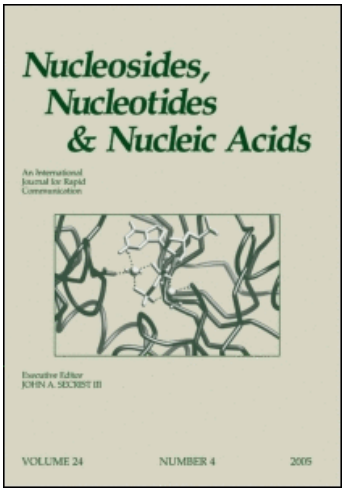

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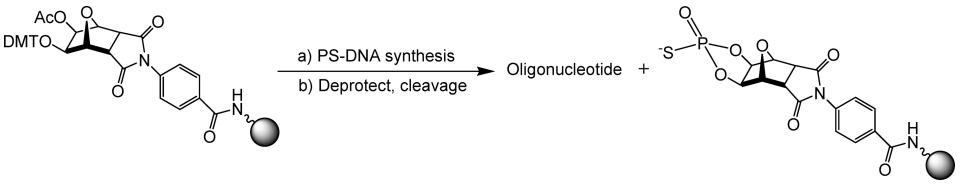
A NOVEL UNIVERSAL LINKER FOR EFFICIENT SYNTHESIS OF PHOSPHOROTHIOATE OLIGONUCLEOTIDES

Zhiwei Wang, Phil Olsen, and Vasulinga T. Ravikumar □ *Isis Pharmaceuticals, Carlsbad, California, USA*

□ *A versatile and conformationally preorganized universal linker molecule is reported here for efficient synthesis of phosphorothioate oligonucleotides. With respect to nucleoside loaded support, comparable yield and quality based on ion-pair LC-MS are obtained for both deoxy and 2′-O-methoxyethyl modified phosphorothioate oligonucleotides. No 3′-phosphate or phosphorothioate monoester or any modification of universal molecule still attached to oligonucleotide was observed.*



Keywords Oligonucleotides; universal linker; solid support; phosphorothioate; antisense

INTRODUCTION

Currently syntheses of DNA and RNA oligonucleotides and their various analogs are routinely performed on automated synthesizers designed by various commercial manufacturers. These syntheses scale range from nanomolar to almost up to a mole. All these syntheses have one starting material in common, that is, they use a solid support derivatized with a nucleoside corresponding to 3′-end of oligonucleotide attached through a cleavable linker such as succinyl or oxalyl group.[1–3] This necessitates making of all these various solid supports and their corresponding nucleoside linker derivatives. In addition, 192- or 384-well plate synthesizers are capable of synthesizing multiple oligonucleotides at a time. This requires placing of

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appropriate solid support in the well and enough chances exist for human error to cause failure of potentially the complete plate. For synthesis of novel oligonucleotides, a continuously growing number of supports carrying other modified nucleosides and 3′-terminal modifiers are required. Thus, a universal linker molecule with no specified nucleoside attached to a solid support would solve these problems. Several universal linker molecules have been reported in literature.[4–20] Most of them have some limitation or other (e.g., addition of a salt, heating at high temperatures, expensive scale up of the linker molecule, affording low yield, or containing traces of 3′-modification). Thus, there is a real need to design one that is efficient, leaves no traces of 3′-modification (as judged by LC-MS), and yields are comparable to standard chemistry.

RESULTS AND DISCUSSION

Synthesis of Universal Linker and Loading to Solid Support

In our earlier communication,[21,22] we proposed a novel universal linker molecule **1** for efficient synthesis of high quality oligonucleotides (Figure 1). By design this molecule contains a cyclic anhydride and on loading to an amino-derivatized solid support such as CPG or GE's PS200 Primer Support undergoes ring opening to liberate a free carboxyl group, which is subsequently capped by reacting with *n*-propyl amine in presence of HBTU. A further capping of hydroxyl group with acetic anhydride is done to ensure potential loss of acetate group during reaction with *n*-propylamine. This procedure involves multiple steps and may not be economical for scale up. Being an anhydride, it is difficult to quantify the purity by high performance liquid chromatography (HPLC) as it undergoes hydrolysis under the analytical conditions. Thus, it would be desirable if the two carboxyl groups are masked in the form of a stable cyclic imide and still possessed a carboxyl group for attachment to solid support. Such a concept was incorporated in design of universal solid support **2** (Figure 2).

