cosolvent was not observed during the reaction, presumably due to the slight acidity provided by $[(CH_3CN)_4Cu]PF_6$ in CH_2Cl_2/MeOH. Phosphitylation of compounds **28** and **29** afforded the corresponding phosphoramidites **30** and **31** in 50 and 83% yields, respectively. The phosphoramidites **30** and **31** were subjected to standard oligonucleotide synthesis and deprotection conditions to obtain modified oligonucleotides **46** and **56** with overall yield 60 and 64%, respectively, based on HPLC analysis.

Conjugation of Ligands to Oligonucleotide in Solution Using the Click Reaction. To explore the validity of the CuAAC reaction for small-molecule conjugation to RNA we used alkyne-modified uridine derivatives. Since the sense strand of the model luciferase siRNA used lacked a uridine at the 5′-end, we decided to append the modified uridine monomers to the 5′- or 3′-end of the sense strand as an additional nucleotide. Solid-support bound alkyne−oligonucleotide scaffolds **32a**−**36a** with a modified alkyne at the 5′-end, and the alkyne−oligonucleotide scaffolds **37a** and **38a** with alkynes at the 3′-end of the sense strand, shown in Table 1, were synthesized using the corresponding monomers according to the standard oligoribonucleotide synthesis conditions. Substitution of uridine at position 12 of the sense strand with 2′- and 3′-*O*-propargyluridine afforded the scaffolds **39a** and **40a** on the controlled pore glass support (CPG). Successive coupling of phosphoramidite **4** on CPG **5** followed by synthesis of the desired sequence on the support under standard oligoribonucleotide synthesis conditions afforded the solid-support bound oligonucleotide scaffold **41a** with three 2′-*O*-propargyl-5-methyluridine moieties at the 3′-end of the sense strand (Table 1). An aliquot of each support-bound alkyne−oligonucleotide scaffold was deprotected under standard RNA deprotection conditions and analyzed by analytical HPLC and LC−MS to establish the integrity of each modified oligonucleotide synthesized (Table S2, Supporting Information). We used, in this study, the alkyne−oligonucleotides **32a**−**41a** shown in Table 1 for evaluating the feasibility and usefulness of CuAAC reaction for small-molecule conjugation to siRNA.

We first evaluated the CuAAC reaction for ligand conjugation to alkyne−oligonucleotide scaffolds in solution. In order to perform solution-phase CuAAC reactions on alkyne-functionalized oligonucleotides, the oligonucleotide scaffold **32** (Table S2, Supporting Information) was obtained from solid-support bound precursor oligonucleotide **32a** (Table 1) by following standard oligonucleotide deprotection and purification protocols. The integrity of the propargylated oligonucleotide **32** was confirmed by LC−MS analysis. Reaction of purified alkyne−oligonucleotide **32** with excess unprotected azide **26a** ($GalNAc^1$-azide, Figure 2) in the presence of catalytic amounts of $CuSO_4 \cdot 5H_2O$ and 3 molar excess of sodium ascorbate (azide/$CuSO_4 \cdot 5H_2O$/sodium ascorbate = 1:0.4:3 molar equiv) in MeOH/H_2O (1:1, v/v) did not yield the desired conjugate **56** (data not shown). Significant degradation of the oligonucleotide **32** was observed both at room temperature overnight and at elevated temperature (60 °C) after 60 min. Presumably chelation of copper ions to the phosphate backbone and 2′-hydroxyl groups of unprotected oligonucleotide caused degradation. This was not unexpected as copper ion-mediated degradation of DNA oligonucleotides has been reported in the literature.[57,58]

At higher molar equivalents of $CuSO_4$ (10 molar equiv) the reaction remained sluggish, and that made mass

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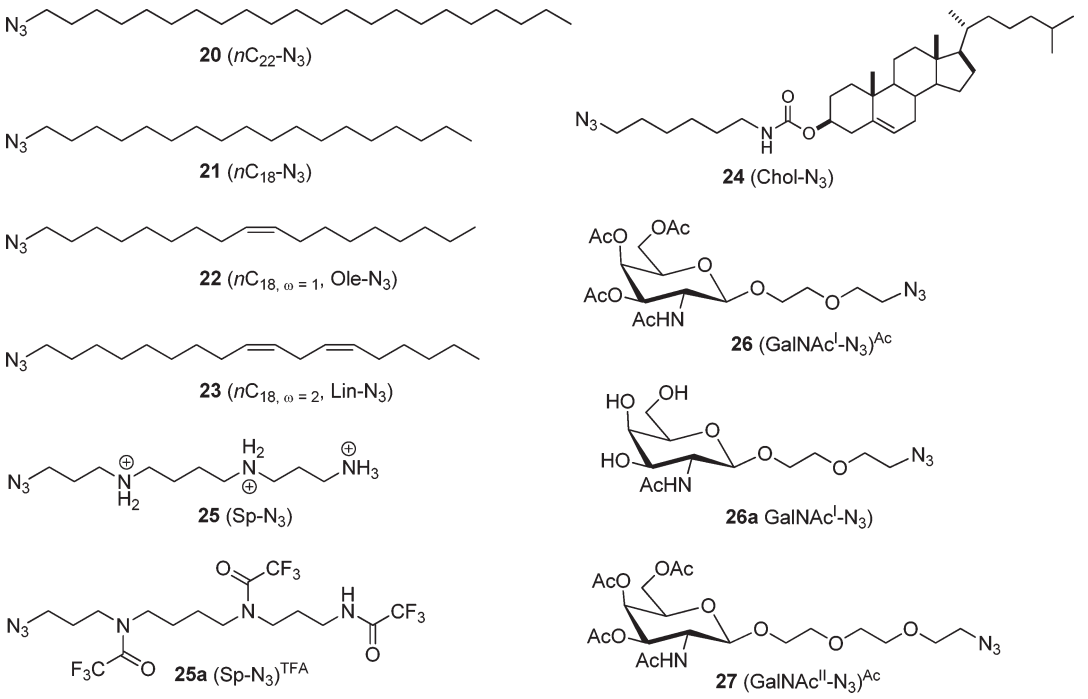
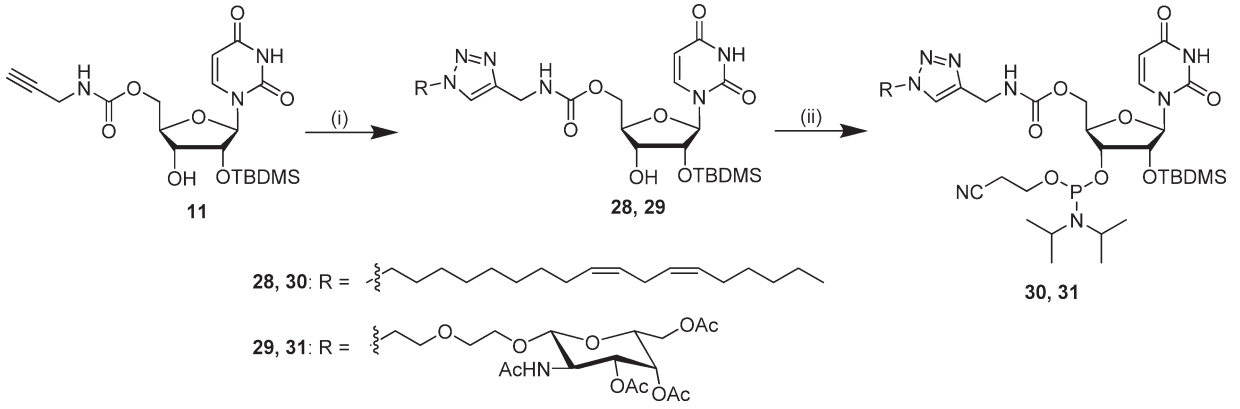


FIGURE 2. Azide monomers used for click reactions.

SCHEME 3. Syntheses of 5′-Linoleyl and *N*-Acetylgalactosaminyl Uridine Click Monomers[a]



