Aminooxy Click Chemistry as a Tool for Bis-homo and Bis-hetero Ligand Conjugation to Nucleic Acids

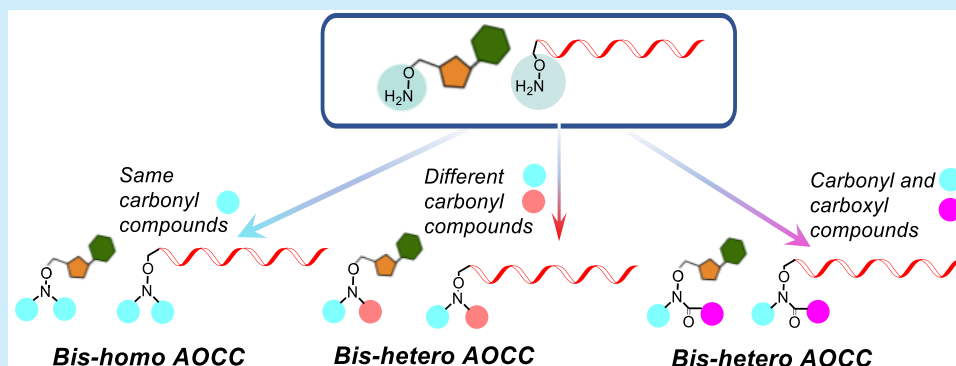Dhrubajyoti Datta, Shohei Mori, Mimouna Madaoui, Kelly Wassarman, Ivan Zlatev, and Muthiah Manoharan*

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ABSTRACT: An aminooxy click chemistry (AOCC) strategy was used to synthesize nucleoside building blocks for incorporation during solid-support synthesis of oligonucleotides to enable bis-homo and bis-hetero conjugation of various biologically relevant ligands. The bis-homo aminooxy conjugation leads to bivalent ligand presentation, whereas the bis-hetero conjugation allows the placement of different ligands with either the same or different chemical linkages. This facile synthetic methodology allows introduction of two different ligands with different biological functions simultaneously.

Aminooxy or *O*-amino groups (-*O*-NH$_2$) act as stronger nucleophiles than amines, due to the second lone-pair-bearing oxygen adjacent to the nucleophilic nitrogen. Moreover, their low basicity allows them to form more stable imines than Schiff bases.[1] This phenomenon was first observed by Jencks and Carriuolo in 1960,[2] and Edwards and Pearson called it the "alpha effect" in 1962.[3] Aminooxy-functionalized sugars, nucleosides, and peptides have attracted interest as they can be easily synthesized and used for oxime ligation[4−8] and *N*-oxyamide functionalization.[9−11] A bis-functionalized aminooxy group was used for sugar modification in oligonucleotide chemistry,[12] and nucleosides with a 5′-aminooxy group have been used in the synthesis of oligonucleotides with modified backbones;[13] both syntheses involved very reactive formaldehyde.

Conjugation chemistry has invigorated oligonucleotide therapeutics by enabling incorporation of moieties that modulate pharmacokinetics and that result in delivery into particular cell types, as demonstrated by the clinical success of small interfering RNAs (siRNAs) conjugated to triantennary *N*-acetylgalactosamine (tri-GalNAc), the ligand for the hepatocyte-specific receptor asialoglycoprotein.[14] In other examples, conjugation to lipophilic molecules like cholesterol[15] and fatty acids[16] results in broad tissue distribution and cellular uptake in the liver and central nervous system;[17,18] folic acid

conjugation results in cancer cell targeting; and conjugation to cyclic RGD peptides results in uptake into cells that express integrins.[19]

