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UnyLinker: An Efficient and Scaleable Synthesis of Oligonucleotides Utilizing a Universal Linker Molecule: A Novel Approach To Enhance the Purity of Drugs

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Abstract:

A novel universal linker (UnyLinker) molecule which has a conformationally rigid and chemically stable bridge head ring oxygen atom carrying a conventional 4,4′-dimethoxytrityl (DMT) and succinyl groups locked in a syn orientation has been developed to carry out oligonucleotide synthesis efficiently and smoothly. The geometry of the vicinal syn oxygen functionalized group allows fast and clean cleavage under standard aqueous ammonia deprotection conditions to afford high-quality oligonucleotides. No base modification is observed, based on the ion-pair HPLC−UV−MS (IP-HPLC−UV−MS) method with detection limit of <0.1%. A class of impurities formed by branching from the exocyclic amino group of nucleosides loaded onto a solid support has been eliminated by the use of this method. Examples demonstrating the versatile nature of this molecule are shown by syntheses of different chemistries such as 2′-deoxy, 2′-*O*-methyl, 2′-*O*-methoxyethyl, Lock nucleic acids (LNA), 2′-α-fluoro nucleic acids (FANA), conjugates such as 5′-phosphate monoester and biotin, and phosphate diester and phosphorothioate backbone modifications. This molecule was loaded onto several commercial solid supports and used in both gas-sparged and packed-bed automated DNA/RNA synthesizers. Large-scale syntheses (up to 700 mmol) of multiple phosphorothioate first- and second-generation antisense drugs on GE-Amersham's OligoProcess synthesizer are demonstrated further, showing that this chemistry could be used for efficient synthesis of multiple oligonucleotide drugs using a single raw material, thereby eliminating a difficult to characterize nucleoside-loaded polymer matrix used as a starting material. A mechanism for deprotection and cleavage of the linker molecule to liberate the free oligonucleotide is proposed. Characterization of the cyclic byproduct formed during release of the oligonucleotide is presented. The exo-syn configuration of the dihydroxy structure of the UnyLinker molecule is conclusively established by X-ray crystallography studies. A novel method to remove the last traces of osmium used during the synthesis of the UnyLinker molecule to reach undetectable levels (<1 ppm) is also described.

Introduction

Oligonucleotide-based drugs (antisense, aptamer, DNA decoys, CpG-based immunomodulatory drugs, siRNA, etc.) are advancing rapidly in the clinic, and two drugs have already been approved by the Food and Drug Administration and other international regulatory agencies.[1,2] Many more drugs are in phase II trials, and few are in potentially pivotal phase III trials.[3] These drugs are currently synthesized using solid-phase chemistry via the phosphoramidite approach[4] where a solid support containing the first nucleoside attached through a cleavable linker is used as the polymer-matrix and β-cyanoethyl protected phosphoramidites of various nucleosides are used as the building blocks. The synthesis is performed in an automated DNA/RNA synthesizer using various commercially available solid supports. Thus, four solid supports are required for synthesis of oligodeoxyribonucleotides. The advancement of antisense therapeutics has resulted in the use of RNA−DNA−RNA chimeric molecules wherein the RNA wings consists of 2′-*O*-alkyl RNA nucleosides (2′-*O*-methoxyethyl or 2′-*O*-methyl).[5] In addition, other modified nucleosides such as 4′-thio, Locked nucleic acid, 2′-α-fluoro nucleic acids are also being evaluated for various applications. This has necessitated the need to have several additional prederivatized solid supports.

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(1) Vitravene and Macugen are two oligonucleotide-based drugs approved by the FDA.

