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2′-Lipid-modified oligonucleotides via a 'Staudinger–Vilarrasa' reaction

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ABSTRACT

We report a new access to 2′-amido-2′-deoxyuridine via a Staudinger–Vilarrasa coupling reaction for the preparation of lipid-modified oligonucleotides. One or two lipidic moieties were inserted within the oligonucleotidic sequence (LONs) leading to a repertoire of original antagomir-like molecules targeting micro RNA (miRNA or miR). Melting temperature (*T*m) experiments revealed that the stability of the duplexes depends on the lipid position and the number of lipid moieties inserted within the oligonucleotide sequence. Single lipid conjugations of positions 11 and 19 of LONs targeting miR-122 do not destabilize the duplexes.

Recently, much attention has been given to the design of hybrid molecules with a bi-functionality based upon the combination of nucleic acids and amphiphile features.[1] Of the many possible combinations, lipid-oligonucleotide conjugates (LONs) nicely illustrate the interest in developing hybrid structures. Interestingly, lipid conjugation of oligonucleotides (ONs) has been used to facilitate the cellular uptake.[2–5] With regard to LONs conjugates, two ON-based antisense therapeutic strategies have been investigated. The first one involves LON antisense sequences that have been selected to bind to messenger RNA (mRNA) and block the genetic expression at the translational step.[5] Previously, a second approach had been developed.[6] This second strategy take advantage of cholesterol-based oligonucleotide conjugates, called antagomirs, which are specific inhibitors of non-coding RNA (micro RNAs) involved in the regulation of gene expression.[7] Antagomirs, which are very attractive and promising therapeutic material to target miRNAs via complementary hybridization, can be tuned to modulate their cellular uptake and biological activity.

A motivator of this research is to expand the repertoire of the chemical structure of antagomirs reported recently. To the best of our knowledge, there is no previous example of modified antagomirs featuring lipid moieties inserted within the sequence.

Here in, we describe an efficient and versatile access to a series of antagomir analogues. The synthesis of a 2′-lipid-amido uridine phosphoramidite via a Staudinger–Vilarrasa reaction (Scheme 1) is developed. The lipid phosphoramidite is further coupled to the oligonucleotides by using a solid supported synthesis, in which the ON is elongated in the 3′–5′ direction. The first series of LONs proposed show a complementary sequence to miR-122, which is a micro RNA involved in the control of the replication of HCV and in the regulation of lipid metabolism, conjugated with either one or two lipid moieties covalently attached at the 2′ position. Indeed, specific silencing of miRNAs such as miR-122 could become a therapeutic strategy against HCV infections or metabolic diseases.[8–10]

A usual strategy for synthesizing LONs is the preparation of modified nucleoside followed by conversion to the corresponding phosphoramidite suitable for automated ON synthesis. Different approaches have been investigated for preparing hydrophobic phosphoramidite building blocks including sugar[11] and bases[12] modifications or inter-nucleoside phosphate functionalization.[13]

The antisense activity of antagomirs or antagomir-like molecules requires the high specificity of Watson and Cricks hybridization between antagomir and miRNA complementary sequences. Hence, the lipid conjugation of the ONs must preserve the duplex stability. Several groups have reported that the modifications of the carbohydrate moiety interfere less with base pairing than those on backbone[14,15] or base sites.[16,17] Interestingly, the 2′-modification of the ribose, which is compatible with the 3′–5′ solid-phase synthesis, is known to induce minimal distortions in ON structures and enhances ON resistance against nucleases.[18]

A large number of ON derivatives bearing 2′-O-tethered modifications have been reported.[19–23] Less described are those involving 2′-carbamate,[24] 2′-ureido, or 2′-amido modifications.[25,26] Concerning the later, they usually result from N-acylation of the corresponding 2′-amino-2′-deoxyribonucleoside, which is previously prepared in five steps through the reduction of the

Abbreviations: CH_2Cl_2, dichloromethane; DIEA, diisopropyl ethylamine; DMF, dimethylformamide; DMAP, 4-(dimethylamino)pyridine; DMTr–Cl, 4,4′-dimethoxytrityl chloride; h, hour(s); HOSu, *N*-hydroxy succinimide; o/n, overnight; LiF, lithium fluoride; P*n*Bu_3, tri-*n*-butylphosphine; Py, pyridine; RT, room temperature; SiO_2, silicium dioxide; TEA, triethylamine; THF, tetrahydrofuran; RNA, ribonucleic acid; TMEDA, *N*,*N*,*N*′,*N*′-tetramethylethylene diamine; TMS–N_3, trimethylsilyl azide.