The biocleavable oxime linkage has been used in oligonucleotide conjugation.[1,5,6,8,20] Here, to facilitate bis-homo and bis-hetero conjugation, we developed a "click-type" aminooxy-based chemistry that we call aminooxy click chemistry (AOCC). The reported reaction meets the Sharpless definition of click chemistry:[21] The reaction is modular and wide in scope and gives very high yields, and product isolation is simple. Two different strategies were envisioned: AOCC conjugate monomers could be synthesized as amidites and incorporated during solid-phase oligonucleotide synthesis, or precursors of aminooxy groups could be introduced into the oligonucleotide strands and functionalized postsynthetically. In the first approach, demonstrated in this report, a combination of two different aldehydes and a combination of an aldehyde

with an acid were used for the bis-hetero ligation (Figure 1). Ligands with different biological functions were introduced, demonstrating the broad scope of the strategy.
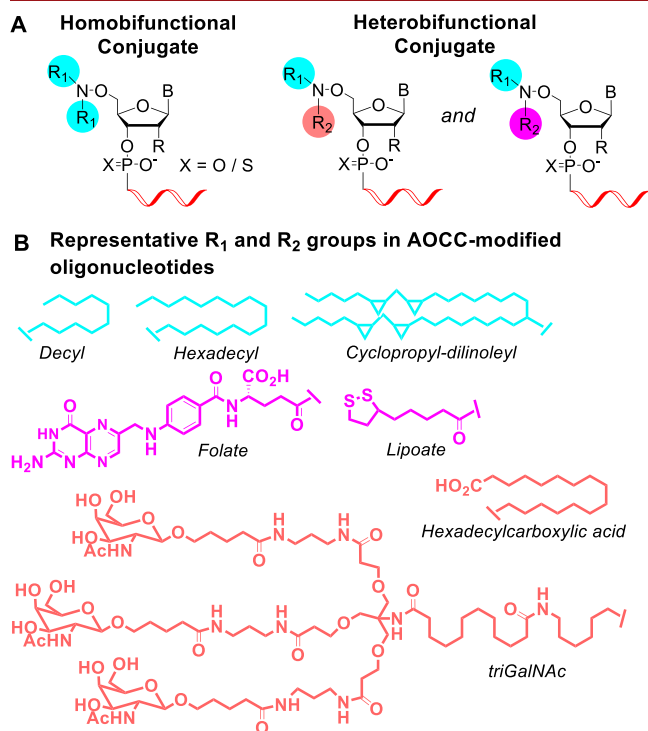

Figure 1. (A) Schematic of 5′-end modification of oligonucleotides with AOCC-mediated bis-homo and bis-hetero conjugation. (B) Representative targeting and pharmacokinetic-modifier ligands.

For construction of building blocks to enable bis-homo and bis-hetero ligand conjugation, we synthesized nucleosides containing *N*-hydroxyphthalimido (OPhth), the precursors of *O*-amino. To generate 5′-OPhth-modified nucleosides, 2′-*O*-methyl (2′-OMe) and locked nucleic acid (LNA) nucleosides with protected 3′-OH were used. Mitsunobu reaction of compounds **1**[22] and **8** afforded the OPhth-containing scaffolds **2** and **9**, respectively.

From these precursors, we first synthesized bis-homo ligand-containing modified nucleosides by generating an aminooxy linkage. Various aldehydes and ketones were used for facile oxime bond formation through a 5′-aminooxy functionality. For the oximes derived from aldehydes, an identical aldehyde was then attached to the aminooxy nitrogen atom through reductive amination. To illustrate this, **2** was deprotected in the presence of *N*-methylhydrazine to afford **3**, which contains the aminooxy group. Reaction of **3** with carbonyl compounds [e.g., citral, decanal, hexadecanal, dilinoleyl aldehyde, cyclopropyl-dilinoleyl ketone, and protected tri-GalNAc aldehyde **3S** (Scheme S1 and Table S1 in the Supporting Information)] resulted in oximes **4a–f** either under basic or acidic conditions. The *E*- and *Z*-isomers of these oximes eluted together during column purification. The oxime was reduced with sodium cyanoborohydride under acidic conditions to afford the substituted aminooxy amine, which was reacted again with the same aldehyde. Thus, oximes **4a**, **4b**, and **4c** were reacted with citral, decanal, and hexadecanal, respectively, under reductive amination conditions to produce compounds **5a**, **5b**, and **5c**. The 3′-OTBS groups of **5a–c** were removed by

treatment with tetrabutylammonium fluoride (TBAF) to obtain **6a–c** with free 3′-OH groups. Compounds **6a–c** were then phosphitylated to afford bis-homo-AOCC-modified pyrimidine nucleoside phosphoramidite building blocks **7a–c** (Scheme 1). Analogous purine compounds were synthesized using the same route.