(2) (a) Antisense strategies: Crooke, S. T. *Curr. Mol. Med.* **2004**, *4*, 465. (b) Progress in antisense technology: Crooke, S. T. *Ann. Rev. Med.* **2004**, *55*, 61. (c) Crooke, S. T. Progress in Antisense Technology. In *Drug Delivery Systems in Cancer Therapy*; Brown, D. M., Ed. Humana Press: Totowa, NJ, 2004. (d) Crooke, S. T.; Twain, M. Antisense Therapy. In *Cytokine Handbook,* 4th ed., Thomson, A. W., Lotze, M. T., Eds.; Elsevier Sciences: London, U. K., 2003. (e) Dias, N.; Stein, C. A. Antisense Oligonucleotides: Basic Concepts and Mechanisms. *Mol. Cancer Ther.* **2000**, *1*, 47. (f) Bennett, C. F. Efficiency of Antisense Oligonucleotide Drug Discovery. *Antisense Nucleic Acid Drug Dev.* **2002**, *12*, 215. (g) Kurreck, J. *Eur. J. Biochem.* **2003**, *270*, 1628.

(3) (a) A number of second-generation phosphorothioate oligonucleotides are in various stages of preclinical and clinical trials against APOB-100, PTP-1B, VLA4, TRPM2, survivin, STAT-3. eIF-eE, GCGR, GCCR, SOD1, SGLT2, CRP, etc. for the treatment of a variety of diseases such as cancer, psoriasis, diabetes, asthma, arthritis, multiple sclerosis, etc. (b) About 40 drugs are being evaluated in the clinic. Isis Pharmaceuticals, Inc. (www.isispharm.com) has the largest portfolio of antisense oligonucleotide products in clinical trials. Visit www.coleypharm.com for CpG-based oligonucleotides in clinical trials.

(4) (a) Beaucage, S. L.; Iyer, R. P. *Tetrahedron* **1992**, *48*, 2223. (b) Beaucage, S. L.; Iyer, R. P. *Tetrahedron* **1993**, *49*, 1925. (c) Beaucage, S. L. Oligonucleotide Synthesis. In *DNA and Aspects of Molecular Biology*; Kool, E. T., Vol. Ed.; Comprehensive Natural Products Chemistry; Barton, D. H. R.; Nakanishi, K., Eds.; Pergamon Press: Elmsford, NY, 1999; *Vol. 7.05,* 105.

(5) Altmann, K.-H.; Dean, N. M.; Fabbro, D.; Freier, S. M.; Geiger, T.; Haner, R.; Husken, D.; Martin, P.; Monia, B. P.; Muller, M.; Natt, F.; Nicklin, P.; Phillips, J.; Pieles, U.; Sasmor, H.; Moser, H. E. *Chimia* **1996**, *50*, 168.

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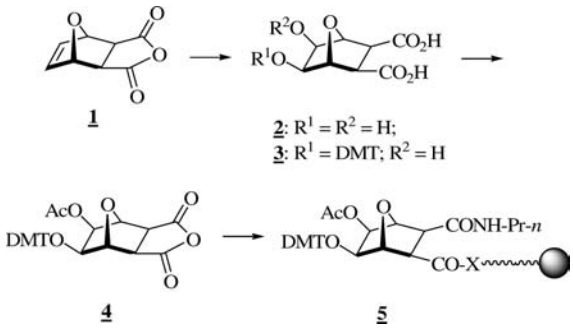
Scheme 1



An important aspect in the development of any drug is the cost of manufacturing, and this assessment is even more important for a non-small-molecule drug such as a phosphorothioate oligonucleotide where multiple drugs are advancing in human clinical evaluation to treat large patient populations suffering from chronic diseases such as diabetes and cholesterol. This novel therapeutic approach may soon lead to a necessity to manufacture several hundreds of kilograms of drug. Recently, we have examined several aspects of oligomerization chemistry with an aim to reduce cost, improve quality, and make the chemistry more rugged, greener, and safe.[6–9] Since the antisense approach is a platform technology, it is a given that this approach of drug discovery is faster, better, and cheaper for testing against several diseases. Thus, in a relatively short period of time multiple drugs have advanced to human clinical trials, and for various reasons some of them may fail to become an approved drug. Since not all drugs contain the same 3′-nucleoside, a clinical failure will lead to waste of a potentially large inventory of expensive solid support containing that particular loaded nucleoside.

In addition, the nucleoside-loaded solid support is considered as a starting material and not a raw material.[10] According to FDA guidelines a starting material has to be well characterized, and it is almost impossible with existing technology to characterize the nucleoside-loaded polymer matrix and show consistency in quality. Thus, it would be a real advantage if we can eliminate this starting material and still make the drug on a solid support. One solution is to use a solid support loaded with a molecule which could act as a linker between the support and the oligonucleotide to be synthesized.