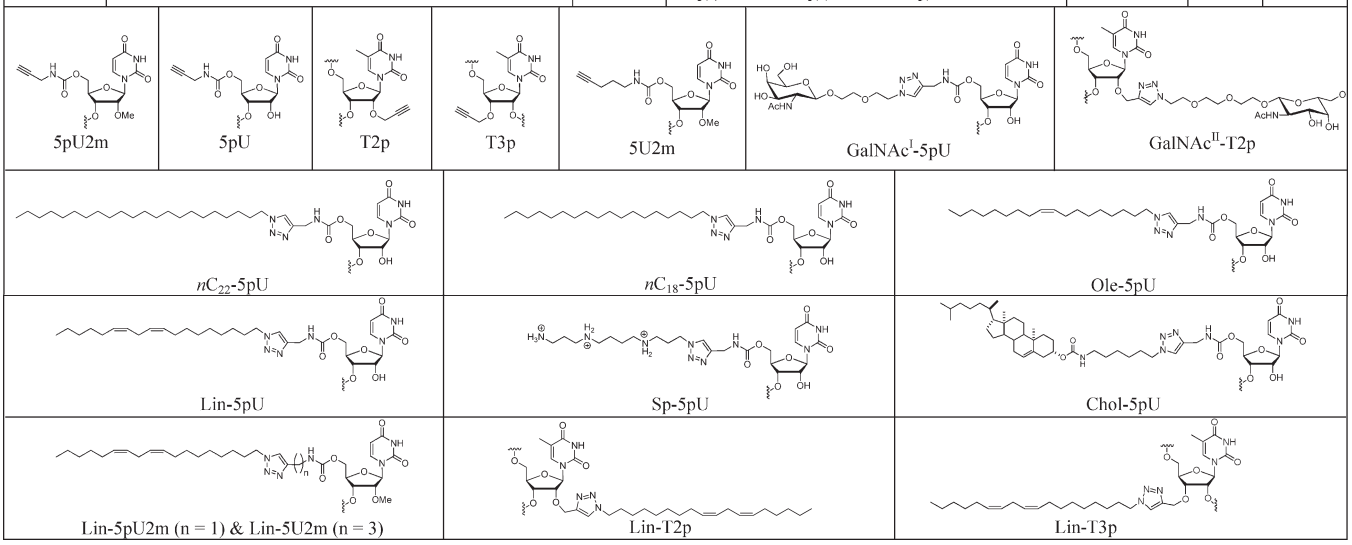
[a]Key: (i) **23** or **26**, $[(CH_3CN)_4Cu]PF_6$ (20 mol %), Cu powder (20 mol %), CH_2Cl_2/MeOH (4:1, v/v), rt, 4−18 h, yields **28** (98%) and **29** (88%); (ii) 2-cyanoethyl *N*,*N*-diisopropylchlorophosphoramidite, DIEA, CH_2Cl_2, rt, 4 h, yields **30** (50%) and **31** (83%).

spectral analysis of the product very difficult. After desalting, the chelated copper ion interfered with HPLC and LC−MS analysis. Treatment with EDTA prior to desalting did not remove copper ions completely from the product (data not shown). Use of powdered metallic copper[25] under heating (60 °C, 30 min) resulted in quantitative reaction; however, the product was contaminated with copper ions. The observed *m*/*z* peak values corresponded to copper adducts (Figure S1, Supporting Information). Replacing hydrophilic azide **26a** with hydrophobic 1-docosyl azide (**20**) in solution-phase conjugation to purified alkyne−oligonucleotide **32** in the presence of $CuSO_4$ and sodium ascorbate was also not effective. Moreover, solvents compatible with the unprotected highly hydrophilic oligonucleotide and hydrophobic alkyl azide that could facilitate an efficient azide−alkyne cycloaddition reaction were difficult to identify. Recently, copper wire has been successfully used as an efficient replacement for copper salt for the CuAAC reaction of 2′-alkyne/azido-modified adenosine with azides or alkynes respectively and also for the preparation of chimera of complementary oligonucleotides functionalized with an alkyne and an azide.[12] The authors reported green coloration of the reaction mixture during prolonged stirring at 35 °C suggesting contamination/release of copper ion to the reaction mixture.

Microwave-assisted CuAAC click conjugation has been successfully used for multiple labeling of solid-support

TABLE 1. Click Reaction of Alkyne−oligonucleotide Scaffolds with Small Molecule Azides 20−27

Alkyne-Oligonucleotide Scaffold on solid-support		Click reaction product				
Precursor	Sequence 5'-3'	Product[a]	Sequence 5'-3'	Post-click conversion[c]	Mass	
					Calc.	Found
32a	[**(5pU)**CUUACGCUGAGUACUUCGAdTdT]-(CPG)	**42**	(***n*C$_{22}$-5pU**)CUUACGCUGAGUACUUCGAdTdT	98 %	7346	7344
32a	[**(5pU)**CUUACGCUGAGUACUUCGAdTdT]-(CPG)	**43**	(***n*C$_{18}$-5pU**)CUUACGCUGAGUACUUCGAdTdT	95 %	7290	7288
32a	[**(5pU)**CUUACGCUGAGUACUUCGAdTdT]-(CPG)	**44**	**(Ole-5pU)**CUUACGCUGAGUACUUCGAdTdT	89 %	7288	7285
32a	[**(5pU)**CUUACGCUGAGUACUUCGAdTdT]-(CPG)	**45**	**(Chol-5pU)**CUUACGCUGAGUACUUCGAdTdT	96 %	7549	7547
32a	[**(5pU)**CUUACGCUGAGUACUUCGAdTdT]-(CPG)	**46**[b]	**(Lin-5pU)**CUUACGCUGAGUACUUCGAdTdT	93 %	7286	7284
33a	[**(T2p)**CUUACGCUGAGUACUUCGAdTdT]-(CPG)	**47**	**(Lin-T2p)**CUUACGCUGAGUACUUCGAdTdT	Quantitative	7257	7255
34a	[**(T3p)**CUUACGCUGAGUACUUCGAdTdT]-(CPG)	**48**	**(Lin-T3p)**CUUACGCUGAGUACUUCGAdTdT	97 %	7257	7256
35a	[**(5pU2m)**CUUACGCUGAGUACUUCGAdTdT]-CPG	**49**	**(Lin-5pU2m)**CUUACGCUGAGUACUUCGAdTdT	91 %	7301	7299
36a	[**(5U2m)**CUUACGCUGAGUACUUCGAdTdT]-(CPG)	**50**	**(Lin-5U2m)**CUUACGCUGAGUACUUCGAdTdT	84 %	7329	7326
37a	[CUUACGCUGAGUACUUCGAdTdT**(T2p)**]-(CPG)	**51**	CUUACGCUGAGUACUUCGAdTdT**(Lin-T2p)**	Quantitative	7257	7255
38a	[CUUACGCUGAGUACUUCGAdTdT**(T3p)**]-(CPG)	**52**	CUUACGCUGAGUACUUCGAdTdT**(Lin-T3p)**	90 %	7257	7255
39a	[CUUACGCUGAG**(T2p)**ACUUCGAdTdT]-(CPG)	**53**	CUUACGCUGAG**(Lin-T2p)**ACUUCGAdTdT	Quantitative	6951	6949
40a	[CUUACGCUGAG**(T3p)**ACUUCGAdTdT]-(CPG)	**54**	CUUACGCUGAG**(Lin-T3p)**ACUUCGAdTdT	91 %	6951	6949
32a	[**(5pU)**CUUACGCUGAGUACUUCGAdTdT]-(CPG)	**55**	**(Sp-5pU)**CUUACGCUGAGUACUUCGAdTdT	Quantitative	7235	7235
32a	[**(5pU)**CUUACGCUGAGUACUUCGAdTdT]-(CPG)	**56**	**(GalNAc^{I}-5pU)**CUUACGCUGAGUACUUCGAdTdT	[d]	7329	7327
41a	[CUUACGCUGAGUACUUCGAdTdT**(T2p)(T2p)(T2p)**]-(CPG)	**57**	CUUACGCUGAGUACUUCGAdTdT**(GalNAc^{II}-T2p)(GalNAc^{II}-T2p)(GalNAc^{II}-T2p)**	86%	8816	8816



[a]The products **42**−**56** were obtained via CuAAC reaction of the corresponding azide and the alkyne-oligoribonucleotide precursor on solid support unless otherwise noted. All reactions were performed under microwave conditions except for compound **55**. Typically, a heterogeneous mixture of the solid-support bound alkyne−oligoribonucleotide, the azide, $CuSO_4 \cdot 5H_2O$, sodium ascorbate, and TBTA (1:3:0.4:3:3 molar equiv, respectively) in H_2O/MeOH/THF (1.2 mL, 2:2:1 v/v) was microwave irradiated at 60 °C in an Explorer-48 reactor for 45 min to obtain the corresponding conjugate. The 5′-oligoamine-conjugated oligonucleotide **55** was prepared under thermal conditions (1.5 equiv of $CuSO_4 \cdot 5H_2O$, 5 equiv of sodium ascorbate, 1:1 *t*-BuOH/H_2O, 50 °C, overnight). [b]The oligonucleotides **46** and **56** were also directly synthesized from the phosphoramidite **30** and **31** with 60 and 64% overall yield, respectively, under solid-phase synthesis conditions. [c]Reaction conversion was calculated from the ratio of product peak intensity to precursor peak intensity analyzed by reversed-phase high-performance liquid chromatography (RP-HPLC). [d]Reaction conversion was not determined due to difficulty in calculating the percentage conversion after solution-phase conjugation of the azide **26** with purified oligonucleotide **32** obtained after deprotection of **32a**.

bound T_{12} DNA oligomer with carbohydrates.[59] A variant of this approach in solution phase in the presence of tris(benzyltriazolylmethyl)amine (TBTA),[21,60] a copper(I)-stabilizing agent, afforded the desired oligoribonucleotide conjugate **42**. The microwave-assisted CuAAC reaction of the alkyl azide **20** and purified alkyne−oligonucleotide **32** in the presence of a large excess of $CuSO_4 \cdot 5H_2O$ (24 molar equiv to alkyne), sodium ascorbate, and TBTA yielded the desired product **42**. After irradiation for 45 min at 60 °C no starting material was detected by HPLC analysis (Figure S2, Supporting Information). The yields of the solution phase conjugations with and without microwave assistance were difficult to obtain reliably using HPLC methods due to incomplete reaction and degradation of the oligonucleotides. In addition, incompatible hydrophilic oligonucleotides and hydrophobic alkyl azide in a cosolvent in the presence of copper salt made the HPLC analysis more difficult. Furthermore, additional purification steps were required to remove TBTA and to minimize copper ion contamination from the product.

