Article

S-Acyl-2-Thioethyl: A Convenient Base-Labile Protecting Group for the Synthesis of siRNAs Containing 5'-Vinylphosphonate

Mehran Nikan, Wenyu Li, Garth A. Kinberger, Punit P. Seth, Eric E. Swayze and Thazha P. Prakash *

Ionis Pharmaceuticals Inc., 2855 Gazelle Ct, Carlsbad, CA 92008, USA; mnikan@ionisph.com (M.N.); lwyl99@yahoo.com (W.L.); gkinberg@yahoo.com (G.A.K.); pseth@ionisph.com (P.P.S.); eswayze@ionisph.com (E.E.S.)

*Correspondence: tprakash@ionisph.com; Tel.: +1-760-603-2590

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Abstract: We recently reported that (E)-5'-vinylphosphonate (5'-VP) is a metabolically-stable phosphate mimic for siRNA and demonstrated that 5'-VP improves the potency of the fully modified siRNAs in vivo. Here, we report an alternative synthesis of 5'-VP modified guide strand using S-pivaloyl-2-thioethyl (tBu-SATE) protecting group. The tBu-SATE group is readily removed during the final cleavage of the oligonucleotide from the solid support and providing a more convenient route for the synthesis of siRNA guide strand carrying a 5'-vinylphosphonate.

Keywords: SATE; siRNA; vinylphosphonate

1. Introduction

RNA interference (RNAi) is a powerful tool for gene regulation and modulation of disease causing genes [1,2]. Potential of this technology for developing drugs to treat unmet medical needs is proven with recent approval of ONPATTRO™ (patisiran) for the treatment of hereditary transthyretin-mediated amyloidosis. There are several siRNA drugs progressing in the clinic with positive clinical results [3–5]. It is necessary to develop stability enhanced siRNA to capture the broader potential of the RNAi therapeutics.

Judicious placement of nucleic acid modifications in each strand led to the development of siRNA derivatives with both improved potency and stability [6–8]. The 5'-phosphate of siRNA guide strand makes critical H-bonding interactions within the Mid domain of the Argonaute-2 (Ago2) that ensures proper positioning and accurate slicing of target mRNA [9–11]. The 5'-phosphorylated guide strands are prone to enzymatic cleavage of the terminal phosphate due to the action of phosphatases in vivo [12]. We have previously reported the synthesis of a metabolically stable (E)-5'-vinylphosphonate as a phosphate mimic for guide strand (Figure 1, 5'-VP) and its incorporation into the single and double-stranded siRNAs [13]. These modified strands are stable to phosphatase-mediated cleavage and are 5–10 times more potent than the corresponding siRNAs containing a natural phosphate in mice [14]. The X-ray crystal structure of the siRNAs containing 5'-VP in complex with human Ago2 revealed that it maintains the key interactions with Ago2 proteins like the 5'-phosphate moiety [15]. While our reported synthetic strategy was successful in yielding the desired product, further protecting group optimization was required for convenient synthesis of 5'-VP siRNAs.
were achieved by standard automated DNA synthesis using the phosphoramidite method and could be conveniently removed using standard aqueous ammonia treatment. 

Figure 1. Structure of 5'-phosphate (5'-P), (E)-5'-vinylphosphonate (5'-VP), 5'-deoxy-(E)-5'-vinyl phosphonate-2'-O-(2-methoxyethyl)-thymidine-3'-phosphoramidite bearing methyl 1 and tBu-SATE 2 phosphate esters; proposed mechanism (3 and 4) for aqueous ammonia assisted deprotection of tBu-SATE protecting group.

In our previous synthesis of 5'-VP siRNA and ss-siRNA, we utilized 5'-deoxy-(E)-5'-vinyl (dimethylphosphonate)-2'-O-(2-methoxyethyl)-thymidine-3'-phosphoramidite 1 (Figure 1) for incorporation of VP modification at 5'-end of guide strand. The synthesis of siRNAs and ss-siRNA were achieved by standard automated DNA synthesis using the phosphoramidite 1 (Figure 1) and the phosphonate moiety was protected as dimethyl esters [13]. While the methyl groups provided the desired stability during synthesis, their removal required an additional iodosotrimethylsilane (TMSI) treatment prior to deprotection of oligonucleotides from the solid support [7]. TMSI mediated deprotection of the methyl groups required strictly anhydrous conditions. Thus, this protecting-group strategy is not amenable to automation and scale-up when a large oligonucleotide library synthesis is intended. These considerations prompted us to explore an alternate synthetic strategy where the phosphonate protecting group can be readily removed under basic conditions. One such approach using pivaloxygenymethyl protecting group was reported very recently [16].