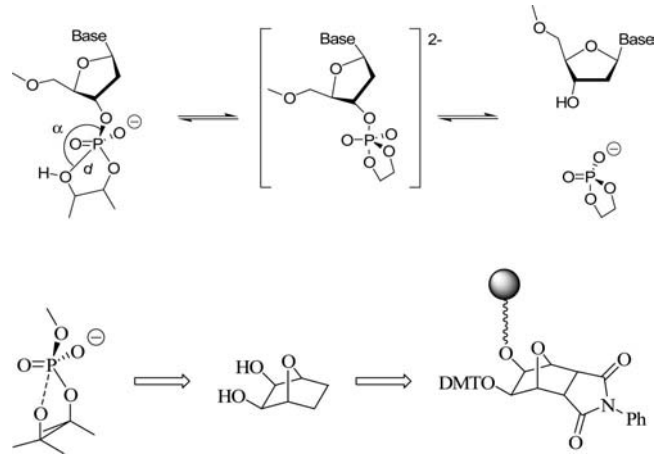


Figure 1. **Pentacoordinated transition state to facilitate cleavage.**

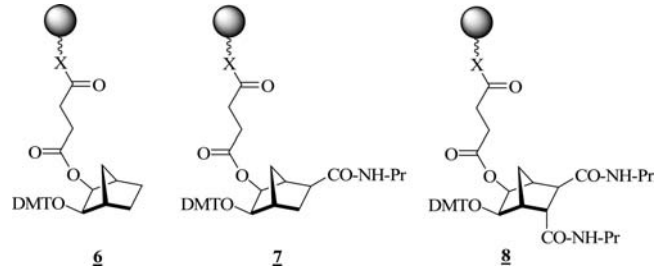


Figure 2. **Molecules evaluated as universal linkers on solid support.**

For high-throughput screening of oligonucleotides, 396-well plate synthesizers capable of synthesizing multiple oligonucleotides at the same time are becoming popular. This requires placing appropriate nucleoside-loaded solid supports in the wells, and enough chances exist for human error to cause failure and discarding 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. Universal supports are also expected to reduce side reactions that take place on the preattached nucleoside of standard supports because universal supports lack any nucleosides attached to them.[9d,11]

To address all these issues, several research groups have proposed the concept of a universal solid support using a *cis*-3,4-dihydroxytetrahydrofuran or a suitably functionalized nucleoside or other acyclic molecule.[12] The oligonucleotides synthesized using these universal solid supports could be used either for research or for pharmaceutical applications where very high standards of drug quality are needed. In our hands, when evaluated for oligonucleotide synthesis and analyzed carefully

(6) (a) Cheruvallath, Z. S.; Wheeler, P. D.; Cole, D. L.; Ravikumar, V. T. *Nucleosides Nucleotides* **1999**, *18*, 485. (b) Cheruvallath, Z. S.; Carty, R. L.; Moore, M. N.; Capaldi, D. C.; Krotz, A. H.; Wheeler, P. D.; Turney, B. J.; Craig, S. R.; Gaus, H. J.; Scozzari, A. N.; Cole, D. L.; Ravikumar, V. T. *Org. Process Res. Dev.* **2000**, *4*, 199. (c) Krotz, A. H.; Gorman, D.; Mataruse, P.; Foster, C.; Godbout, J. D.; Coffin, C. C.; Scozzari, A. N. *Org. Process Res. Dev.* **2004**, *8*, 852. (d) Ravikumar, V. T.; Andrade, M.; Carty, R. L.; Dan, A.; Barone, S. *Bioorg. Med. Chem. Lett.* **2006**, *16*, 2513. (e) Krotz, A. H.; Hang, A.; Gorman, D.; Scozzari, A. N. *Nucleosides Nucleotides Nucleic Acids* **2005**, *24*, 1293.