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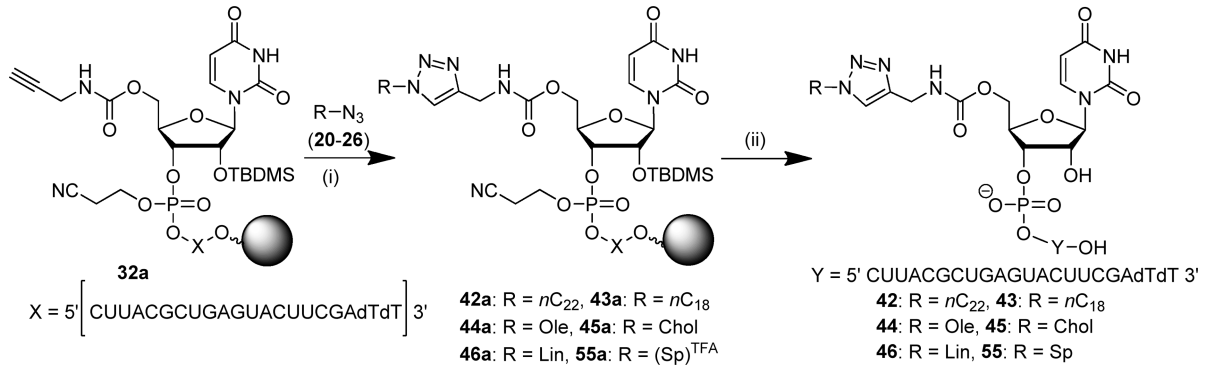
SCHEME 4. CuAAC Reaction of Azides 20−26 with Alkyne−Oligoribonucleotide Scaffold 32a



(i) Alkyne/azide/$CuSO_4 \cdot 5H_2O$/TBTA/sodium ascorbate (1:3:0.4:3:3 mol ratio), H_2O/MeOH/THF (2:2:1, v/v), 1.2 mL total volume, microwave at 60 °C, 45 min, yields **42** (98%), **43** (95%), **44** (89%), **45** (96%), **46** (93%) and **55** (99%); (ii) standard oligonucleotide deprotection. Yield was calculated on the basis of RP-HPLC peak ratio of product to precursor.

TABLE 2. Optimization of CuAAC Reaction Conditions Using Azide 20 and Solid-Support Bound Fully Protected Alkyne−Oligonucleotide 32a

reaction no.	molar ratio[a]	solvent (2:2:1, v/v)[b]	temp (°C)	time (min)	conversion[c] (%)
1	1:16:0.4:3:0	H_2O/MeOH/THF	60	45	no reaction
2	1:16:0.4:3:3	H_2O/MeOH/THF	60	45	71
3	1:3:0.4:3:3	H_2O/MeOH/THF	60	45	99
4	1:3:0.1:3:3	H_2O/MeOH/THF	60	45	no reaction
5	1:3:0.4:3:3	H_2O/MeOH/DMSO	60	45	88
6	1:3:0.4:3:3	H_2O/MeOH/DMF	60	45	79
7	1:3:0.4:3:3	H_2O/MeOH/THF	60	45	57
8	1:3:0.4:3:3	H_2O/MeOH/THF	60	45	60
9	1:3:0.4:3:3	H_2O/MeOH/THF	rt	48 h	quantitative

[a]Alkyne **32a**/azide **20**/$CuSO_4 \cdot 5H_2O$/sodium ascorbate/TBTA. [b]The total reaction volume is 1.2 mL except for reactions 7 and 8. For reaction 7 the volume was 0.24 mL and for 8 it was 3.6 mL. [c]Reaction conversion was monitored by HPLC.

Conjugation of Ligands to Oligonucleotide on Solid Support Using Click Reaction. The conditions used by Morvan and co-workers for the synthesis of various carbohydrate−DNA conjugates by click reaction on solid-support[25,59,61] were optimized for fully protected support-bound alkyne−oligoribonucleotide precursor **32a** (Scheme 4, Table 1). The effects of molar equivalence of azide to alkyne, Cu catalyst, chelator (TBTA), temperature, and solvent for microwave-assisted CuAAC reaction were investigated; the findings are summarized in Table 2 and Figure S3, Supporting Information. The microwave-assisted CuAAC reaction of solid-support bound **32a** with the azide **20** in the presence of TBTA afforded 71% yield of the desired conjugate **42** (Table 2, entry 2 and Figure S4, Supporting Information), whereas in the absence of TBTA no product was recovered (Table 2, entry 1). Addition of TBTA was required for the postsynthetic click reaction to occur on solid-support bound alkyne−oligonucleotide.

We found that 0.4 molar equiv of $CuSO_4 \cdot 5H_2O$ relative to the alkyne were required to achieve almost quantitative conversion to conjugate (Table 2, entry 3). Use of 3 molar equiv of the azide was sufficient for quantitative reaction. THF was the most effective cosolvent tested for dissolving lipophilic molecules in combination with water and methanol to produce higher yields rather than the use of DMF and/or DMSO in place of THF (Table 2, entries 3, 5, and 6). Excessive amounts of lipophilic azides resulted in lower product yields presumably due to aggregation of highly hydrophobic azide **20** (Table 2, entries 2 and 3). The reaction volume was also critical: a solvent volume of 1.2 mL was optimal for the Explorer-48 microwave reactor with lowest volume reaction vessel tested. Attempts to use less or more solvent (Table 2, reactions 7 and 8, total volume of solvent 0.24 and 3.6 mL, respectively) resulted in poor yield. The optimal reaction conditions (Table 2, entry 3) were microwave irradiation at 60 °C for 45 min with 1:3:0.4:3:3 molar ratios of alkyne−oligonucleotide, azide, $CuSO_4 \cdot 5H_2O$, TBTA, and sodium ascorbate in 1.2 mL of H_2O, MeOH, and THF (2:2:1, v/v).

The CuAAC reaction of **32a** with the azide **20** also proceeded to completion in the absence of microwave irradiation in about 48 h at ambient temperature (Table 2, entry 9). HPLC and LC−MS analysis of the product obtained from microwave-assisted and unassisted reactions showed formation of the expected product. The observed rate enhancement of microwave-assisted conjugation reaction corroborated well with the cycloaddition reaction of small molecules.[62,63] When the reaction was performed on a fully protected alkyne−oligonucleotide on solid support, contamination of the product with copper ions was minimized to below the detection limit of our LC−MS analytical capability. Presumably poor availability of lone-pair electrons and lack of negative charges on the phosphate backbone for the fully protected oligonucleotide **32a** minimized its coordination with the metal ion.

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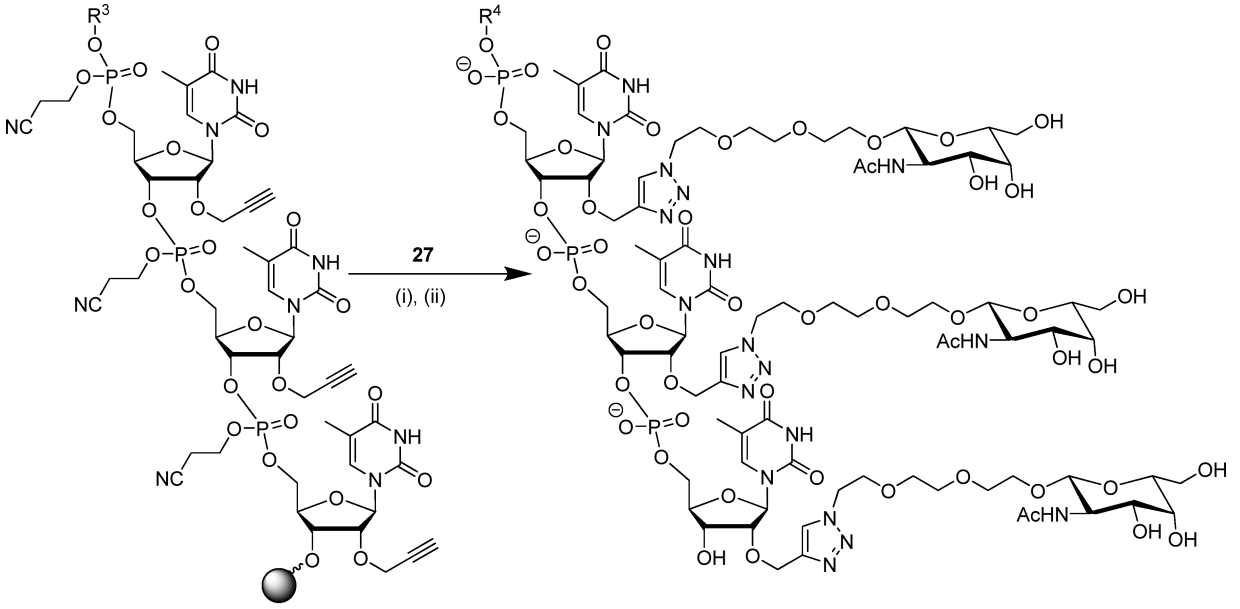
SCHEME 5. Conjugation of GalNAc to the Fully Protected Alkyne−Oligonucleotide 41a[a]



[a](i) Alkyne/azide/$CuSO_4 \cdot 5H_2O$/TBTA/sodium ascorbate 1:9:1.2:9:9 (molar ratio), H_2O/MeOH/THF (2:2:1, v/v, vol. 1.2 mL), microwave at 60 °C, 45 min; (ii) standard oligonucleotide deprotection

TABLE 3. Gene Silencing Activity of siRNAs in Dual Luciferase Assay

siRNA (S/AS[a])	IC_{50}[b] (nM)	T_m[c] (°C)
59/58	0.32	73.4
42/58	0.35	74.4
43/58	0.2	72.6
44/58	0.074	nd
45/58	0.77	nd
46/58	0.062	74.4
47/58	0.35	nd
48/58	0.32	nd
49/58	0.26	nd
50/58	0.38	nd
51/58	1.12	73.4
52/58	1.41	nd
53/58	0.22	72.5
54/58	0.23	69.6

[a]S and AS indicate sense and antisense strands, respectively; the unmodified duplex control was **59/58**. See Table 1 for details of modified sense strands **42**−**54**. [b]IC_{50} values were calculated from the dose−response curves shown in Figure 3; all IC_{50} were determined in the presence of Lipofectamine 2000. [c]2 μM duplex concentration in 0.9% physiological saline solution; nd: not determined.