The tert-S-pivaloyl-2-thioethyl (tBu-SATE) group has been successfully utilized in the synthesis of nucleotide prodrugs and charge neutral oligonucleotides [17–20]. We envisage that tBu-SATE could be a suitable protecting group for installing the vinylphosphonate during solid phase oligonucleotide synthesis and could be conveniently removed using standard aqueous ammonia deprotection as shown in Figure 1 (3 and 4). Herein, we report the synthesis of the 5'-deoxy-(E)-5'-vinyl-bis(tBu-SATE)phosphonate-2'-O-(2-methoxyethyl)-thymidine-3'-phosphoramidite 2 (Figure 1) and its application to synthesize 5'-VP modified guide strand of siRNA. Using phosphoramidite 2 the synthesis of 5'-VP modified siRNA was accomplished using conventional oligonucleotide synthesis methods and tBu-SATE protecting group was efficiently removed during aqueous ammonia treatment (3 and 4, Figure 1).
2. Results and Discussion

2.1. Synthesis of 5′-deoxy-(E)-5′-vinyl-bis(tBu-SATE)phosphonate)-2′-O-(2-methoxyethyl)-thymidine-3′-phosphoramidite 2

Overall strategy for the synthesis of bis(tBu-SATE) protected phosphoramidite 2 is described in Scheme 1. Synthesis started from a known nucleoside 5, which was synthesized using a reported procedure [13]. First N3 position of the thymine of compound 5 was protected as the benzoyl amide by treatment with benzoyl chloride (BzCl) in presence of N,N-diiisopropylethylamine (DIEA) in dichloromethane to afford 6 (79%). The methyl protecting groups of 6 were then removed quantitatively by treatment with TMSI in dichloromethane to afford 7. The compound 7 was first converted to vinylphosphonic dichloride derivative 8 by reacting with oxalyl chloride in dichloromethane containing 2,6-di-tert-butyl-4-methylpyridine and catalytic amount of DMF. The vinylphosphonic dichloride derivative 8 was then reacted with tBu-SATE alcohol 9 [20] to afford bis(tBu-SATE) derivative 10 in 30% yield over two steps. Compound 10 was then treated with triethylamine trihydrofluoride (TREAT-HF) to yield 11 (68%). Finally, 11 was reacted with 2-cyanoethyl-N,N,N′,N′-tetraisoproylphosphorodiamidite (Phosphitylating reagent) in the presence of 1H-tetrazole and N-methylimidazole in DMF to yield compound 2 (82%).

Scheme 1. Synthesis of 5′-deoxy-(E)-5′-vinyl-bis(tBu-SATE)phosphonate)-2′-O-(2-methoxyethyl)-thymidine-3′-phosphoramidite 2.
Figure 2. (A) siRNA 12/13 targeting murine phosphate and tensin homolog (PTEN) containing an alternating 2′-F/2′-O-Me motif with a (E)-5′-VP-2′-O-MOE T; (B) Solid-phase synthesis of siRNA guide strand 13 using bis(tBu-SATE)-protected phosphoramidite 2.

2.2. Synthesis of Oligonucleotides Using 5′-deoxy-(E)-5′-vinyl-bis(tBu-SATE)phosphonate)-2′-O-(2-methoxyethyl)-thymidine-3′-phosphoramidite 2

We synthesized a guide strand of previously described modified siRNA 12/13 (Figure 2) targeting murine PTEN containing an alternating 2′-F/2′-O-Me motif with a (E)-5′-VP-2′-O-MOE T [12]. We first synthesized precursor guide strand 14 lacking 5′-VP on a DNA synthesizer using UnyLinker solid support and 2′-F, 2′-O-Me and 2′-O-MOE modified nucleoside phosphoramidites (Figure 2) [8]. For incorporation of (E)-5′-VP-2′-O-MOE T residue we used phosphoramidite 2 containing bis(tBu-SATE)-protected 5′-VP (Figure 2) [13]. After the synthesis of guide strand 15 was completed the solid support was treated with 50% triethylamine in acetonitrile containing 1M 2-mercaptoethanol for 30 min to remove the cyanoethyl groups from the phosphate and phosphorothioate linkages. The oligonucleotide was then cleaved and deprotected using aqueous ammonia (28–30 wt%) containing 1M 2-mercaptoethanol at 55 °C for 6 h or at 25 °C for 24 h to yield...
guide strand 13 (Figure 2). 2-Mercaptoethanol was used to scavenge the acrylonitrile and episulfide (Figure 1) generated during the deprotection of cyanoethyl groups and from tBu-SATE protecting groups respectively. The guide strand 13 was then purified by high pressure liquid chromatography (HPLC) and characterized by LCMS analysis (Table 1).