(7) (a) Krotz, A. H.; Cole, D. L.; Ravikumar, V. T. *Bioorg. Med. Chem.* **1999**, *7*, 435. (b) Krotz, A. H.; Cart, R. L.; Moore, M. N.; Scozzari, A. N.; Cole, D. L.; Ravikumar, V. T. *Green Chem.* **1999**, 277. (c) Krotz, A. H.; Carty, R. L.; Scozzari, A. N.; Cole, D. L.; Ravikumar, V. T. *Org. Process Res. Dev.* **2000**, *4*, 190.

(8) (a) Capaldi, D. C.; Scozzari, A. N.; Cole, D. L.; Ravikumar, V. T. *Org. Process Res. Dev.* **1999**, *3*, 485. (b) Hayakawa, Y.; Hirata, A.; Sugimoto, J.; Kawai, R.; Sakakura, A.; Kataoka, M. *Tetrahedron* **2001**, *57*, 8823.

(9) (a) Capaldi, D. C.; Gaus, H.; Krotz, A. H.; Arnold, J.; Carty, R. L.; Moore, M. N.; Scozzari, A. N.; Lowery, K.; Cole, D. L.; Ravikumar, V. T. *Org. Process Res. Dev.* **2003**, *7*, 832. (b) Gaus, H.; Olsen, P.; Van Sooy, K.; Rentel, C.; Turney, B.; Walker, K. L.; McArdle, J. V.; Capaldi, D. C. *Bioorg. Med. Chem. Lett.* **2005**, *15*, 4118. (c) Rentel, C.; Wang, X.; Batt, M.; Kurata, C.; Oliver, J.; Gaus, H.; Krotz, A. H.; McArdle, J. V.; Capaldi, D. C. *J. Org. Chem.* **2005**, *70*, 7841. (d) Kurata, C.; Bradley, K.; Gaus, H.; Luu, N.; Cedillo, I.; Ravikumar, V. T.; Van Sooy, K.; McArdle, J. V.; Capaldi, D. C. *Bioorg. Med. Chem. Lett.* **2006**, *16*, 607.

(10) Starting material is any chemical component that contributes an atom to the drug (e.g., phosphoramidite), and a raw material (solvents, activators such as 4,5-dicyanoimidazole) does not.

(11) Cazenave, C.; Bathany, K.; Rayner, B. *Oligonucleotides* **2006**, *16*, 181.

by ion-pair–liquid chromatography−electrospray mass spectroscopy (IP-LC−EMS) at least three of the reported universal solid supports showed that the quality of drug produced is not the same as the quality of drug produced with the standard method and were contaminated with unacceptable levels of the pendent linker molecule. In addition, there was a considerable (ca. 10%) amount of oligonucleotide still attached to the solid support, making it an inefficient approach. Addition of salt such as lithium chloride or heating at high temperatures (75–80 °C) or use of heavy metals or sulfides or use of strong nucleophiles such as 40% aqueous methylamine solution is recommended for release of oligonucleotides from some linker molecules. These conditions may not be practical or may add additional cost or may not be acceptable for large-scale synthesis or for therapeutic applications. The above limitations have thus far outweighed the added convenience of using one solid support material for all sequences, and prederivatized supports are still predominantly preferred. Clearly, while important strides have been made in this area, additional research efforts are warranted.

For a universal solid support to be widely accepted, it has to produce good yield of high-quality oligonucleotides and be applicable for synthesizing different kinds of oligonucleotide analogs. In addition, the universal linker molecule should be easily scaleable and inexpensive at large scales. It should work well across different solid supports, chemistries, and synthesizers. The molecule should be stable to oligomerization conditions and cleaved under standard ammonium hydroxide incubation conditions (55 °C) without use of any additives.

The universal support described in this work has several features that should enable greater acceptance by academics for performing basic research and by industry for development of various therapeutics: (a) the oligonucleotide is released from the linker molecule quantitatively under standard deprotection conditions; (b) the linker molecule is compatible with various modified chemistries as shown by extensive examples of syntheses and analyses; (c) it enables the synthesis of high-quality oligonucleotides (as judged by LC−MS analysis) without any detectable levels of base modification.