Scheme 1. Synthesis of Bis-homo-aminooxy-modified 2′-OMe-uridine Building Blocks[a]
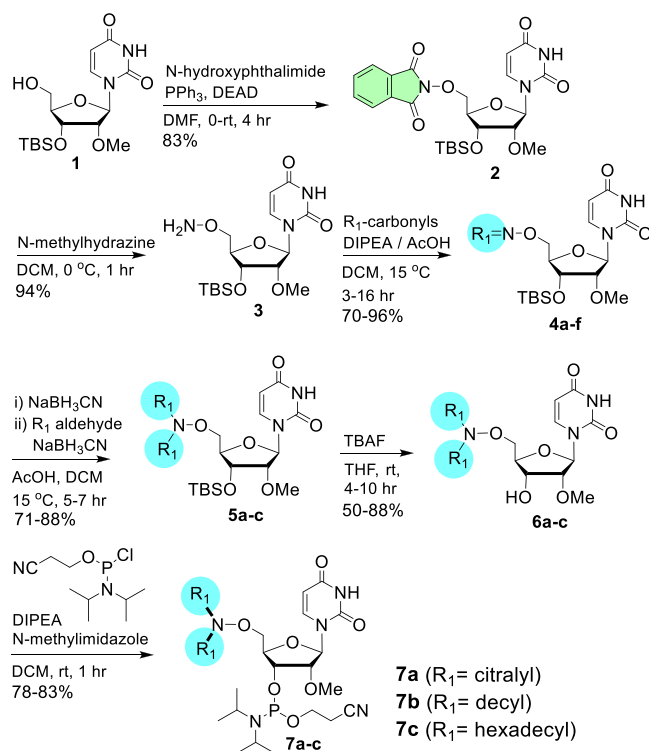
[a]For **4a–7a**, R$_1$ is citralyl; for **4b–7b**, R$_1$ is decyl; for **4c–7c**, R$_1$ is hexadecyl; for **4d**, R$_1$ is dilinoleyl; for **4e**, R$_1$ is cyclopropyl-dilinoleyl; and for **4f**, R$_1$ is protected tri-GalNAc (structure shown in Scheme 3).

The adenosine LNA analogue **9** was prepared using an approach similar to that used for the synthesis of compound **2** from 3′-*O*-silyl-protected LNA-adenosine **8**. The 5′-OPhth of compound **9** was deprotected to afford **10** in the presence of *N*-methylhydrazine. Compound **10** was reacted with hexadecanal to obtain oxime **11**, which was subsequently converted to the bis-homoligated compound **12** using hexadecanal under reductive amination conditions. An additional step resulted in protection of the exocyclic N^6-amine of adenosine with an amidine group to yield **13**, and 3′-*O*-silyl deprotection of **13** produced **14**. Phosphitylation of **14** produced the phosphoramidite **15** (Scheme 2).

To synthesize the bis-hetero building blocks that allow introduction of two different ligands, we initially reacted three lipophilic oximes, **4c**, **4d**, and **4e**, with formaldehyde to afford simple bis-hetero-AOCC analogues **16a**, **16b**, and **16c**, respectively. Additionally, lipophilic oxime **4c** and tri-GalNAc-conjugated oxime **4f** were reacted with methyl-16-oxohexadecanoate and hexadecanal to afford **16d** and **16e**, respectively, under reductive amination conditions. These bis-hetero AOCC products were subsequently desilylated to obtain **17a–e**, which were converted to the respective phosphoramidites **18a–e** (Scheme 3). For the synthesis of