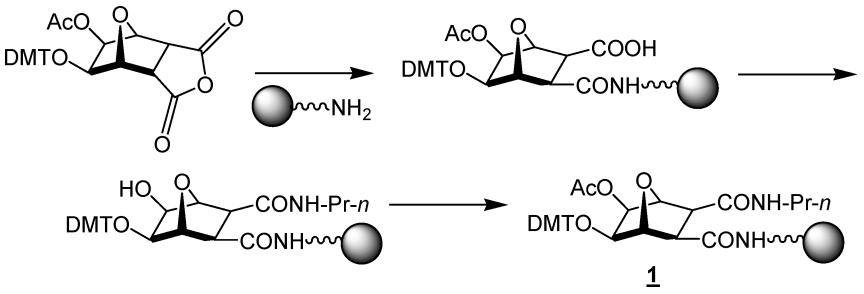


FIGURE 1 Structure of earlier reported universal solid support **1**.

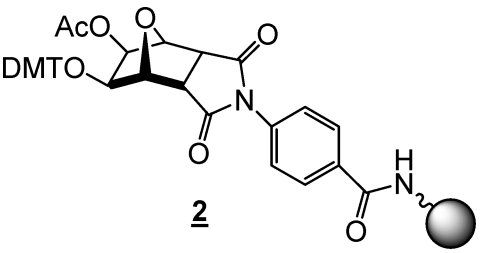
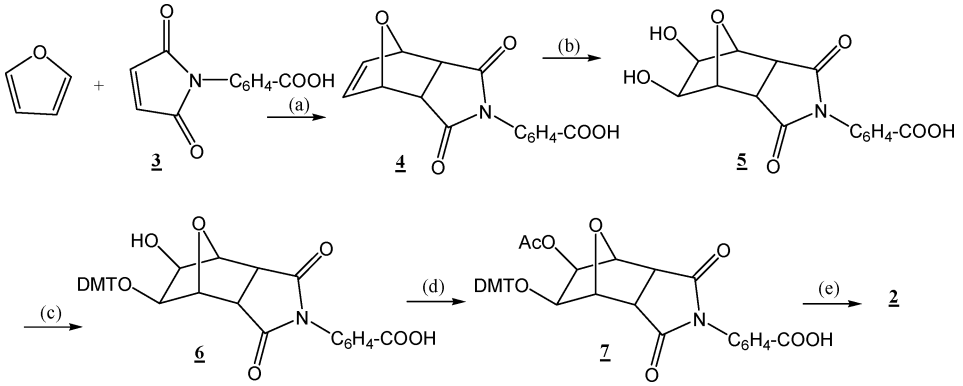


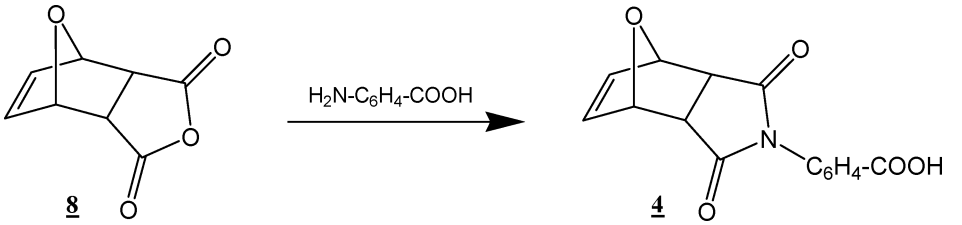
FIGURE 2 Structure of universal solid support **2**.