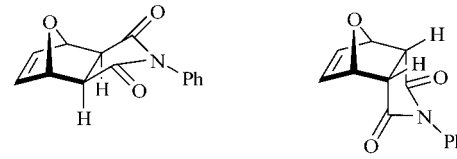


Figure 3. **Exo and endo structures of olefin 9.**

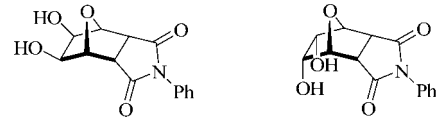


Figure 4. **Exo and endo structures of diol 10.**

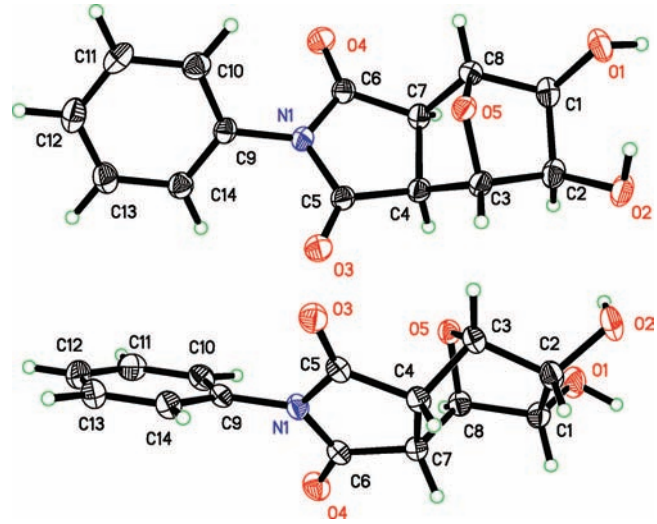


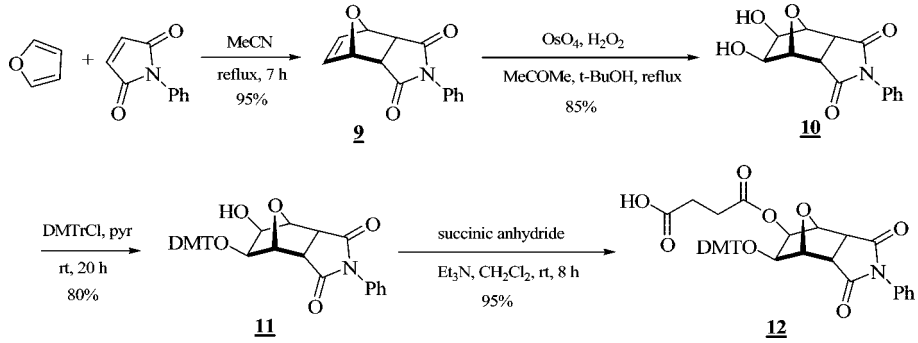
Figure 5. **ORTEP diagrams (different projections) of diol 10.**

Results and Discussion

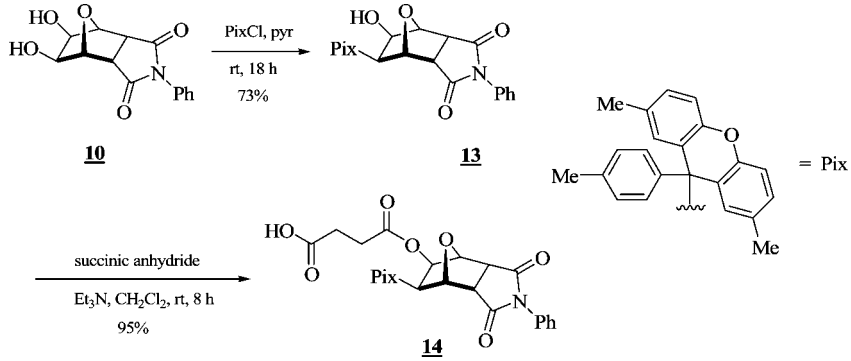
Design of UnyLinker Molecule. During our extensive investigation during this project, we initially designed a molecule **4** which on loading to a solid support and used led to the formation of high-quality oligonucleotides with similar or higher yields compared to those from nucleoside-loaded supports (Scheme 1). However, after completing all the laboratory-scale research work, when we started to scale up, we realized that this molecule failed to scale up efficiently. Isolated yields of two intermediates, **2** and **3**, were low, and it was not considered economical if we were to scale up to quantities of several hundreds of kilograms. In addition, there were some drawbacks in the design of this molecule. During the osmium-catalyzed dihydroxylation step to form **2**, the reaction has to be monitored by ^{1}H NMR (using relatively expensive deuterated pyridine) since there is no UV chromophore present in the molecule. Second, the dicarboxylic acid **3** was highly water soluble even in presence of a hydrophobic group such as 4,4′-dimethoxytrityl. Isolation of the product was found to be difficult