Our evaluation indicated that the microwave-assisted click reaction of solid-support bound alkyne−oligonucleotide scaffold with azide bearing ligands is more efficient and cost-effective for small-scale oligonucleotide−conjugate synthesis than other strategies tested. The CuAAC reaction of solid-support bound alkyne−oligonucleotide also proceeded at ambient temperature in the absence of microwave irradiation; this procedure may prove useful with more reactive azide-bearing ligands than the ones used here.

To further evaluate the scope of the click reaction on immobilized and protected alkyne−oligonucleotide scaffolds, different alkyl azides (**21**−**24**) were reacted with **32a** on solid-support under the optimized conditions. All expected products were obtained in excellent yields (Table 1, **43**−**46**, Figure S5, Supporting Information). A typical HPLC profile of the deprotected precursor alkyne−oligonucleotide **32** and the product **46** after treatment with the linoleyl azide (**23**) indicated quantitative CuAAC reaction on the solid-support under the optimized conditions. The same 5′-conjugated oligonucleotide **46** was also synthesized from the phosphoramidite **30** using standard solid-phase oligonucleotide synthesis and deprotection conditions (Table 1). LC−MS data and HPLC profiles of the products obtained by both routes were identical.

To evaluate the effect of the position of conjugation within the oligonucleotide, the linoleyl azide (**23**) was reacted with alkyne−oligonucleotide scaffolds carrying the alkyne moiety at 3′ or 5′ terminal positions or an internal position at either through the 2′ or 3′ position of the ribose moiety (Table 1, **32a**−**40a**). The CuAAC reaction of oligonucleotide scaffolds modified with 2′-*O*-propargy-5-methylluridine (Table 1, **33a**, **37a**, and **39a**) with azide **23** gave quantitative conversion to the expected products irrespective of the location of the modification on the oligonucleotide. Reaction with the 3′-*O*-propargyluridine scaffolds **34a**, **38a**, and **40a** gave slightly lower yields (~90%) of the product (Table 1, **48**, **52**, and **54**). Reaction with the oligonucleotide scaffold **36a** modified with an alkyne at the 5′-terminus gave only 84% yield of the desired conjugate **50**. The HPLC profiles of the purified products **47**−**54** are shown in Figure 5S, Supporting Information.

The impact of physical properties of small molecule azides on CuAAC reaction on an immobilized oligonucleotide−alkyne scaffold was evaluated. Hydrophilic and cationic small molecule azides were reacted with solid-support bound

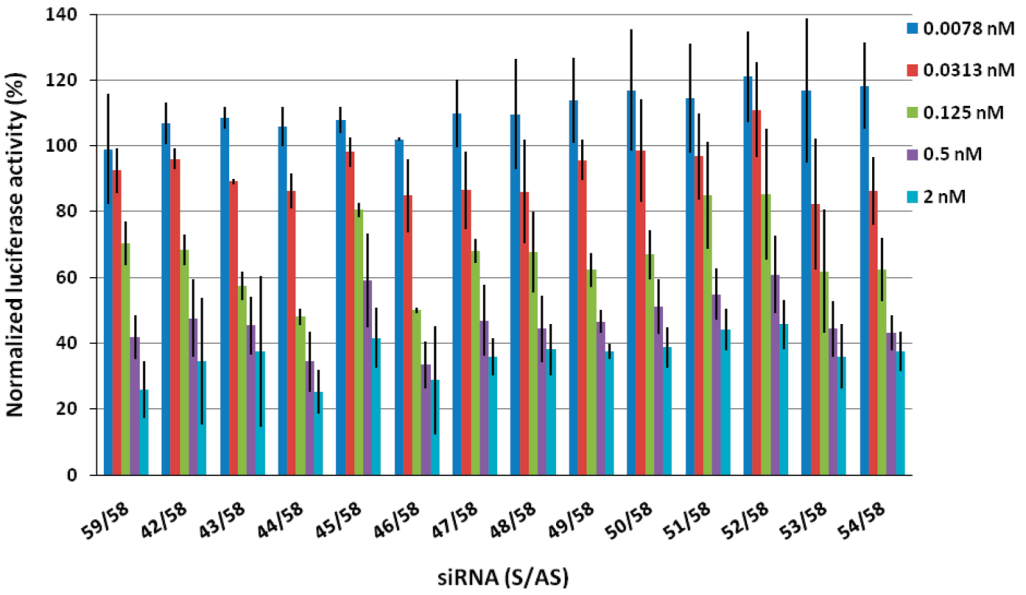


FIGURE 3. Plot of RNAi activity of various lipophile-conjugated luciferase siRNA via click chemistry at different positions on the sense strand in the *Renilla* luciferase mRNA expressed HeLa cell lines. The parent duplex is unmodified control siRNA duplex **59**/**58**, where **58** (UCGAAGUACUCAGCGUAAGdTdT) and **59** (CUUACGCUGAGUACUUCGAdTdT) represent antisense and sense strands, respectively (IC_{50} data in Table 3). The sense strand of each siRNA conjugate was annealed with the unmodified antisense strand **58** to obtain the corresponding siRNA.

oligonucleotide−alkyne **32a** under optimized CuAAC reaction conditions. Reaction with amino azide **25** did not yield the expected product, presumably due to chelation of copper ions to the unprotected amines. The click reaction of base-labile TFA-protected amino azide **25a** with **32a** yielded the desired product **55**. The solid-support bound precursor **32a** was reacted with compound **25a** in the presence of $CuSO_4 \cdot 5H_2O$ (1.5 equiv) and sodium ascorbate (5 equiv) at 50 °C overnight to afford oligonucleotide conjugate **55** quantitatively. The efficiency of the reaction and the identity of the product formed were confirmed by HPLC and LC−MS analysis, respectively (Figure S6, Supporting Information).

Previous studies have demonstrated that conjugation of multivalent GalNAc to drug results in binding of the conjugate with nM affinity to asialoglycoprotein receptor (ASGPR) and internalization into liver hepatocytes.[64] We, therefore, explored the click reaction between GalNAc derivatives **27** carrying an azido group and an RNA oligonucleotide **41a** bearing three alkyl functionalities at the 3′-end as shown in Scheme 5. Analysis of the crude product after deprotection showed that multiple incorporation of GalNAc onto solid-support bound oligonucleotide was efficient. HPLC analysis of the deprotected crude product confirmed formation of 86% of the desired tri-GalNAc product **57** with insignificant amounts of mono- and di-GalNAc conjugate byproduct (Figure S7, Supporting Information).

Dual-Luciferase Assay for RNA Interference Evaluation. A dual-luciferase assay was used to evaluate gene silencing by siRNAs formed from the modified oligonucleotides synthesized. A pGL4 vector containing the *Renilla* and firefly luciferase genes was used for the assay. The siRNA sequences were designed to target the firefly luciferase gene. siRNAs were transfected into cells using Lipofectamine2000. The gene silencing abilities of unmodified siRNA (**59**/**58**) and siRNA conjugates are summarized in Table 3; IC_{50} values were calculated from dose−response curves (Figure 3).

The siRNAs **44**/**58** and **46**/**58** shown in Table 3 with the antisense strand **58** and complementary sense strands comprising of an added extra uridine derivative at the 5′-terminus conjugated to unsaturated lipid such as oleyl (C_{18}, $\omega = 1$) or linoleyl (C_{18}, $\omega = 2$) (**44**, **46**, Table 1) showed 4- to 5-fold improvement in IC_{50} over the control siRNA **59**/**58**. The most active siRNA **46**/**58** had a T_m 1 °C higher than that of the control siRNA **59**/**58**. In contrast, the siRNA **43**/**58** containing saturated stearyl conjugate **43** had an IC_{50} comparable to the unconjugated control. The siRNA formed from the 5′-cholesteryl conjugate (**45**) had a 2-fold lower IC_{50} than the control siRNA **59**/**58**. Interestingly, replacement of the "5pU" sugar linker with '5pU2m' reduced activity of the linoleyl conjugate (compare activities of **49**/**58** and **46**/**58**, Table 3).