Synthesis and loading of universal linker molecule **2** is shown in Scheme **1**. The synthesis starts with Diels-Alder reaction of furan and *N*-(4-carboxy)phenyl maleimide **3** to afford the adduct **4** as a colorless solid in 83% yield. Osmium-catalyzed bis-hydroxylation of olefin **4** was carried out similar to literature procedure to afford diol **5** in 79% isolated yield.[23] Chemoselective protection of diol **5** with 4,4′-dimethoxytrityl chloride in pyridine followed by treatment with acetic anhydride without isolation of the intermediate gave the acetoxy protected compound **7** as colorless amorphous compound in 74% yield. Loading of universal linker molecule **7** to amino-derivatized polymeric solid support was carried our using HBTU as coupling activator to give the appropriate loading (200 μmol/g). The excess sites on solid support were capped using acetic anhydride to give the final product **2.**

An alternative approach to synthesize **4** by reacting exo-oxohimic acid **8** with 4-aminobenzoic acid afforded unacceptable yield of the product (Scheme **2**).[24–26]



SCHEME 1 Synthesis of universal linker loaded support: (a) CH_3CN, reflux, 10 hours; (b) $OsO_4/H_2O_2/H_2O$/acetone/t-BuOH; 35°C, 24 hours; (c) DMT chloride, pyridine, room temperature, 20 hours; (d) Acetic anhydride, pyridine, room temperature, 12 hours, (e) HBTU, CH_3CN, Hunig's base, amino-derivatized support.



SCHEME 2 Attempted synthesis of compound 4.

Phosphorothioate Oligonucleotide Synthesis

To demonstrate this methodology, a 20-mer phosphorothioate oligodeoxyribonucleotide [PS-[GCTGA]-(TTAGAGAGAG)-[GTCCC], ISIS 104838 targeted against tumor necrosis factor-α was chosen as an example. All syntheses were performed on an Amersham Biosciences Akta OligoPilot 100 DNA/RNA synthesizer at approximately 160 μmole scale in a 6.33 mL fixed column using β-cyanoethyl phosphoramidite synthons (1.75 equivalents, 0.2M in CH_3CN). Details of synthesis cycle are given in Table 1. At end of each synthesis, the support was thoroughly dried to determine the crude weight yield[27] and then treated with a solution of triethylamine:CH_3CN (1:1, v/v) at room temperature for 2 hours to remove the β-cyanoethyl protecting groups,[28,29] then treated with 30% aqueous ammonium hydroxide solution for 12 hours at 55°C to effect release from support and base deprotection. Yield (expressed in mg of oligonucleotide/μmole of support)[27] and analytical RP-HPLC (full length determination) data were collected for each synthesis. In addition, the crude material obtained from each synthesis was purified by C_{18} reversed phase HPLC, the final DMT removed and then analyzed by ion-pair liquid chromatography electrospray mass spectrometry (IP-LC-MS). Four syntheses were carried out (standard nucleoside containing succinate linked support as control and universal linker attached support in duplicates). The results are summarized in Table 2 and representative analysis shown in Figure 3.

Mechanism of Release of Oligonucleotide from Solid Support

A reasonable mechanism for release of oligonucleotide is depicted in Scheme 3. At the end of oligonucleotide synthesis, treatment of the

TABLE 1 Synthesis parameters of cycle used on Amersham's Akta OligoPilot 100 synthesizer

Step	Reagent	Volume (ml)	Time (min)
Detritylation	10% dichloroacetic acid/toluene	**72**	**1.5**
Coupling	Phosphoramidite (0.2M), 1 *H*-tetrazole (0.45 m) in acetonitrile	**10, 15**	**5**
Sulfurization	PADS (0.2M) in 3-picoline-CH_3CN (1:1, v/v)	**36**	**3**
Capping	Ac_2 O/pyridine/CH_3CN, NMI/CH_3CN	**24, 24**	**2**

TABLE 2 Comparison of oligonucleotides synthesized using standard succinate (996–73 and 996–80) and universal linker support (996–75 and 996–79). Depurinated species include n-G, n-A/n-G+H_2 O, n-A+H_2 O, 3′-TPT