(12) (a) Kumar, P.; Gupta, K. C. *Helv. Chim. Acta* **2003**, *86*, 59. (b) Kumar, P.; Mahajan, S.; Gupta, K. C. *J. Org. Chem.* **2004**, *69*, 6482. (c) Kumar, P.; Dhawan, R.; Chandra, R.; Gupta, K. C. *Nucleic Acids Res.* **2002**, *30*, e130. (d) Ghosh, P. K.; Kumar, P.; Gupta, K. C. *Ind. J. Chem. Soc.* **1998**, *75*, 206. (e) Kumar, P.; Gupta, K. C. *Nucleic Acids Res.* **1990**, *27*, e2. (f) Kumar, P.; Gupta, K. C. *React. Funct. Polym.* **1999**, *41*, 197. (g) Lyttle, M. H.; Hudson, D.; Cook, R. M. *Nucleic Acids Res.* **1996**, *24*, 2793. (h) Lyttle, M. H.; Dick, D. J.; Hudson, D.; Cook, R. M. *Nucleosides Nucleotides* **1999**, *18*, 1809. (i) Gough, G. R.; Brunden, M. J.; Gilham, P. T. *Tetrahedron Lett.* **1983**, *24*, 5321. (j) deBear, J. S.; Hayes, J. A.; Koleck, M. P.; Gough, G. R. *Nucleosides Nucleotides* **1987**, *6*, 821. (k) Schwartz, M. E.; Breaker, R. R.; Asteriadis, G. T.; Gough, G. R. *Tetrahedron Lett.* **1995**, *36*, 27. (l) Scheuer-Larsen, C.; Rosenbohm, C.; Joergensen, T. J. D.; Wengel, J. *Nucleosides Nucleotides* **1997**, *16*, 67. (m) Nelson, P. S.; Muthini, S.; Vierra, M.; Acosta, L.; Smith, T. H. *Biotechniques* **1997**, *22*, 752. (n) Azhayev, A. V. *Tetrahedron* **1999**, *55*, 787. (o) Scheuer-Larson, C.; Rosenbohm, C.; Jorgensen, T. J. D.; Wengel, J. *Nucleosides Nucleotides* **1997**, *16*, 67. (p) Nelson, P. S.; Muthini, S.; Kent, M. A.; Smith, T. H. *Nucleosides Nucleotides* **1997**, *16*, 1951. (q) Azhayev, A. V.; Antopolsky, M. L. *Tetrahedron* **2001**, *57*, 4977. (r) Zheng, X.; Gaffney, B. L.; Jones, R. A. *Nucleic Acids Res.* **1997**, *25*, 3980. (s) Crea, R.; Horn, T. *Nucleic Acids Res.* **1980**, *8*, 2331. (t) Scott, S.; Hardy, P.; Sheppard, R. C.; McLean, M. J. In *Innovations and Perspectives in Solid-Phase Synthesis*; Epton, R., Ed.; Mayflower Worldwide: Birmingham, U. K., 1994; pp 115. (u) Anderson, E.; Brown, T.; Picken, D. *Nucleosides Nucleotides* **2003**, *22*, 1403. (v) Ferreira, F.; Meyer, A.; Vasseur, J-J.; Morvan, F. *J. Org. Chem.* **2005**, *70*, 9198. (w) Guzaev, A. P.; Manoharan, M. *J. Am. Chem. Soc.* **2003**, *125*, 2380. (x) Krishna Kumar, R.; Guzaev, A. P.; Rental, C.; Ravikumar, V. T. *Tetrahedron* **2006**, *62*, 4528. (y) Wang, Z.; Olsen, P.; Ravikumar, V. T. *Nucleosides Nucleotides and Nucleic Acids* **2007**, *26*, 259. (z) Anderson, K. M.; Jaquinod, L.; Jensen, M. A.; Ngo, N.; Davis, R. W. *J. Org. Chem.* **2007**, *72*, 9875. (aa) Mackie, H. *Glen Res. Rep.* **2001**, 141.