Scheme 2. Synthesis of Bis-homo-Conjugated LNA-adenosine Building Blocks[a]
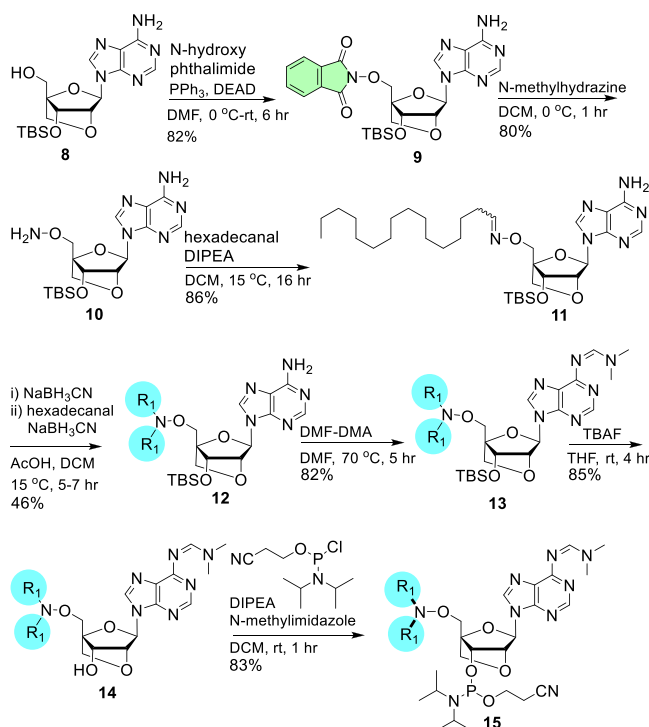
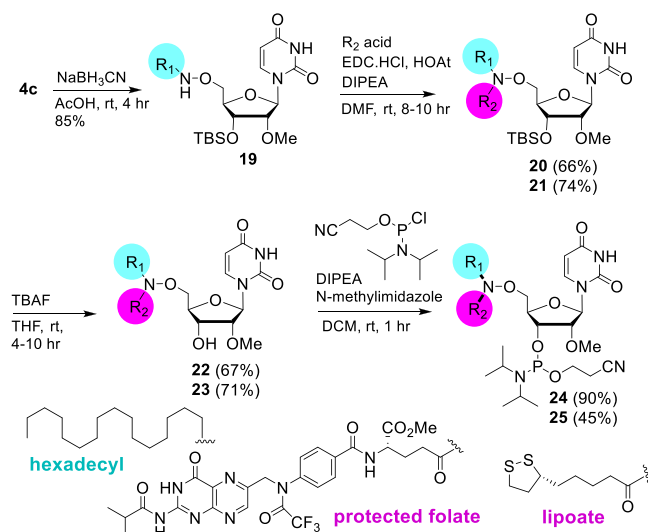

[a]For 12−15, R$_1$ is hexadecyl.

bis-hetero building blocks, bulky ligands like tri-GalNAc or with keto-group-like dilinoleyl and cyclopropyl-dilinoleyl ketones were installed first for facile conversion and better yields.

We also evaluated bis-heteroaminooxy-modified building blocks where the second ligand (R$_2$) was an acid. Previously, this chemistry was used for conjugation to a neutral hydroxamate-modified DNA backbone.[23] For this, 4c was reduced to afford the lipophilic aminooxy amine 19 and coupled with the protected folic acid 7S (Scheme S2 in Supporting Information) or lipoic acid to obtain the bis-hetero conjugates 20 and 21, respectively. The folate conjugation was achieved through the γ-carboxyl group.[24] The amide coupling reaction was done using standard amide coupling conditions.[25,26] The 3′-OTBS groups of 20 and 21 were deprotected to produce 22 and 23, respectively, which were converted to the phosphoramidites 24 and 25 in moderate to good yields (Scheme 4).