| Table 1. Synthesis scale, deprotection condition and analytical data of guide strand 13 synthesized using tBu-SATE vs. methyl protecting groups. |
|-----------------|------------------|-----------------|------------------|-----------------|------------------|------------------|
| Comp. | Protecting Group | Deprotection Temperature | Mol. Wt. Calculated | Mol. Wt. Found | Scale µmol | %UV Purity |
| 13   | SATE            | 55 °C             | 7300.4             | 7299.3         | 2               | 99.3            |
| 13   | SATE            | 25 °C             | 7300.4             | 7299.1         | 2               | 98.6            |
| 13   | Me              | 55 °C             | 7300.4             | 7299.1         | 2               | 99.1            |

For comparison we also synthesized guide strand 13 using phosphoramidite 1 containing 5′-VP methyl esters [13]. Synthesis was accomplished on a DNA synthesizer according to the reported procedure [13]. Conventional deprotection condition (aqueous ammonia at 55 °C) was not able to hydrolyze both methyl esters of the 5′-VP moiety. A post-synthetic treatment of solid support bearing guide strand 13 with a solution of TMSI in dichloromethane was needed to completely remove methyl groups. In contrast, for the guide strand 13 containing 5′-VP bis(tBu-SATE) esters, conventional aq. ammonia treatment completely removed tBu-SATE esters from the 5′-VP moiety. Thus, tBu-SATE approach simplifies the synthesis of 5′-VP modified oligonucleotides and eliminated the need for moisture sensitive and inconvenient TMSI treatment. The overall isolated yield obtained for the synthesis of guide strand 13 using phosphoramidites containing methyl or tBu-SATE esters were similar. Our results suggest that tBu-SATE is a convenient protecting group for the solid-phase synthesis of 5′-VP modified oligonucleotides.

3. Materials and Methods

General

All reactions were carried out under an argon atmosphere using anhydrous solvents unless otherwise stated. Anhydrous solvents and reagents were purchased from commercial sources and used without further purification. Chromatography was performed on Biotage SP1 system equipped with UV detector and SNAP prepacked columns (Biotage LLC, Charlotte, NC, USA). Analytical thin-layer chromatography (TLC) was performed using silica gel 60 F254 (Merck KGaA, Darmstadt, Germany) using UV lamp (UVP, Upland, CA, USA) a visualizing agent and developed by a solution of p-anisaldehyde (6 mL), H2SO4 (8.3 mL), CH3COOH (2.5 mL) in C2H5OH (227 mL) followed by charring. 1H-NMR spectra were recorded on a Bruker 300 MHz instrument (Billerica, MA, USA) using residual solvent as reference. 31P-NMR spectra are not calibrated and δ is reported relative to 85% phosphoric acid. Yields refer to chromatographically isolated yields. Compound 1 was synthesized using the reported procedure [13].

Compound 2: To a solution of 11 (0.51 g, 0.651 mmol) in DMF (2.0 mL) were added 1H-tetrazole (36 mg, 0.521 mmol), 1-methylimidazole (13 mg, 0.163 mmol), followed by 2-cyanoethyl N,N,N′,N′-tetraisopropylphosphordiamidite (0.31 mL, 0.977 mmol). The reaction was complete after 2 h as determined by TLC using 70% ethyl acetate/hexanes as the eluent. The reaction mixture was diluted with saturated NaHCO3 and partitioned against ethyl acetate. The aqueous layer was then extracted with ethyl acetate. The ethyl acetate layers were combined, dried over Na2SO4, filtered and concentrated. The resulting crude compound was purified by silica gel column chromatography and eluted with 70% ethyl acetate/hexanes to yield 2 (0.5 g, 82%). 31P-NMR (121 MHz, CDCl3): δ 150.54–150.09 (d, 1P), 18.07–17.32 (d, 1P); LR MS (ESI) calcd for C44H66N4O13P2S2 [M − H]− m/z = 983.3, found 982.1.
Compound 6: To a solution of 5 (5.0 g, 7.72 mmol) in CH₂Cl₂ (35 mL) were added DIEA (2.69 mL, 15.43 mmol) and benzoylchloride (1.12 mL, 9.65 mmol) and stirred at room temperature overnight. Reaction mixture was concentrated by rotary evaporation under reduced pressure and the residue was purified by silica gel column chromatography by a gradient of hexaneseethyl acetate to ethyl acetate and filtered and concentrated. The reaction mixture was dissolved in CH₂Cl₂ (4.56 g, 20.48 mmol) and TMSI (1.28 mL, 12.53 mmol) and stirred at room temperature. Reaction was completed after 30 min as determined by LC-MS (6130 Quadruple LC/MS, Agilent Technologies, Santa Clara, CA, USA). The reaction mixture was concentrated under reduced pressure and residue dissolved in CH₂Cl₂ and filtered and concentrated. The crude product was purified by silica gel column chromatography (ethyl acetate to 50% acetone/CH₂Cl₂) to afford 6 (3.32 g, 31%). P-NMR (121 MHz, CDCl₃): δ 7.84–7.95 (m, 2H), 7.59–7.77 (m, 4H), 7.33–7.55 (m, 9H), 6.96 (s, 1H), 6.57–6.78 (m, 1H), 5.80–6.01 (m, 2H), 4.65 (t, 1H), 3.93–4.16 (m, 5H), 3.31–3.65 (m, 5H), 3.29 (s, 3H), 1.84 (s, 3H), 1.09 (s, 9H); 31P-NMR (121 MHz, CDCl₃): δ 10.2; LR MS (ESI) calcd for C₃₅H₄₀N₂O₁₂PS₂Si [M - H]⁻ m/z = 735.2, found 735.1.