Scheme 2. **Synthesis of UnyLinker succinate 12**



Scheme 3. **Synthesis of pixyl-derivatized UnyLinker 14**



and cumbersome. Third, the final molecule **4** is an anhydride, and the purity was difficult to quantify by HPLC as it underwent hydrolysis under the analysis conditions. In addition, loading of **4** to yield solid support **5** is not straightforward. The cyclic anhydride ring opens during loading, liberating a free carboxyl group that needs to be capped. This protection is done by reacting with an amine in the presence of an activator such as HBTU. However, it will be difficult to ensure all carboxyl groups are capped. The presence of a free carboxyl group was found to hinder oligomerization yield particularly during coupling when low excess (1.7 equiv) of amidites was used. Because of these various hurdles in the use of this molecule (particularly not being able to scale up economically) we were forced to abandon this route. Subsequently, we started investigating the design of a new and better molecule that retained all the key features of the above molecule while still solving all the issues to make it a versatile chemistry for various applications.

In the design of a universal linker molecule, both the angle α and the distance *d* are important for efficient hydrolysis. Optimal values are α equals 90° or 180° and *d* equals 3 Å. In syn conformation of hydroxyl groups, *d* is reduced and α is increased. Because of this stabilized transition state, more rapid hydrolysis is expected (Figure 1).

We have not thoroughly investigated the necessity of two carboxyl groups attached to the bicyclic ring system although we know that the bridge head oxygen is needed for efficient synthesis since linker molecules based on norbornylene (**6**), 5-norbornene-2-carboxylic acid (**7**), and 5-norbornene-2-*endo*-3-*exo*-dicarboxylic acid (**8**) did not perform efficiently (Figure 2).

Assuming that the two carbonyl groups are desired, it would be desirable if they are masked in the form of a cyclic imide and also possess a UV chromophore. Thus, we had to design a new molecule that includes all of these needed features. We report here that a vicinal diol-based linker molecule, UnyLinker (**12**) has all the salient features of a good universal linker. We hypothesized that locking the two vicinal C−O bonds of an alkyl 2-hydroxyethyl phosphate in a syn-periplanar conformation could make the distance between the 2′-O and P(V) atoms appropriate for efficient attack and release of the oligonucleotide. We proposed **12** to be an optimally designed molecule for efficient synthesis of oligonucleotides. Thus, UnyLinker **12** was synthesized and loaded onto various solid supports and evaluated for the synthesis of multiple modified oligonucleotides in different designed automated synthesizers. The molecule has been scaled up to multikilogram quantities without any hurdle and successfully loaded onto polymeric supports on very large scales.

Synthesis of UnyLinker (12). The synthesis starts with the Diels–Alder reaction of furan and *N*-phenyl maleimide to afford the adduct **9** as a colorless crystalline solid (95%). Bis-hydroxylation of olefin **9** was carried out using hydrogen peroxide and a catalytic amount of osmium tetroxide (85%). Chemoselective protection of diol **10** with 4,4′-dimethoxytrityl chloride in pyridine gave the product **11** as a colorless, amorphous solid (80%). Treatment with succinic anhydride in the presence of triethylamine in methylene chloride afforded **12** as a colorless to pale-yellow amorphous solid in high (>60%) overall yield based on *N*-phenylmaleimide (Scheme 2).