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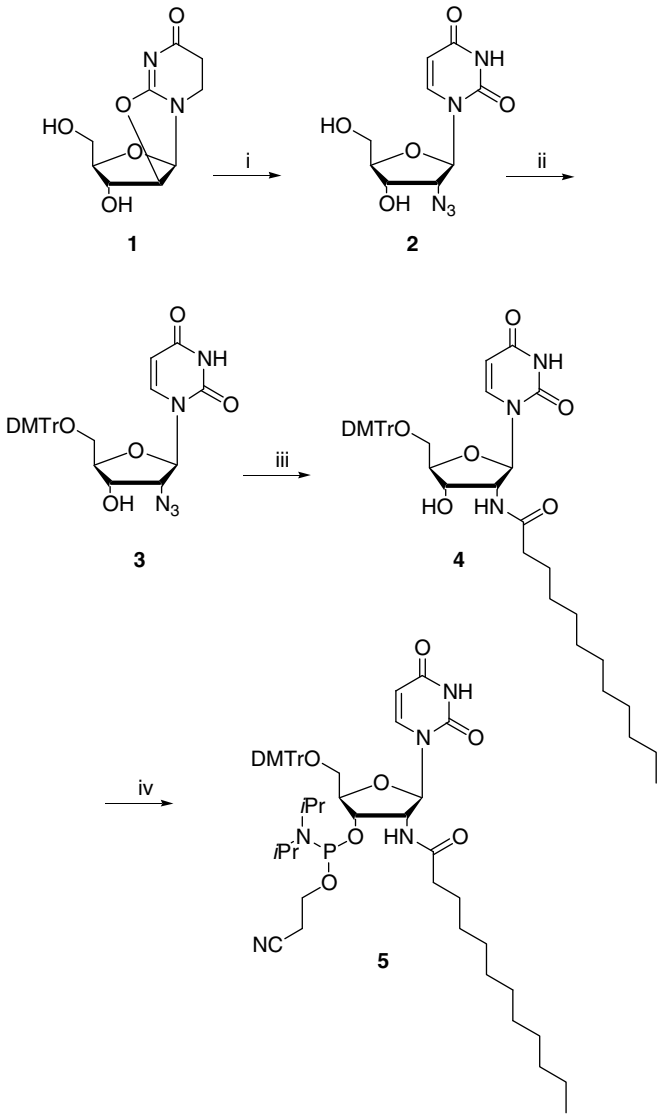

Scheme 1. Reagents and conditions: (i) LiF, TMS–N_3, TMEDA, DMF, 105 °C, 48 h, 89%; (ii) DMTr–Cl, triethylamine, DMAP, Py, rt, o/n; SiO_2, 51%; (iii) HOSu fatty ester of dodecanoic acid 2 equiv, $PnBu_3$ 2 equiv, THF, 0 °C then rt in 30 min, 3 h, CH_2Cl_2/$NaHCO_3$ workup; SiO_2, 90%; (iv) chloro(2-cyanoethoxy)(*N*,*N*-diisopropylamino)phosphine, DIEA, dichloromethane, 1 h, 68% (taking into account the amount of H-phoshonate in our sample).

2′-azido analogue or through the intramolecular cyclization of a trichloroacetamidate.[27] Herein, we report a new synthetic pathway for synthesizing a 2′-amido-2′-deoxyuridine based on a Staudinger–Vilarrasa coupling reaction.[28]

Briefly stated, uridine was reacted with diphenyl carbonate and sodium bicarbonate in DMF to provide cyclic anhydro intermediate **1**. The five-membered ring cycle of the anhydrouridine **1** was then opened in the presence of trimethyl silyl azide (Scheme 1).[29] The 5′ hydroxyl position of the resulting azido derivative **2,** which was first described in 1971 by Moffat and co-workers[30] was reacted with dimethoxytritylchloride in the presence of DMAP to give the key precursor 5′-(dimethoxytrityl)-2′-azido-2′-deoxyuridine **3**.

In order to introduce the lipid part to the ribose 2′ position through an amide linker, a Staudinger-based reaction was performed. The classical Staudinger transformation is a potentially biorthogonal reaction, which occurs between a phosphine and an organic azide. It produces an aza-ylide intermediate that hydrolyzes spontaneously in aqueous conditions to yield the corresponding phosphine oxide and primary amine which can be later N-acylated.[28] The Staudinger–Vilarrasa variant added an activated carboxylic acid to the organic azide and the phosphine, leading in one step to the corresponding carboxamide under very mild conditions.[28]

In this work, lauric acid was used as hydrophobic segment for the carboxamide synthesis. Preliminary to the coupling reaction, the carboxylic acid was converted into its *N*-hydroxysuccinimide ester derivative. The optimized protocol of this coupling step consisted in mixing the activated fatty acid and **3** at 0 °C. The coupling begins with a dropwise addition of freshly distilled $n$$Bu_3P$ to the reaction mixture, and then rising the temperature to ambience within approximately 30 min. Thanks to this quick temperature increase, we did not observe any trace of the acyltriazene byproduct.[31,32] As confirmed by COSY NMR and MS analysis, this reaction provides the expected amide derivative **4** (Scheme 1).

This four-step procedure used readily available starting materials and is an improvement over the N-acylation route to 2′-amido-2′-deoxuridine;[27] also, it is amenable to the preparation of a large number of derivatives. The following step is the synthesis of the requisite 3′-phosphoramidite building block **5** suitable for automated oligonucleotide synthesis.

Compound **5** was further coupled to the ON chain using a classical supported synthesis under standard conditions. The resulting LONs feature either one or two lipid-modified nucleoside combined with 2′OMe nucleoside and phosphodiester backbones. LONs were cleaved from solid support and deprotected after an overnight treatment in 30% aqueous ammonia at 55 °C.