Finally, we tested the "click"-like behavior of the AOCC through a sequential one-pot reductive amination of 10 in the presence of two different carbonyl compounds. The LNA-A intermediate 10 with a 5′-aminooxy group was reacted first with hexanal. Then, without isolation of the oxime, the reductive amination step was performed with methyl-16-oxohexadecanoate to afford the bis-hetero product 26 in moderate yield. We did not observe the formation of the bis-homo-AOCC product upon incorporation of two units of hexanal/methyl-16-oxohexadecanoate. Similarly, a sequential one-pot reaction with tetrahydro-4H-pyran-4-one and hexadecanal produced the bis-hetero product 27 in moderate yield. Here also, the order of addition for the carbonyl compounds was important. For 27, addition of the ketone, tetrahydro-4H-pyran-4-one, followed by the aldehyde hexade-

Scheme 3. Synthesis of Bis-hetero-aminooxy-modified 2′-OMe-uridine Building Blocks[a]
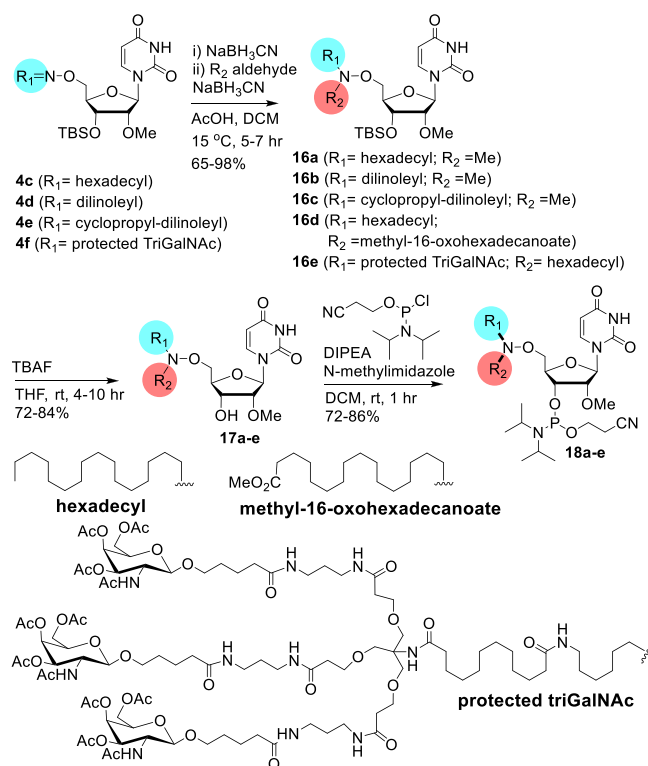
[a]For 16a−18a, R$_1$ is hexadecyl, and R$_2$ is CH$_3$; for 16b−18b, R$_1$ is dilinoleyl, and R$_2$ is CH$_3$; for 16c−18c, R$_1$ is cyclopropyl-dilinoleyl, and R$_2$ is CH$_3$; for 16d−18d, R$_1$ is hexadecyl, and R$_2$ is methyl-16-oxohexadecanoate; for 16e−18e, R$_1$ is protected tri-GalNAc, and R$_2$ is hexadecyl.

Scheme 4. Synthesis of Bis-hetero-aminooxy-modified 2′-OMe-uridine Building Blocks[a]

[a]For 19, R$_1$ is hexadecyl; for 20, 22, and 24, R$_1$ is hexadecyl, and R$_2$ is protected folate; and for 21, 23, and 25, R$_1$ is hexadecyl, and R$_2$ is lipoate.

canal was more efficient than the reverse order. Amidine protection of 26 and 27 with DMF-DMA afforded 28 and 29, which were then desilylated with TBAF to yield 30 and 31,

respectively. The phosphitylation reaction of **30** and **31** furnished the bis-heteroaminooxy-modified LNA-adenosine building blocks **32** and **33** in good yields (Scheme 5).