The IC_{50}'s calculated for "Lin-5pU" **46**/**58** and "Lin-5pU2m" **49**/**58** were 0.062 and 0.26 nM, respectively. siRNAs constituted from strands with the linoleyl moiety conjugated through the 2′- (**47**) or 3′- (**48**) position of the ribosugar ("T2p" and "T3p" linkers, Tables 1 and 3) at the 5′-end of the sense strand showed comparable IC_{50}s to the control siRNA **59**/**58**. The 3′ ("T3p") and 2′ ("T2p") modified siRNAs had comparable activity in all cases: **47**/**58** vs **48**/**58**, **51**/**58** vs **52**/**58**, and **53**/**58** vs **54**/**58**). Introduction of linoleyl to the 3′-end of the sense strand through either the "T2p" (**51**) or "T3p" (**52**) linker considerably reduced siRNA activity.

Placement of the bulky residues "Lin-T2p" or "Lin-T3p" in the central region of the sense strand (**53**, **54**) led to a small decrease in thermal stability (T_m) of the corresponding duplexes **53**/**58** and **54**/**58** compared to the control **59**/**58** (Table 3). In a recent study, we showed that local destabilization of the central region of the sense strand can enhance the potency of corresponding siRNA.[65] Although in the current

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example, the linoleyl conjugates were also placed in the central region (position 12) of the sense strand, we only observed a minor improvement in potency. Presumably, the decrease in thermal stability was too small to have a significant effect on the potency of modified siRNAs. It also is interesting to note that insertion of an extra modified nucleotide to the 5′-end of the sense strand did not hamper the activity of the siRNA, whereas addition of a bulky nucleotide to the 3′-end of the sense strand was not well tolerated.

Conclusions

In this work, we developed and compared various approaches to conjugate small molecules with a range of physicochemical properties to oligoribonucleotides using click chemistry. We have demonstrated that the microwave-assisted CuAAC conjugation of azido-functionalized small-molecule ligands to solid-support bound oligoribonucleotide allowed for efficient synthesis of oligoribonucleotide−heteromolecule conjugates with the potential for high-throughput applications. The procedure described here allows rapid and economical synthesis of a wide variety of conjugates. Using the alkyne−oligonucleotide scaffolds described here, any azido-functionalized ligand can be introduced to a desired site within the oligonucleotide sequence and at selected positions on the sugar moiety (2′, 3′, or 5′ position) or in the selected sites of nucleobase. Although we demonstrated the conjugation using uridine derivatives, the approach will be applicable to other nucleosides, employing the reported derivatization protocols.[66−69] We have optimized the reaction conditions for conjugation of azides derived from hydrophobic, hydrophilic, and cationic compounds to alkyne−oligonucleotide scaffolds. Further optimization of this conjugation strategy for siRNA therapeutic screening with several ligands is in progress. The very recent demonstration of strain-promoted copper free click conjugation of ligands to RNA that is completely devoid of heavy metal ions is another promising approach for RNA oligonucleotide conjugation chemistry.[70,71] However, because of simplicity of the chemistry involved and the versatility of the CuAAC reaction on solid-support bound alkyne-oligonucleotide, it is a promising high-throughput strategy for oligonucleotide conjugate synthesis for biological screening. Our laboratory is presently engaged in optimizing both copper-assisted[72] and copper-free[70] azide−alkyne click reactions for synthesis of RNA conjugates.

Experimental Section

Materials. All reagents and solvents obtained from commercial suppliers were used without further purification. All reactions were carried out under argon in oven-dried glassware. Thin-layer chromatography was carried out on glass-backed silica-gel 60 F_{254} plates. Column chromatography was performed using silica gel (60 Å, 230 × 400 mesh). Chemical shifts are given in parts per million (ppm); J values are given in hertz (Hz). All spectra were internally referenced to the appropriate residual undeuterated solvent.

2′,3′-*O*-Isopropylidene-5′-*O*-propargylcarbamoyluridine (9). 2′,3′-*O*-Isopropyrideneuridine (10.00 g, 35.18 mmol) in pyridine (350 mL) was treated with phenyl chloroformate (4.8 mL, 38.70 mmol) at room temperature for 2 h. Propargylamine (7.3 mL, 175.89 mmol) was added, and the reaction mixture was stirred at room temperature for 18 h. The reaction was monitored by TLC (R_f = 0.60; developing solvent CH_2Cl_2/MeOH, 12:1, v/v). Solvents and volatiles were removed *in vacuo*. The residue was suspended in CH_2Cl_2 (250 mL) and washed with satd aq $NaHCO_3$ solution followed by standard workup. The residue was purified by flash chromatography on silica gel (98:2 CH_2Cl_2/MeOH, v/v) to obtain **9** (11.73 g, 91%) as a white foam: ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.41 (br, 1H), 7.73 (m, 1H), 7.65 (d, J = 8.0 Hz, 1H), 5.80 (d, J = 2.4 Hz, 1H), 5.62 (d, J = 8.0 Hz, 1H), 5.03 (dd, J = 6.4, 2.4 Hz, 1H), 4.74 (dd, J = 6.4, 3.1 Hz, 1H), 4.27−4.14 (m, 2H), 4.08 (m, 1H), 3.77 (m, 2H), 3.11 (m, 1H), 1.48 (s, 3H), 1.28 (s, 3H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 163.2, 155.5, 150.3, 142.5, 113.2, 101.8, 92.2, 84.1, 83.4, 81.2, 80.8, 73.1, 64.2, 29.8, 27.0, 25.1; MS calcd for $C_{16}H_{20}N_3O_7$ (MH^+) 366.13, found 366.12.

3′-*O*-Acetyl-2′-*O*-methyl-5′-*O*-propargylcarbamoyluridine (13). Compound **13** (5.03 g, 99%) was obtained as a white foam from compound **12** (4.00 g, 13.3 mmol), phenyl chloroformate (2.15 mL, 14.64 mmol), and propargylamine (3.21 mmol, 66.5 mmol) as described for the synthesis of **9**. The residue after workup was purified by flash chromatography on silica gel (98:2 CH_2Cl_2/MeOH, v/v) to obtain compound **13**: ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.49 (s, 1H), 7.91 (t, J = 5.7 Hz, 1H), 7.62 (d, J = 8.1 Hz, 1H), 5.87 (d, J = 6.6 Hz, 1H), 5.72 (d, J = 8.1 Hz, 1H), 5.22−5.16 (m, 1H), 4.26−4.12 (m, 4H), 3.85−3.79 (m, 2H), 3.28 (s, 3H), 3.18 (t, J = 2.3 Hz, 1H), 2.10 (s, 3H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 169.7, 162.9, 155.5, 150.6, 140.3, 102.7, 85.9, 81.2, 79.8, 79.4, 73.4, 70.5, 63.7, 58.2, 29.8, 20.6; MS calcd for $C_{16}H_{20}N_3O_8$ (MH^+) 382.12, found 382.00.

5′-*O*-Propargylcarbamoyluridine (10). Compound **9** (11.73 g, 32.10 mmol) in MeOH (20 mL) was refluxed with 80% AcOH (200 mL) for 18 h. The solution was concentrated *in vacuo*. The residue was coevaporated with methanol three times and then redissolved in 50 mL of anhydrous MeOH. The MeOH solution was sonicated for 30 min, and a white precipitate was obtained. The precipitate was filtered on a suction funnel and washed with CH_2Cl_2 (1 L). The white residue obtained was dried at 40 °C in a vacuum oven for 18 h to obtain compound **10** (10.34 g, 99%) ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.35 (br, 1H), 7.79 (m, 1H), 7.60 (d, J = 7.8 Hz, 1H), 5.78 (d, J = 5.5 Hz, 1H), 5.65 (d, J = 7.8 Hz, 1H), 5.42 (d, J = 5.5 Hz, 1H), 5.27 (d, J = 4.9 Hz, 1H), 4.33−3.72 (m, 7H), 3.13 (s, 1H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 172.1, 163.0, 155.7, 150.7, 140.7, 102.1, 87.8, 81.7, 81.2, 73.1, 72.5, 69.9, 64.0, 29.8; HRMS calcd for $C_{13}H_{16}N_3NaO_7$ (MNa^+) 348.0808, found 348.0812.

5′-*O*-Propargylcarbamoyl-2′-*O*-(*tert*-butyldimethylsilyl)uridine (11). Compound **10** (10.34 g, 28.30 mmol) in anhydrous THF (30 mL) was treated with silver nitrate (5.77 g, 34.00 mmol), pyridine (17.0 mL, 209.4 mmol), and TBDMS-Cl (4.27 g, 28.30 mmol)

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at room temperature for 18 h. The reaction mixture was diluted with CH_2Cl_2 (300 mL) and washed with water. The organic layer was dried over anhydrous Na_2SO_4 and then concentrated *in vacuo*. The residue was purified by chromatography on neutral alumina (98:2 CH_2Cl_2/MeOH, v/v) to obtain **11** (2.45 g, 20%, R_f = 0.15 on aluminum gel TLC) as a pure isomer: ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.41 (br, 1H), 7.93−7.71 (m, 1H), 7.65 (d, J = 8.1 Hz, 1H), 5.83 (d, J = 5.1 Hz, 1H), 5.72 (dd, J = 8.1, 2.1 Hz, 1H), 5.23 (d, J = 6.0 Hz, 1H), 4.32−4.22 (m, 2H), 4.17 (m, 1H), 4.09−3.98 (m, 1H), 3.95−3.87 (m, 1H), 3.87−3.82 (m, 2H), 3.22−3.13 (m, 1H), 0.88 (d, J = 1.5 Hz, 12H), 0.09 (s, 3H), 0.07 (s, 3H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 163.4, 163.4, 156.1, 151.0, 140.7, 102.6, 88.4, 82.5, 81.7, 75.1, 73.7, 70.0, 64.2, 30.3, 26.2, 26.1, 18.5, 18.3, −4.1, −4.3, −4.6, −4.7; MS calcd for $C_{19}H_{29}N_3NaO_7Si$ (MNa^+) 462.17, found 462.10.