Compound 7: To a solution of 6 (3.82 g, 20.48 mmol) in CH₂Cl₂ (15 mL) were added pyridine (1.67 mL, 20.00 mmol) and TMSI (1.28 mL, 12.53 mmol) and stirred at room temperature. Reaction was completed after 30 min as determined by LC-MS (6130 Quadruple LC/MS, Agilent Technologies, Santa Clara, CA, USA). The reaction mixture was concentrated under reduced pressure and residue dissolved in CH₂Cl₂ and filtered and concentrated. The crude product was purified by silica gel column chromatography (ethyl acetate to 50% acetone/CH₂Cl₂) to afford 7 (3.5 g, 31%). P-NMR (121 MHz, CDCl₃): δ 7.81–7.94 (m, 2H), 7.56–7.77 (m, 4H), 7.30–7.53 (m, 9H), 6.97 (s, 1H), 6.49–6.72 (m, 1H), 5.89–6.01 (m, 2H), 4.62 (t, 1H), 4.01 (t, 1H), 3.32–3.61 (m, 5H), 3.28 (s, 3H), 1.84 (s, 3H), 1.09 (s, 9H); 31P-NMR (121 MHz, CDCl₃): δ 10.2; LR MS (ESI) calcd for C₃₅H₄₀N₂O₁₂PS₂Si [M + H]⁺ m/z = 735.2, found 735.1.

Compound 10: To a solution of 7 (2.5 g, 31 mmol) in CH₂Cl₂ (8.4 mL) were added 2,6-di-tert-butyl-4-methylpyridine (4.20 g, 20.48 mmol), oxalyl chloride (0.78 g, 6.15 mmol) and DMSO-D₅-[M + H]⁺ m/z = 783.3.}

Compound 11: To a solution of 10 (1.0 g, 0.98 mmol) in THF (3.0 mL) was added TREAT-HF (1.2 mL, 7.34 mmol) and stirred at room temperature overnight. The reaction mixture was concentrated under reduced pressure and residue obtained was purified by silica gel column chromatography using a gradient of ethyl acetate to 50% acetone/CH₂Cl₂ to afford 11 (0.52 g, 68%). 1H-NMR (300 MHz, DMSO-d₆): δ 7.92–8.04 (m, 2H), 7.75–7.86 (m, 1H), 7.70 (s, 1H), 7.56–7.66 (m, 2H), 6.73–6.95 (m, 1H), 6.00–6.20 (m, 1H), 5.85 (d, 1H), 5.45 (d, 1H), 4.40 (t, 1H), 4.12–4.26 (m, 2H), 3.93–4.10 (m, 4H), 3.60–3.82 (m, 2H), 3.48 (m, 2H), 3.24 (s, 3H), 3.11 (m, 4H), 1.89 (s, 3H), 1.19 (s, 18H); 31P-NMR (121 MHz, DMSO-d₆): δ 22.24; LR MS (ESI) calcd for C₃₅H₄₀N₂O₁₂PS₂Si [M - H]⁻ m/z = 783.2, found 783.3.