Scheme 5. Sequential One-Pot AOCC Reaction for the Synthesis of Bis-hetero-aminooxy-modified LNA-adenosine Building Blocks[a]
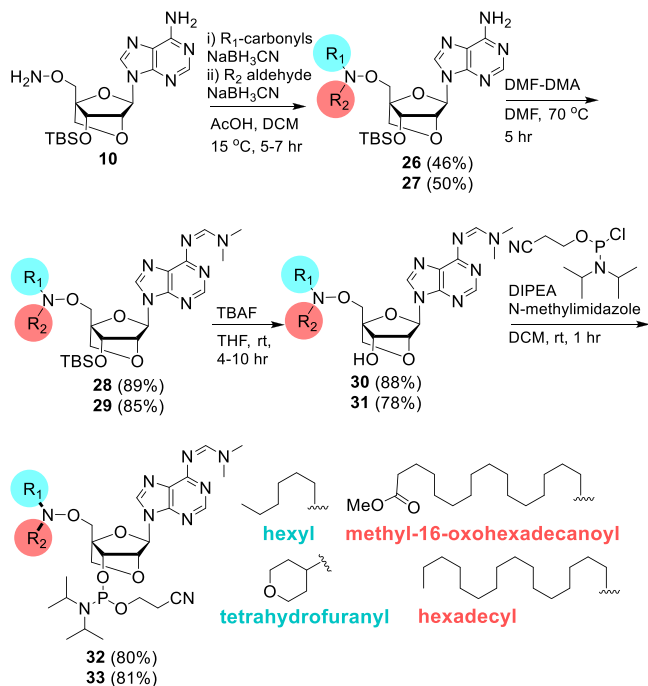
[a]For **26**, **28**, **30**, and **32**, R$_1$ is hexyl, and R$_2$ is methyl-16-oxohexadecanoyl. For **27**, **29**, **31**, and **33**, R$_1$ is tetrahydrofuranyl, and R$_2$ is hexadecyl.

To demonstrate the representative first-generation oxime conjugates, we synthesized a lipophilic oxime building block. Intermediate **4c** was desilylated with TBAF to afford compound **34** in good yield. Compound **34** was phosphitylated to obtain the starting monomer for oxime conjugation, the 5′-oxime-hexadecyl conjugate **35** (Scheme 6).

Scheme 6. Synthesis of the Oxime C16 Lipid Building Block (35)
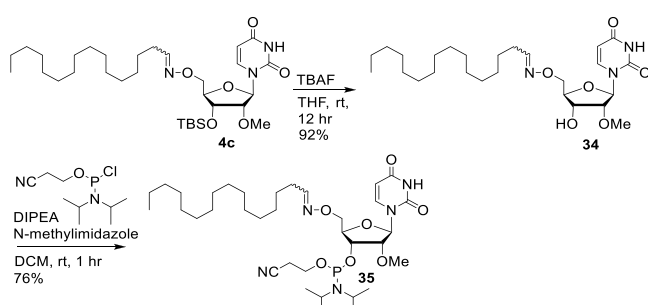
The AOCC−conjugate building blocks were incorporated at the 5′ ends of oligonucleotides using standard oligonucleotide synthesis conditions. The oligonucleotides used were the sense strands of siRNAs targeting the *SOD1*, *β-cat*, and *Apo-B* mRNAs (Table 1). In these oligonucleotides, the modified monomers replaced the U nucleotide during solid-phase

Table 1. Sequences, mRNA Targets, Chemistry, and Ligand Features of siRNA Sense Strand Oligonucleotides (ON) with AOCC Building Blocks at the 5′ End[a]

ON	Sequence 5′-3′	Target	Ligand(s)
1	**I**•a•uuuu*AaUCC*ucacucua•a•a	*SOD1*	**C16 oxime**
2	**II**•a•uuuu*AaUCC*ucacucua•a•a	*SOD1*	**C10/C10**
3	**III**•a•uuuu*AaUCC*ucacucua•a•a	*SOD1*	**C16/lipoate**
4	**IV**•a•uuuu*AaUCC*ucacucua•a•a	*SOD1*	**cyclo-propyldi-linoleyl/Me**
5	**V**•a•*CuGuUgGA*Uu*GaUuCgA*•a•*A*	*β-cat*	**C16/folate**
6	**VI**•g•*UgAcAaAUAuGgGcAuC*•a•*A*	*Apo-B*	**triGal-NAc/C16**