5′-*O*-Propargylcarbamoyl-2′-*O*-(*tert*-butyldimethylsilyl)uridine-3′-*O*-(*N,N*-diisopropyl)phosphoramidite (1). Compound **11a** (452 mg, 1.02 mmol) in anhydrous CH_2Cl_2 (12 mL) was treated with DIEA (612 μL, 3.41 mmol) and 2-cyanoethyl *N,N*-diisopropylchlorophosphoramidite (380 μL, 1.71 mmol) at room temperature for 6 h. The reaction mixture was diluted with CH_2Cl_2 (100 mL) and washed with brine (25 mL). The organic layer was dried over sodium sulfate and concentrated *in vacuo*. The residue was purified by flash chromatography on silica gel (50:50:0.5 hexane/EtOAc/TEA, v/v) to obtain the phosphoramidite **1** (469 mg, 71%, R_f = 0.6) as a white foam: ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.51−11.33 (m, 1H), 7.96−7.71 (m, 1H), 7.71−7.53 (m, 1H), 5.91−5.79 (m, 1H), 5.76−5.62 (m, 1H), 4.39−4.08 (m, 5H), 3.87−3.53 (m, 6H), 3.20−3.05 (m, 1H), 2.85−2.69 (m, 2H), 1.28−1.04 (m, 12H), 0.92−0.72 (m, 9H), 0.16−0.10 (m, 6H); ^{31}P NMR (162 MHz, DMSO-d_6) δ 149.99, 148.44; HRMS calcd for $C_{28}H_{47}N_5O_8PSi$ (MH^+) 640.2931, found 640.2927.

2′-*O*-Methyl-5′-*O*-propargylcarbamoyluridine-3′-*O*-(*N,N*-diisopropyl)phosphoramidite (2). The phosphoramidite **2** (1.18 g, 2.18 mmol, 87%, R_f = 0.1) was obtained as a white foam from compound **15** (1.00 g, 2.51 mmol) and 2-cyanoethyl *N,N*-diisopropylchlorophosphoramidite (1.12 mL, 5.01 mmol) in the presence of DIEA (1.75 mL, 10.02 mmol) in anhydrous CH_2Cl_2 (25 mL) for 6 h as described above for synthesis of **1**. The residue after workup was purified by flash chromatography on silica gel (3:7 hexane/EtOAc, v/v) to obtain the phosphoramidite **2**: ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.43 (br, 1H), 7.95−7.74 (m, 1H), 7.72−7.52 (m, 1H), 5.98−5.76 (m, 1H), 5.76−5.58 (m, 1H), 4.40−3.90 (m, 5H), 3.86−3.47 (m, 6H), 3.33 (br, 3H), 3.18−2.84 (m, 1H), 2.83−2.70 (m, 2H), 1.28−1.00 (m, 12H); ^{31}P NMR (162 MHz, DMSO-d_6) δ 155.02, 154.38; HRMS calcd for $C_{23}H_{35}N_5O_8P$ (MH^+) 540.2223, found 540.2220.

2′-*O*-Methyl-5′-*O*-(pentynyl)carbamoyluridine-3′-*O*-(*N,N*-diisopropyl)phosphoramidite (3). The phosphoramidite **3** (800 mg, 70%, R_f = 0.2) was obtained as a white foam from compound **16** (750 mg, 2.03 mmol) and 2-cyanoethyl *N,N*-diisopropylchlorophosphoramidite (878 μL, 2.76 mmol) in the presence of DIEA (1.38 mL, 7.52 mmol) in anhydrous CH_2Cl_2 (20 mL) for 18 h as described above for synthesis of **1**. The residue after workup was purified by flash chromatography on silica gel (30:70:0.5 hexane/EtOAc/TEA, v/v) to obtain the phosphoramidite **3**: ^{1}H NMR (400 MHz, CD_3CN) δ 9.04 (br, 1H), 7.58−7.45 (m, 1H), 5.97−5.85 (m, 1H), 5.85−5.73 (m, 1H), 5.73−5.57 (m, 1H), 4.46−4.12 (m, 4H), 3.95−3.58 (m, 5H), 3.57−3.36 (m, 3H), 3.32−3.09 (m, 2H), 2.82−2.65 (m, 2H), 2.26−2.17 (m, 2H), 2.16−2.13 (m, 1H), 1.83−1.61 (m, 2H), 1.31−1.11 (m, 12H); ^{31}P NMR (162 MHz, CD_3CN) δ 155.71, 155.47; HRMS calcd for $C_{25}H_{39}N_5O_8P$ (MH^+) 568.2536, found 568.2550.

5′-*O*-(4,4′-Dimethoxytrityl)-3′-*O*-propargyl-5-methyluridine-3′-*O*-(*N,N*-isopropyl)phosphoramidite (6). The phosphoramidite **6** (183 mg, 51%) was obtained as a white foam from compound **19** (270 mg, 0.45 mmol) and 2-cyanoethyl *N,N*-diisopropylchlorophosphoramidite (200 μL, 0.88 mmol) in the presence of DIEA (325 μL, 1.75 mmol) in anhydrous CH_2Cl_2 (3 mL) for 6 h as described above for the synthesis of **1**. The residue after workup was purified by flash chromatography on silica gel (4:6 hexane/EtOAc, v/v) to obtain the phosphoramidite **6**: ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.42−11.41 (m, 1H), 7.60−7.17 (m, 10H), 6.97−6.79 (m, 4H), 5.92−5.88 (m, 1H), 4.67−4.58 (m, 1H), 4.46−4.24 (m, 3H), 4.26−3.94 (m, 2H), 3.76 (m, 7H), 3.56 (m, 3H), 3.40−3.14 (m, 2H) 2.77−2.69 (m, 2H), 1.50−0.84 (m, 15H); ^{31}P NMR (162 MHz, DMSO-d_6) δ 156.06, 154.99; MS calcd for $C_{43}H_{51}N_4NaO_9P$ (MNa^+) 821.32, found 821.30.

2′-*O*-TBDMS-5′-*O*-[1-linoleyl-(1,2,3-triazo-3-yl)methyl]-carbamoyluridine-3′-*O*-(*N,N*-diisopropyl)phosphoramidite (30). The phosphoramidite **30** (463 mg, 50%) was obtained as a white foam from compound **28** (730 mg, 1.00 mmol) and 2-cyanoethyl *N,N*-diisopropylchlorophosphoramidite (335 μL, 1.50 mmol) in the presence of DIEA (525 μL, 3.00 mmol) in anhydrous CH_2Cl_2 (10 mL) for 4 h as described above for the synthesis of **1**. The residue after workup was purified by flash chromatography on silica gel (60:30:0.5 hexane/EtOAc/TEA, v/v) to obtain the phosphoramidite **30**: ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.43−11.42 (1H, br), 7.91−7.89 (2H, m), 7.66−7.59 (1H, m), 5.86−5.84 (1H, m), 5.70−5.66 (1H, m), 5.38−5.27 (3H, m), 4.32−4.13 (8H, m), 3.83−3.56 (4H, m), 2.80−2.72 (4H, m), 2.04−1.99 (3H, m), 1.79−1.76 (2H, m), 1.33−1.11 (30H, m), 0.87−0.82 (12H, m), 0.05−0.02 (6H, m); ^{31}P NMR (162 MHz, DMSO-d_6) δ 149.98, 148.37; MS calcd for $C_{46}H_{79}N_8NaO_8PSi$ (MNa^+) 953.54, found 953.40.

Compound 31. The phosphoramidite **31** (403 mg, 83%) was obtained as a white foam from compound **29** (400 mg, 0.44 mmol) and 2-cyanoethyl *N,N*-diisopropylchlorophosphoramidite (150 μL, 0.67 mmol) in the presence of DIEA (230 μL, 1.33 mmol) in anhydrous CH_2Cl_2 (4 mL) for 4 h as described above for the synthesis of **1**. The residue after workup was purified by flash chromatography on silica gel (EtOAc then 2:98:0.5 MeOH/CH_2Cl_2/TEA, v/v) to obtain the phosphoramidite **31**: ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.37 (br, 1H), 7.94−7.88 (m, 1H), 7.89−7.76 (m, 2H), 7.65−7.58 (m, 1H), 5.81−5.72 (m, 1H), 5.68−5.58 (m, 1H), 5.25−5.18 (m, 2H), 4.99−4.92 (m, 1H), 4.57−4.43 (m, 3H), 4.30−4.11 (m, 5H), 4.05−3.98 (m, 4H), 3.97−3.84 (m, 2H), 3.84−3.67 (m, 3H), 3.64−3.44 (m, 3H), 2.16−2.05 (m, 3H), 2.05−1.93 (m, 3H), 1.93−1.82 (m, 3H), 1.82−1.74 (m, 3H), 0.91−0.71 (m, 10H), 0.09−0.05 (m, 6H); ^{31}P NMR (162 MHz, DMSO-d_6) δ 148.98, 148.37; MS calcd for $C_{46}H_{74}N_9NaO_{18}PSi$ (MNa^+) 1123.17, found 1123.30.