Compound 13: The synthesis of modified oligonucleotides was performed on an ABI 394 synthesizer (Biolytic Lab Performance Inc., Fremont, CA, USA) on a 2 µmol scale. A 0.1 M solution of 2′-F, 2′-O-Me, and 2′-O-MOE, modified nucleoside phosphoramidites in anhydrous acetonitrile (CH₃CN) and were used for the synthesis. Phosphoramidite 2 was dissolved in 30% dichloromethane in anhydrous CH₃CN (0.1 M) and used for the oligonucleotide synthesis. The modified oligonucleotides were synthesized on VIMAD UnyLinker™ (AM Chemicals, Oceanside, CA, USA) solid support. Dichloroacetic acid (6%) in toluene was used as detritylating reagent. 4,5-Dicyanoimidazole in the presence of N-methylimidazole in CH₃CN was used as activator during the coupling step. During the coupling step, nine equivalents of phosphoramidite solutions were delivered and the coupling was carried out for 10 min. Phosphorothioate linkages were introduced by sulfurization with a 0.05 M solution of DDTT (3-(dimethylamino-methylidene)amino)-3H-1,2,4-dithiazole-3-thione) in 1:1 pyridine/CH₃CN for a contact time of 3 min. Phosphate diester linkages were incorporated
via oxidation of phosphate triesters using a solution of tert-butyl hydroperoxide/CH$_3$CN/water (10:87:3) for a contact time of 12 min. All other steps in the protocol supplied by the manufacturer were used without modification. After the desired sequence was assembled, the solid-support bound oligonucleotide was washed with 1:1 TEA/CH$_3$CN for 30 min. All other steps in the protocol supplied by the manufacturer were used without modification. After the desired sequence was assembled, the solid-support bound oligonucleotide was washed with 1:1 TEA/CH$_3$CN for 30 min. The solid-support bound oligonucleotide was suspended in aqueous ammonia (28–30 wt%) containing 1M 2-mercaptoethanol (used 1 mL/µmol of solid support) and either heated at 55 °C (13, Table 1) for 6 h or kept at room temperature for 24 h (13, Table 1) to complete cleavage from support. The unbound oligonucleotide was then filtered and the support was rinsed and filtered with water:ethanol (1:1) followed by water. The filtrate was combined and concentrated to dryness. The residue obtained was purified by HPLC (Gilson Inc., Middleton, WI, USA) on a reverse phase column (Waters X-Bridge C-18 5 µm, 10 × 250 mm, A = 5 mM tributylammonium acetate in 5% aqueous CH$_3$CN, B = CH$_3$CN, 0 to 90% B in 80 min, flow 3 mL min$^{-1}$, λ = 260 nm). Fractions containing full-length oligonucleotides were pooled together (assessed by LC/MS analysis > 95%) and the tributylammonium counter ion was exchanged to sodium by HPLC on a strong anion exchange column (GE Healthcare Bioscience, Pittsburgh, PA, USA) Source 30Q, 30 µm, 2.54 × 8 cm, A = 100 mM ammonium acetate in 30% aqueous CH$_3$CN, B = 1.5 M NaBr in A, 0–40% of B in 60 min, flow 14 mL min$^{-1}$). The residue was desalted by HPLC on reverse phase column to yield the oligonucleotide in an isolated yield of 15–20% based on solid-support loading. The oligonucleotides were characterized by ion-pair-HPLC-MS analysis with Agilent 1100 MSD system (Agilent Technologies, Santa Clara, CA, USA).

4. Conclusions

In this study, we designed and synthesized 5′-deoxy-(E)-5′-vinyl-phosphonate)-2′-O-(2-methoxyethyl)-thymidine-3′-phosphoramidite with a base-labile tBu-SATE protected vinylphosphonate in good yield. To examine the compatibility of the tBu-SATE-protecting group under solid-phase oligonucleotide synthesis conditions, we synthesized a guide strand of fully modified siRNA containing (E)-5′-vinylphosphonate using this phosphoramidite. The tBu-SATE-protected phosphoramidite was successfully incorporated at the 5′-terminus of a 2′-F/2′-O-Me guide strand of siRNA using solid-phase DNA synthesis reagents and methods. Moreover, the tBu-SATE group was conveniently removed under aqueous ammonia mediated deprotection conditions. The yield and purity of resulting oligonucleotides were identical to the one obtained using previously reported phosphoramidite containing vinylphosphonate methyl esters [13]. The strategy described in this report simplify the synthesis and deprotection of the siRNAs containing a metabolically stable (E)-5′-vinylphosphonate and eliminates the need for any additional steps or reagents.

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Sample Availability: Samples of the compounds 1, 5 and 13 are available from the authors.

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