LONs were further purified by using Reverse Phase-HPLC (RP C4) (Table 1). The retention times in HPLC analysis for the unconjugated ON and LONs, respectively, gave an indication about the lipophilic potentials of the compounds (see Supplementary data).

In an effort to realize drug-design around the promising therapeutic antagomir material, we prepared anti-miR-122 oligonucleotides featuring a lipidic part within the sequence. Different positions in the miR-122 complementary oligonucleotide were

Table 1
Characterization of LONs by RP-HPLC elution times and MALDI mass spectrometry

Entry	Oligonucleotide[a] miR-122: (GUUUGUGGUAACAGUGUGAGGU)	Elution time (min)	Mass calculated (Da)[b] $(M–H)^-$	Mass found (Da) MALDI $(M–H)^-$	*Tm* (°C)
ON-1	2′OMe(CAAACACCAUUGUCACACUCCA) control	11.5[c]	7196	7196	61
LON-2	2′OMe(CAAACACCAUU11GUCACACUCCA)	38.0	7359	7369	61
LON-3	2′OMe(CAAACACCAUUGU13CACACUCCA)	32.7	7359	7361	56
LON-4	2′OMe(CAAACACCAUUGUCACAC U19CCA)	37.0	7359	7362	61
LON-5	2′OMe(CAAACACCAUU11GUCACAC U19CCA)	37.3	7527	7530[d]	56

[a] Sequences are written from 5′ to 3′.
[b] Calculations with all the phosphate groups protonated. Accuracy: +/−0.1%.
[c] Conditions used for ON-1 and LONs purification are not the same (see Supplementary data).
[d] Data obtained from ESI MS (see Supplementary data).

investigated (Table 1): u_{11} (**LON-2**), u_{13} (**LON-3**), u_{19} (**LON-4**), or u_{11}/u_{19} (**LON-5**).

To evaluate the effect of LONs on miR-122/anti-miR-122 duplexes, hybridization experiments were performed. Melting temperatures (*T*m) of the duplexes are listed in Table 1. For comparative analysis, Tm of anti-miR-122 lacking the hydrophobic part was also determined. Hybridization of the 2′-conjugated oligonucleotides to their RNA complements, miR-122, reveals, firstly, that large lipid modifications are well tolerated at the 2′ position despite the amido linker. Indeed, substitutions involving nitrogen, like 2′-amino, 2′-carbamate, or 2′-amido push the equilibrium of the ribose from the 3′-*endo* conformation toward a more DNA-like 2′-*endo* (S-type) conformation.[25] Depending on the structural context, these shifts in sugar pucker can destabilize modified helices compared to 2′O-modified ONs, which favor the 3′-*endo* (N-type) conformation that is characteristic of RNA, leading to stabilizing effects on RNA/RNA and DNA/RNA duplexes.

Secondly, it is noteworthy that, depending on its location, a 2′-lipid modification incorporated within the oligonucleotide sequence can be envisioned without destabilizing the anti-miR/miR duplex.

Interestingly, **LON-2** possessing a lipid modified u_{11} has a similar Tm as the unconjugated control indicating that the lipid modification on this position does not destabilize the duplex. This observation is in agreement with antagomir-122 mismatch studies, which revealed that a single mismatch at position 11 did not affect the inhibitory effect on miR-122.[9] Accordingly, it is not surprising that u_{11} lipidic modification does not affect the Tm. In contrast, u_{13} modified oligonucleotide **LON-3** decreases the anti-miR122/miR-122 duplex stability, confirming the fact that although anti-miR can exhibit high sequence specificity, discrimination at the single nucleotide level is position-dependent.

It is also interesting to note that while u_{11} and u_{19} 2′-modified oligonucleotides (**LON-2** and **LON-4**), taken separately, do not affect the stability of anti-miR-122/miR-122 duplex, oligonucleotide **LON-5** featuring simultaneous lipid-modified u_{19} and u_{11} leads to a destabilization of 6 °C. While we do not have any structural data, one possible explanation is that the hydrophobic parts attracted themselves introducing a kink in the helix, which generates steric distortions.

In conclusion, we have synthesized the first example of an antagomir strand featuring a fatty acid in place of a cholesterol moiety. Interestingly, this lipid modification is incorporated internally in the oligonucleotide sequence and not at the 3′-end (up to two modifications within the same strand). Lipid conjugation of the 2′ position was realized via a Staudinger–Vilarrasa reaction. This straightforward procedure was used instead of a standard coupling procedure as a key-step to functionalize our building block with the fatty acid. Melting temperature (*T*m) experiments revealed that the stability of the duplexes depends on the lipid position and the number of lipid moieties inserted within the oligonucleotide sequence. For the targeted miRNA (miR-122) lipid modifications of positions 11 and 19 do not destabilize the duplexes, whereas the concomitant lipid conjugation on the same position leads to decreases of 6 °C of the *T*m. Such materials being likely to be of interest for multiple applications ranging from chemistry to medicine, biological assays are currently in progress.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.tetlet.2008.09.078.

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