Support used	Crude yield mg/μmole	Crude yield OD/μmole	Purified full length (%) (IP-LC-MS)	(Depurinated species) (%) (IP-LC-MS)	n-1	Full length + PO
MOE-meC succinyl linker (996–73)	7.3	113	91	1.7	1.3	96
Universal linker **2** (996–75)	7.3	102	92	1.3	2.6	94
MOE-meC succinyl linker (996–80)	7.5	109	90	1.7	1.0	97
Universal linker **2** (996–79)	7.4	107	92	1.5	2.6	95

support with triethylamine:acetonitrile removes the cyanoethyl protecting group and generates the phosphorothioate diester charged backbone. Subsequent treatment with ammonium hydroxide liberates the vicinal hydroxyl masked by acetate group which undergoes an intramolecular attack on the

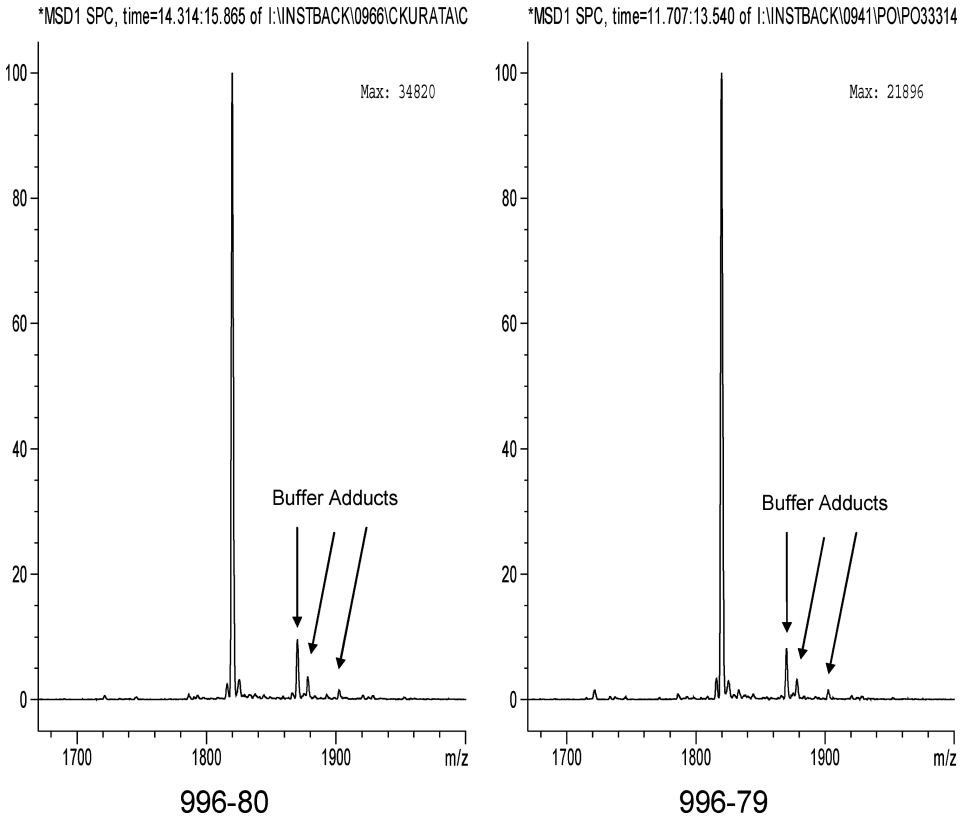
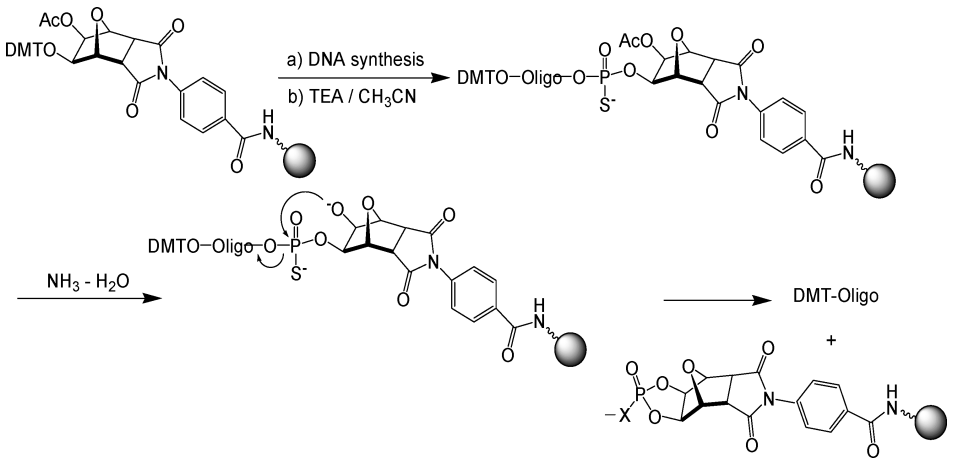