3′-*O*-Acetyl-2′-*O*-methyl-5′-*O*-(4-pentynyl)carbamoyluridine (14). 5-Hexynoic acid (3.11 mL, 28.1 mmol) in DMF (150 mL) was treated with diphenylphosphoryl azide (10.7 mL, 56.2 mmol) and triethylamine (8.12 mL, 56.2 mmol) at ambient temperature to 100 °C for 6 h. Then 3′-*O*-acetyl-2′-*O*-methyluridine (**12**, 2.0 g, 7.04 mmol) was added to the reaction mixture, and stirring was continued at 100 °C for 18 h. Progress of the reaction was monitored by TLC. The reaction mixture was cooled to ambient temperature, diluted with EtOAc, and washed with brine. The organic layer was dried over sodium sulfate and then concentrated *in vacuo*. The residue was purified by flash chromatography on silica gel (98.5:1.5 CH_2Cl_2/MeOH, v/v) to obtain **14** (2.07 g, 71%, R_f = 0.2) as a white foam: ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.49 (s, 1H), 7.64 (d, J = 8.1 Hz, 1H), 7.44 (t, J = 5.6 Hz, 1H), 5.87 (d, J = 6.4 Hz, 1H), 5.72 (d, J = 8.1 Hz, 1H), 5.26−5.09 (m, 1H), 4.28−4.04 (m, 4H), 3.28 (s, 3H), 3.11−2.96 (m, 2H), 2.79 (t, J = 2.5 Hz, 1H), 2.21−2.11 (m, 2H), 2.13 (s, 3H), 1.61−1.46 (m, 2H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 169.6, 162.95, 155.7, 150.6, 140.3, 102.7, 86.15, 83.9, 79.8, 79.5, 71.5, 70.5, 63.3, 58.2, 28.3, 20.6, 15.2; MS calcd for $C_{18}H_{23}N_3O_8$ (MH^+) 410.15, found 410.00.

2′-*O*-Methyl-5′-*O*-propargylcarbamoyluridine (15). Compound **13** (4.35 g, 14.49 mmol) was treated with 0.5 M NaOMe in methanol (200 mL) for 4 h. The solution was concentrated *in vacuo*. The residue was purified by flash chromatography on silica gel (95:5 CH_2Cl_2/MeOH, v/v) to obtain **15** (2.75 g, 67%, R_f = 0.3) as a white foam: ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.41 (br, 1H), 7.97 (dd, J = 5.7 Hz, 1H), 7.60 (d, J = 8.1 Hz, 1H), 5.87 (d, J = 5.5 Hz, 1H), 5.68 (d, J = 8.1 Hz, 1H), 5.37 (d, J = 5.8 Hz, 1H), 4.28−4.02 (m, 3H), 4.04−3.95 (m, 1H), 3.91−3.84 (m, 1H), 3.84−3.75 (m, 2H), 3.46 (s, 3H), 3.21 (dd, J = 2.2 Hz, 1H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 163.0, 155.6, 150.5, 140.4, 102.3, 86.0, 82.1, 81.4, 81.3, 73.3, 68.6, 63.9, 57.5, 29.8; MS calcd for $C_{14}H_{18}N_3O_7$ (MH^+) 340.11, found 340.10.

2′-*O*-Methyl-5′-*O*-(4-pentynyl)carbamoyluridine (16). Compound **14** (296 mg, 0.72 mmol) was treated with 7 N NH_3 in methanol (5 mL) at ambient temperature for 4 h. The solution was concentrated *in vacuo* and then coevaporated with CH_2Cl_2 to remove traces of methanol. The residue was dissolved in a minimal amount of EtOAc/hexane/CH_2Cl_2 and sonicated for 5 min to give a white precipitate. The precipitate was filtered, washed with hexane, and dried over a suction funnel. Drying in a vacuum oven at 40 °C for 18 h gave compound **16** (173 mg, 65%): ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.41 (s, 1H), 7.61 (d, J = 8.1 Hz, 1H), 7.36 (t, J = 5.5 Hz, 1H), 5.86 (d, J = 5.4 Hz, 1H), 5.67 (d, J = 8.1 Hz, 1H), 5.35 (d, J = 5.9 Hz, 1H), 4.26−3.81 (m, 5H), 3.35 (s, 3H), 3.13−2.98 (m, 2H), 2.78 (t, J = 2.4 Hz, 1H), 2.22−2.08 (m, 2H), 1.69−1.49 (m, 2H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 162.9, 155.8, 150.5, 140.4, 102.2, 86.1, 83.9, 82.2, 81.5, 71.4, 68.6, 63.5, 57.5, 28.3, 15.2; MS calcd for $C_{16}H_{22}N_3O_7$ (MH^+) 368.14, found 368.3; HRMS calcd for $C_{16}H_{21}N_3NaO_7$ (MNa^+) 390.1277, found 390.1283.

General Procedure for the Propargylation of the 2′/3′-Hydroxyl Group of Ribonucleoside Derivatives. 5′-*O*-DMTr-nucleoside (1.00 molar equiv), dibutyltin(II) oxide (1.10 molar equiv), tetrabutylammonium iodide (0.50−2.00 molar equiv), and propargyl chloride (2.00 molar equiv) were added to the solution of benzene/CH_3CN (1:1, 0.2 M). The suspension was microwave irradiated at 100 °C for 1−6 h. Aqueous workup followed by flash silica gel chromatography gave the desired propargylated nucleosides.

5′-*O*-(4,4′-Dimethoxytrityl)-3′-*O*-propargyl-5-methyluridine (19): ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.37 (br, 1H), 7.59−7.18 (m, 10H), 6.91 (m, 4H), 5.80−5.69 (m, 1H), 5.63 (d, J = 5.99 Hz, 1H), 4.46−4.20 (m, 4H), 4.18−3.99 (m, 1H), 3.72 (s, 6H), 3.56−3.46 (m, 1H), 3.27−3.15 (m, 2H), 1.37 (d, J = 0.8 Hz, 3H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 163.6, 158.2, 158.2, 150.6, 144.6, 135.5, 135.3, 135.0, 129.7, 128.0, 127.6, 126.8, 113.3, 109.5, 87.9, 86.0, 80.6, 80.1, 77.6, 76.0, 72.3, 62.9, 58.0, 55.1, 31.0, 22.1, 14.0, 11.6; HRMS calcd for $C_{34}H_{34}N_2NaO_8$ (MNa^+) 621.2213, found 621.2214.

2′-*O*-(*tert*-Butyldimethylsilyl)-5′-*O*-[1-linoleyl(1,2,3-triazo-3-yl)-methyl]carbamoyluridine (28). Compound **11** (100 mg, 0.17 mmol) in CH_2Cl_2/MeOH (4:1, v/v) (2 mL) was treated with **23** (59 mg, 0.17 mmol), tetrakis(acetonitrile)copper(I) hexafluorophosphate (13 mg, 0.03 mmol), and copper (2 mg, 0.03 mmol) at ambient temperature for 3 h. The solution was concentrated in vacuo. The residue was purified by flash chromatography on silica gel (1:3 hexane/EtOAc, v/v) to obtain **28** (122 mg, 98%) as a white foam: ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.35 (s, 1H), 7.91 (s, 1H), 7.82 (br, 1H), 7.60 (d, J = 7.8 Hz, 1H), 5.78 (d, J = 5.0 Hz, 1H), 5.63 (d, J = 7.8 Hz, 1H), 5.46−5.24 (m, 4H), 5.17 (br, 1H), 4.35−4.16 (m, 6H), 4.16−4.08 (m, 1H), 4.06−3.96 (m, 1H), 3.93−3.82 (m, 1H), 2.73 (dd, J = 6.2 Hz, 2H), 2.16−1.90 (m, 2H), 1.90−1.66 (m, 2H), 1.60−1.03 (m, 16H), 1.04−0.54 (m, 12H), 0.33−0.30 (m, 9H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 161.7, 154.7, 139.1, 128.6, 128.5, 126.6, 101.0, 86.7, 80.9, 73.6, 68.4, 62.3, 48.1, 39.0, 38.8, 38.6, 38.0, 37.8, 34.8, 29.7, 28.6, 27.8, 27.5, 27.4, 27.2, 25.5, 25.4, 24.7, 24.4, 24.0, 20.8, 16.7, 12.7, −6.0, −6.4; MS calcd for $C_{37}H_{63}N_6O_7Si$ (MH^+) 730.45, found 731.20.