[a]Uppercase italicized and lowercase letters represent 2′-F and 2′-OMe nucleosides, respectively. Phosphorothioate (PS) linkages are indicated by the "•" symbol. AOCC-derived conjugates in the context of 2′-OMe-uridine are **I** to **VI** for **ON1**−**ON6**, respectively, and are shown in Figure 2.

synthesis under standard conditions; the oligonucleotide conjugates were obtained with good coupling efficiencies and yields even with large ligands like folate and tri-GalNAc in combination with C16 (Figure 2). After deprotection and cleavage from the support under standard conditions, the conjugates were purified and characterized. As this was, to our knowledge, the first lipoic acid conjugation to oligonucleotides, we confirmed that there was no oxidation or degradation of disulfide-containing rings (Figure 2, ON3).
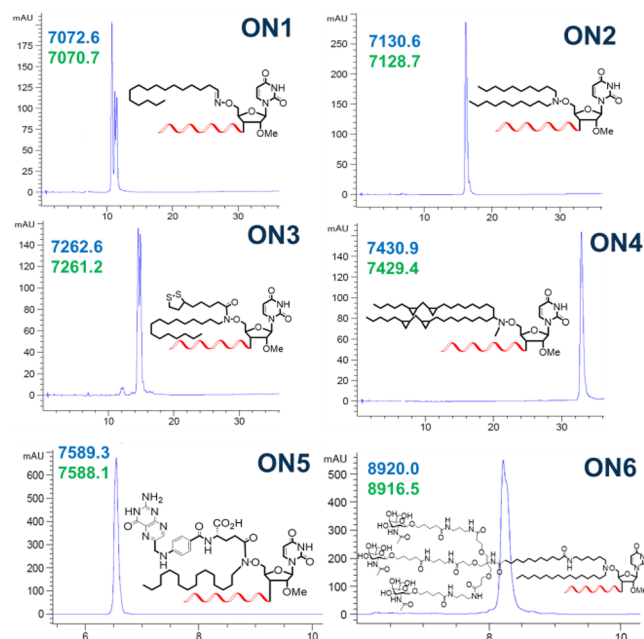
Figure 2. HPLC elution profiles (details in SI) of AOCC-modified oligonucleotides ON1−ON6 with calculated (blue) and observed (green) masses. The multiplicity of peaks is due to diastereomeric chiral phosphorothioates and/or oxime rotamers.

In summary, we have synthesized several nucleoside monomer building blocks equipped with the highly reactive nucleophilic aminooxy functionalities. These groups were converted into bis-homo and bis-hetero building blocks that were functionalized with two of the same or two different moieties, respectively. Both bis-homo- and bis-heterotype nucleoside conjugates were successfully incorporated at the 5′-ends of oligonucleotides. The ability to incorporate two different functionalities should prove especially useful in improving the therapeutic potential of all classes of therapeutic oligonucleotides. For example, siRNAs with single targeting agents like GalNAc, folate, and lipids enable tissue-type targeting. Use of these moieties as bis-hetero conjugates with the appropriate choice of a second ligand could improve tissue-specific delivery, cellular uptake, endosomal release, and pharmacodynamics.[27,28] To date, conjugation of ligands has been performed mostly at the 3′ end[14,15] of the sense strand of the siRNA. The strategy reported here will make 5′-end conjugation easier from the oligonucleotide synthesis and purification perspective. Conjugation at the 5′-position of the siRNA sense strand may improve antisense strand selection by the RNA interference machinery, thus eliminating sense-strand-mediated off-target effects,[29] and should also enhance resistance to 5′-exonucleases.[30] Work to test these possibilities is in progress.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.orglett.2c00988.

Experimental and compound characterization (PDF)

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Author Contributions

The manuscript was written through contributions of all authors.

Notes

The authors declare the following competing financial interest(s): All authors are employees of Alnylam Pharmaceuticals.

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