FIGURE 3 IP-LC-MS of purified 20-mer phosphorothioate oligonucleotide using standard succinyl linker support (996–80) and using universal linker support (996–79).



SCHEME 3 Mechanism for release of oligonucleotide from solid support.

phosphorothioate to liberate the oligonucleotide. A cyclic five-membered molecule is formed as a by-product.

EXPERIMENTAL

N-(4-Carboxy)phenyl-tetrahydro-4,7-epoxyisobenzopyrrole-1,3-dione (4): A three-necked, round-bottomed flask (2 L) equipped with a magnetic stirring bar, a heating mantle, a reflux condenser, and a dropping funnel, was charged with a solution of *N*-(4-carboxy)phenylmaleimide (**3**) (217 g; 1.0 mol) in acetonitrile (700 mL). Following the addition of furan (100 mL), the stirred solution was heated at reflux for 5 hours. Then the reaction mixture was cooled to room temperature when colorless solid precipitates out. The material was filtered, washed with acetonitrile (0.5 L). The filtrate solution was concentrated to afford more of product which also was filtered and washed with acetonitrile (0.3 L). The combined solid portions was dried under high vacuum at room temperature overnight to afford 237 g (83%) of desired product. ^{1} H NMR (DMSO-d_6, 300 MHz) δ: 3.02 (s, 2H), 5.24 (s, 2H), 6.64 (s, 2H), 7.32 d, 2H), 8.08 (d, 2H). ^{13} C NMR (DMSO-d_6, 75.5 MHz) δ: 47.55, 80.83, 126.69, 129.95, 130.51, 135.74, 136.60, 166.57, 175.37. MS (ESI, m/z): 284.1.

N-(4-Carboxy)phenyl-5,6-dihydroxy-hexahydro-4,7-epoxy-isobenzopyrrole-1,3-dione (5): The content of a 1 g sealed vial of osmium tetroxide was dissolved in purified *tert*-butyl alcohol (0.2 L). The pale green solution was treated with 3–5 drops of 30% hydrogen peroxide and allowed to remain at room temperature for 1 day. If the solution became dark, the dropwise addition of 30% hydrogen peroxide was repeated until the pale green color persisted.

This solution is stable for at least 1 year at room temperature. Each mL contains 2×10^{-5} mole of osmium tetroxide.

A 5-L three-necked flask fitted with a mechanical stirrer, reflux condenser with ice-water cooling, and a heating mantle was charged with a solution of olefin (**4**) (0.285 kg, 1 mol) in acetone (2.5 L). Thirty percent hydrogen peroxide solution (0.5 L) was added followed by osmium tetroxide solution prepared earlier (0.18 L). (Warning: For larger scales the reaction could be exothermic.) Slow addition (1–2 hours) of osmium tetroxide solution is recommended. Gentle refluxing of reaction mixture with stirring was maintained for 7–8 hours. During this period, reaction color changed from brown to pale brown to colorless and solid started crashing out. Vigorous stirring was maintained throughout the period. TLC indicated disappearance of starting material. The reaction mixture was cooled to room temperature and the precipitated solid filtered. The solid was washed with ether (2 L) and dried in vacuum oven at room temperature overnight. The filtrate solution was concentrated and ether (1 L) was added when additional solid precipitated out which was filtered and washed with ether (0.3 L). The combined solid product was dried in an oven at 45°C for 2 days to afford a total of 252 g (79%) of colorless product (**5**). ^{1} H NMR (DMSO-d_6, 300 MHz) δ: 2.96 (s, 2H), 3.75 (s, 2H), 4.20 (s, 2H), 7–17 (d, 2H), 7.84 (d, 2H), 12.92 (broad, 1H). ^{13} C NMR (DMSO-d_6, 75.5 MHz) δ: 45.58, 71.77, 84.11, 126.64, 129.92, 130.51, 135.83, 166.55, 175.91. MS (ESI, m/z): 318.