Compound 29. Compound **11** (500 mg, 1.14 mmol) in CH_2Cl_2/MeOH (4:1, v/v) was treated with **26** (460 mg, 1.14 mmol), tetrakis(acetonitrile)copper(I) hexafluorophosphate (42 mg, 0.11 mmol), and copper (7 mg, 0.11 mmol) at room temperature for 18 h. The solution was concentrated *in vacuo*. The residue was directly loaded on flash silica gel column without aqueous workup and purified (eluent: 9:1 CH_2Cl_2/MeOH, v/v) to obtain **29** (800 mg, 88%) as a white foam: ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.37 (s, 1H), 7.90 (s, 1H), 7.84 (dd, J = 14.7, 7.6 Hz, 2H), 7.61 (d, J = 8.1 Hz, 1H), 5.78 (d, J = 5.0 Hz, 1H), 5.64 (d, J = 8.0 Hz, 1H), 5.25−5.18 (m, 2H), 4.96 (dd, J = 11.2, 3.3 Hz, 1H), 4.57−4.43 (m, 3H), 4.30−4.11 (m, 5H), 4.05−3.98 (m, 4H), 3.97−3.84 (m, 2H), 3.84−3.67 (m, 3H), 3.64−3.44 (m, 2H), 2.09 (s, 3H), 1.99 (s, 3H), 1.89 (s, 3H), 1.76 (s, 3H), 0.83 (s, 9H), 0.03 (d, J = 11.8 Hz, 6H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 170.0, 169.9, 169.6, 169.3, 162.9, 155.9, 150.5, 144.8, 140.2, 123.1, 102.1, 100.9, 87.9, 82.0, 74.7, 70.4, 69.9, 69.5, 69.2, 68.8, 68.2, 66.7, 63.5, 61.4, 49.3, 25.6, 22.8, 20.49, 20.42, 20.41, 17.8, −4.8, −5.2; HRMS calcd for $C_{37}H_{58}N_7O_{17}Si$ (MH^+) 900.3658, found 900.3660.

Loading of 5′-*O*-(4,4′-Dimethoxytrityl)-3′-*O*-propargyl-5-methyluridine on CPG Support (7). Compound **19** (286 mg, 0.48 mmol) in anhydrous CH_2Cl_2 (50 mL) was treated with succinic anhydride (231 mg, 0.68 mmol) and DMAP (111 mg, 0.91 mmol) at ambient temperature for 18 h. The solution was concentrated under reduced pressure. The residue was chromatographed on silica (eluent: TEA/MeOH/CH_2Cl_2, 5:5:90) to obtain the pure succinate (383 mg, 0.49 mmol, 99%, R_f = 0.6). The succinate was dissolved in anhydrous DMF (50 mL), and DIEA (320 μL, 1.84 mmol) and HBTU (191 mg, 0.50 mmol) were added. The solution was agitated briefly for 2 min, lcaa-CPG (pore size 500 Å NH_2, loading of 140 μmol/g, 3.57 g) was added to the reaction mixture, and the slurry was agitated on a wrist-action shaker for 4 h at ambient temperature. The CPG was filtered, washed with CH_2Cl_2/MeOH (9:1, v/v, 400 mL), and dried over suction funnel for 15 min. The remaining amino residues on the CPG were capped by pyridine/Ac_2O/TEA (75:25:5, v/v, 100 mL) treatment for 15 min. The CPG was filtered, washed with CH_2Cl_2/MeOH (9:1, v/v, 600 mL), and then dried over a suction funnel. Finally, the CPG was dried under vacuum at ambient temperature for 18 h to give **7** (4.08 g CPG, loading: 80 μmol/g).

General Procedure for Solid-Phase Synthesis of Modified siRNA Targeting the Firefly Luciferase Gene. Oligonucleotide sequences bearing alkyne moieties at desired sites (3′ or 5′ termini or internal position, Table 1) and other functional groups on the 5′-terminal position (entry **46** and **56** in Table 1) were synthesized on an ABI 394 DNA synthesizer using standard phosphoramidite chemistry with commercially available 5′-*O*-(4,4′-dimethoxytrityl)-3′-*O*-(2-cyanoethyl-*N*,*N*-diisopropyl)-phosphoramidite monomers of uridine (U), 4-*N*-acetylcytidine (C^{Ac}), 6-*N*-benzoyladenosine (A^{Bz}), and 2-*N*-isobutyrylguanosine (G^{iBu}), 2′-*O*-*tert*-butyldimethylsilyl-protected phosphoramidites, and 5′-*O*-(4,4′-dimethoxytrityl)-2′-deoxythymidine-3′-*O*-(2-cyanoethyl-*N*,*N*-diisopropyl) phosphoramidite (dT). Extended nucleotide coupling times (30 min) were applied at steps incorporating modified phosphoramidites (alkyne monomers **1**, **2**, **3**, **4**, **6**, and triazoyl monomers **30** and **31**). After synthesis, a small portion of the oligonucleotide bound to CPG was treated with 100 μL of methylamine solution (40 wt % in water) in a 1 mL microtube at 65 °C for 10 min. The mixture was cooled on dry ice for 5 min and the solid suspension was spun down. Of the supernatant, 80 μL was decanted into another microtube and heated with 120 μL of TEA·3HF at 65 °C for 12 min. The purity of crude oligonucleotides was analyzed by RP-HPLC or anion-exchange high-performance

liquid chromatography (IEX-HPLC) and the mass was confirmed by LC−MS analysis prior to postsynthetic CuAAC reaction.

General Procedure for Click Reaction on Solid Support. To a 0.6 μmol solid-supported alkyne−RNA phosphotriester was added a mixture of an azide (3 equiv by alkyne, 1.8 μmol, 36 μL of a 50 mM solution in THF), freshly prepared $CuSO_4 \cdot 5H_2O$ (0.4 equiv, 0.24 μmol, 5 μL of a 50 mM solution in H_2O), freshly prepared sodium ascorbate (3 equiv, 1.8 μmol, 37 μL of a 50 mM solution in H_2O), and TBTA (3 equiv, 1.8 μmol, 37 μL of a 50 mM solution in THF). Water/MeOH/THF (2:2:1 v/v) was added to obtain a total volume of 1200 μL. The resulting preparation was heated in a sealed glass tube in an Explorer-48 microwave synthesizer at a 100 W and a 30 s premixing time. The temperature was monitored with an internal infrared probe and held at 60 °C over 45 min. The solution was removed, and the CPG supports were washed with THF and MeOH then dried. The product on the CPG supports were cleaved and deprotected by treating the solid-support with 100 μL of methylamine solution (40 wt % in water) at 65 °C for 10 min. The mixture was cooled on dry ice for 5 min and the solid suspension was spun down. Of the supernatant, 80 μL was decanted into another microtube and heated with 120 μL of TEA·3HF at 65 °C for 12 min. The product was analyzed by LC-MS and by RP-HPLC (C4 column, 150 × 3.9 mm i.d., 5 μm, 300 Å) using a linear gradient of 0 to 70% B over 24 min at 30 °C at a flow rate of 1 mL/min (buffer A: 50 mM TEAA, pH 7.0; buffer B: CH_3CN). Completion of the CuAAC reaction was estimated by comparing the purity of starting material alkyne-RNAs and their corresponding triazole products by RP-HPLC. After base and sugar deprotection, oligonucleotides were purified by RP-HPLC with C4 column (300 × 7.8 mm i.d., 15 μm, 300 Å) using a linear gradient of 0 to 90% B in 40 min at room temperature at a flow rate of 3 mL/min (buffer A: 50 mM TEAA, pH 7.0; buffer B: CH_3CN).

Duplex Annealing. For duplex annealing, 1 mM stock solutions of oligonucleotides were prepared in deionized water. For the cell culture experiments, 0.05 mM stock solutions of siRNA duplexes were prepared by mixing equimolar amounts of complementary sense and antisense strands in PBS buffer. The solutions were heated at 95 °C for 5 min and then allowed to slowly cool to room temperature. To ensure low variability in duplex concentration, the optical densities of the final duplex solutions in PBS buffer were measured and the duplex preparation was repeated if the optical density was not within a 10% range of a reference sample (parent duplex).

Cell Culture. HeLa SS6 cells, stably transfected with the luciferase plasmids, were grown at 37 °C in 5% CO_2 in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS) and 0.5 mg/mL Zeocin and 0.5 μg/mL puromycin (selective for cells transfected with plasmids). The cells were maintained in exponential growth phase.

Luciferase Assay. The cells were plated in 96-well plates (0.1 mL medium per well) and were at about 90% confluence at transfection. The cells were grown for 24 h, and the culture medium was changed to Opti-MEM, 0.5 mL per well. Transfection of siRNAs was carried out with Lipofectamine 2000 as described by the manufacturer for adherent cell lines at siRNA concentrations ranging from 0.002 to 8.0 nM. The final volume was 150 μL per well. The cells were harvested 24 h post transfection and lysed using passive lysis buffer (PLB), 100 μL per well. The luciferase activities of the samples were measured using a Victor FL Luminometer with 20 μL of sample and 75 μL each of Luciferase assay reagent II and Stop & Glo reagent. The inhibitory effects of the siRNAs were expressed as normalized ratios between the activities of the reporter (firefly) luciferase gene and the control (*Renilla*) luciferase gene relative to untreated controls (no siRNA, but with Lipofectamine 2000). Values represent the mean of triplicates. The potency of the siRNAs was determined by calculating the IC_{50} values from dose−response curves using XL-Fit software.

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Supporting Information Available: Yields of 2′/3′-*O*-propargylated 5′-*O*-DMTr nucleosides under optimized microwave-assisted reaction conditions; synthetic scheme of alkyl azides **20**−**24**, scheme and synthetic procedures for azides **25** and **25a**; NMR spectra of nucleoside derivatives and azides **24**, **25**, and **25a**; LC−MS characterization of alkyne−oligonucleotide scaffolds **32a**−**41a**, and HPLC profiles of click reaction of alkyne−oligonucleotides with azides and purified oligonucleotide conjugates. This material is available free of charge via the Internet at http://pubs.acs.org.