N-(4-Carboxy)phenyl-5-(4,4′-dimethoxytriphenylmethyloxy)-6-acetoxy-hexahydro-4, 7-epoxyisobenzopyrrole-1,3-dione (7): The dihydroxy compound (**5**) (31.9 g; 0.1 mol) was taken in a 1-L round-bottomed flask and coevaporated with anhydrous pyridine (0.2 L). This step was repeated one more time to render the material anhydrous. Pyridine (0.3 L) was added and stirred using magnetic stirrer at room temperature. 4,4′-dimethoxytrityl chloride (50 g, 0.15 mol; 1.5 equivalents) was slowly added as solid over a period of 2 hours. Solution was stirred overnight. TLC indicated disappearance of starting material. To this acetic anhydride (20 mL) was added and stirred for 7 hours. All volatiles were removed and extracted with ethyl acetate (1 L) and the extract washed with water, brine, and concentrated. The remaining crude material was purified by flash silica gel chromatography using hexane-ethyl acetate. One percent triethylamine was used through out purification to afford 0.49 kg (74%) of product (**7**). ^{1}H NMR ($CDCl_3$, 300 MHz) δ: 2.02 (s, 3H), 2.78 (s, 1H), 3.03 (d, 1H), 3.55 (s, 6H), 3.91 (d, 1H), 4.41 (s, 1H), 5.05 (d, 1H), 6.72–7.26 (m, 15H), 7.77 (d, 2H). ^{13}C NMR (DMSO-d_6, 75.5 MHz) δ: 20.90, 44.94, 45.92, 55.05, 74.37, 75.30, 81.72, 87.17, 113.62, 125.93, 127.50, 129.55, 129.64, 135.40, 135.90, 136.55, 145.10, 158.51, 168.35, 170.14, 174.92, 175.39. MS (ESI, m/z): 662.3.

Loading of universal linker (7) to solid support: To a 500-mL round-bottom flask was successively added amino-functionalized polystyrene solid support

(50 g), (7.28 g, 11 mmol), HBTU (4.17 g, 11 mmol), Hunig's base (5.75 mL, 33 mmol) and anhydrous acetonitrile (0.3 L). The resulting mixture was stirred for 18 hours at room temperature. An aliquot sample was taken, filtered, washed with acetonitrile, dried, and loading determined by UV. It was found to be 199 μmol/g. The reaction mixture was filtered, washed with acetonitrile (150 mL × 5), and dried. Capping: To a 1-L three-necked round-bottom flask was successively added loaded support, DMAP (1 g), CAP A (0.2 L) and CAP B (0.2 L). The resulting mixture was stirred for 16 hours at room temperature. Then it was filtered, washed with acetonitrile (150 mL × 5), and thoroughly dried (52 g). Final loading determined by UV was found to be 198 μmol/g.

Oligonucleotide Synthesis

Oligonucleotide syntheses were performed on a GE Amersham's Akta 100 DNA/RNA synthesizer by the phosphoramidite coupling method. Appropriate amount of solid supports were packed in the column for synthesis. Ten percent dichloroacetic acid in toluene was used for deblocking of dimethoxytrityl (DMT) groups from 5′-hydroxyl group of the nucleotide.[30,31] Extended detritylation condition (twice the column volume and contact time as the normal cycle) was used to remove the DMT group from the secondary hydroxyl group of the universal linker molecule. 4,5-Dicyanoimidazole (0.7 M) in presence of *N*-methylimidazole (0.1 M) or 1 *H*-tetrazole (0.45 M) in acetonitrile was used as activator during coupling step.[32,33] 1.75 equivalents of amidites (both deoxy and 2′-*O*-methoxyethylribonucleosides) and a ratio of 1:1 (v/v) of amidite to activator solution was used during coupling step. Amidite and activator solutions were prepared using low-water CH_3CN (water content <30 ppm) and were dried further by addition of activated 4 Å molecular sieves (∼50 g/l).[34] Phosphorothioate linkages were introduced by sulfurization with a 0.2 M solution of phenylacetyl disulfide in acetonitrile in 3-picoline or pyridine (1:1 v/v) for a contact time of 2–3 minutes.[35–37] Phosphate diester linkages were incorporated via oxidation of phosphite triesters using a solution of iodine/tetrahydrofuran/pyridine/water for 2 minutes. Capping reagents were made to the recommended Amersham Biosciences receipe: Cap A: *N*-methylimidazole-CH_3CN (1:4 v/v), Cap B: acetic anhydride-pyridine-CH_3CN (2:3:5, v/v/v). Details of synthesis cycle are given in Table 1.

At the end of synthesis, the support-bound DMT-on oligonucleotide was treated with a solution of triethylamine and acetonitrile (1:1, v/v) for 2 hours to remove acrylonitrile formed by deprotection of cyanoethyl group from phosphorothioate triester.[13] Subsequently, the solid support containing oligonucleotide was incubated with concentrated aqueous ammonium hydroxide at 55°C for 13 hours to complete cleavage from support,

elimination of UnyLinker molecule to liberate 3′-hydroxy group of oligonucleotide and deprotection of base-protecting groups.

HPLC Analysis and Purification of Oligonucleotides

Analysis and purification of oligonucleotides by reversed phase high performance liquid chromatography (RP-HPLC) was performed on a Waters Novapak C_{18} column (3.9 × 300 mm) using a Waters HPLC system (600E System Controller, 996 Photodiode Array Detector, 717 Autosampler, Foster City, CA, USA). For analysis an acetonitrile (A)/0.1M triethylammonium acetate gradient was used: 5–35% A from 0–10 minutes, then 35–40% A from 10–20 minutes, then 40–95% A from 20–25 minutes, flow rate = 1.0 mL/min/50% A from 8–9 minutes, 9–26 minutes at 50% flow rate = 1.0 mL/min, t_R(DMT-off) 10–11 minutes, t_R(DMT-on) 14–16 minutes. The DMT-on fraction was collected and was evaporated in vacuum, redissolved in water, and the DMT group was removed as described below.

Dedimethoxytritylation

An aliquot (30 μl) was transferred into an Eppendorff tube (1.5 ml), and acetic acid (50%, 30 μl) was added. After 30 minutes at room temperature sodium acetate (2.5M, 20 μl) was added, followed by cold ethanol (1.2 ml). The mixture was vortexed and cooled in dry ice for 20 minutes. The precipitate was spun down with a centrifuge, the supernatant was discarded and the precipitate was rinsed with ethanol and dried under vacuum.

CONCLUSION

A novel, conformationally preorganized nonnucleosidic universal solid support for oligonucleotide synthesis has been developed. The solid support featured two chemically equivalent hydroxyl groups locked in *syn*-periplanar orientation and orthogonally protected with 4,4′-dimethoxytrityl and acetyl groups. The solid support was tested in preparation of multiple phosphorothioate oligonucleotides of 18–20-mer in length containing 2′-deoxy and 2′-*O*-methoxyethylnucleoside residues at the 3′-terminus. Upon completion of chain assembly, the support-bound oligonucleotide material was treated with concentrated ammonium hydroxide, which removed the *O*-acetyl protection. The deprotected hydroxyl group then effected the transesterification of a phosphorothioate linkage between the solid support and the 3′-terminal nucleoside residue to result in a facile release of the oligonucleotide to the aqueous solution.

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