Synthesis of Diels–Alder Adduct (9). Furan-based Diels–Alder adducts have found extensive use in organic synthesis; the resulting 7-oxanorbornene is often elaborated and/or ring-opened, providing routes to a wealth of valuable synthetic

targets.[13] Thus, Diels–Alder addition with *N*-phenylmaleimide gave the adduct **9**.[14] Depending on the condition used, exo or a mixture of endo and exo isomers could be obtained (Figure 3). Since we wanted to have a single thermodynamically more stable exoisomer, we refluxed the reaction mixture in MeCN as solvent. Similar results were obtained when toluene or xylene was used as solvent. As expected, ^{1}H NMR of the product showed a singlet for the C-5/C-6 protons (δ 3.0 ppm) corresponding to the exo isomer, with the absence of a coupled signal around δ 3.8 ppm attributable to the endo isomer. The 7-oxabicyclo[2.2.1]heptane ring system is conformationally rigid with a dihedral angle (H−C_5−C_6−H) of almost 90° for the exo isomer.[14a]

Synthesis of Dihydroxy Compound (10). The dihydroxylation of *N*-phenylmaleimide−furan adduct has received little attention although such reactions with other Diels–Alder adducts and other olefins have been reported extensively in the literature.[15–17] The adduct **9** was cis-dihydroxylated using the method described by Milas[18] which involves oxidation using a catalytic amount of osmium tetroxide in the presence of hydrogen peroxide in *tert*-butyl alcohol. Excellent yields were obtained by refluxing for 8 h to obtain the desired product as colorless, amorphous powder.

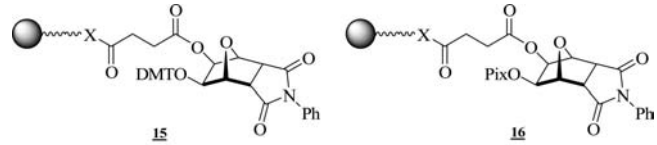
Stereochemistry of *cis*-Glycol 10. It has been established that hydroxylation with osmium tetroxide produces *cis*-glycol.[19] However, in the present case two *cis*-glycols are possible, viz. **10a** and **10b** (Figure 4). A choice can be made by applying Alder's rule of exoaddition.[20] Since the cis dihydroxylation proceeds[21] via the osmate ester of glycol, steric consideration for this intermediate strongly suggests the dihydroxylated compound has the configuration shown in **10a** i.e. the *exo-cis*-glycol.[18] For the formation of this product, there is less steric interference between osmium tetroxide and the oxygen bridge than between the reagent and the two-carbon bridge.

X-ray Crystallography Studies of Diol 10. The structure of diol **10** was confirmed to be *exo*,*exo-cis*-8,9-dihydroxy-4-phenyl-10-oxa-4-aza-tricyclo[5,2,1,$0^{2,6}$]decane-3,5-dione based on X-ray crystallography. Single crystals suitable for X-ray structure analysis were obtained by slow evaporation from a concentrated solution in dimethylformamide:methanol at 298 K.

Refinement. The H atoms bonded to O1 and O2 were found in a difference Fourier map and refined with free coordinates and isotropic *U* parameters. The C-bound H atoms were placed in idealized positions and refined as riding on their parent C atom, with the following constraints: 0.93 %A for aromatic CH, 0.98 %A for methine CH. Isotropic *U* parameters were fixed at *U*Ĩsõ(H) = 1.2*U*∼eq̃(carrier atom) for aromatic CH and methine CH (Figure 5).

Monoprotection of Diol and Succinylation. Efficient monoprotection of diol **10** with 4,4′-dimethoxytrityl chloride depends on its quality. Generally the diol is coevaporated twice with pyridine to ensure it is anhydrous. Otherwise, more than 1.2 equiv of DMT chloride is needed and renders the product purification difficult. In addition to pyridine, other bases such as lutidine and *syn*-collidine were found to perform equally efficiently for chemoselective protection. Subsequent treatment of **11** with succinic anhydride in dichloromethane afforded the UnyLinker succinate **12** as its triethylammonium salt. Formation of **12** was found to be slow when ethyl acetate was used as solvent.

Synthesis of Pixyl-Derivatized UnyLinker (14). Earlier, we observed that removing of DMT group from the secondary hydroxyl of **5** with dichloroacetic acid in an organic solvent such as toluene was relatively slow compared to removing the



Supports: **15a** = CPG; **15b** = HL30; **15c** = PS200; **15d** = OligoPrep; **15e** = NittoPhase; **16** = HL30

Figure 6. **UnyLinker succinate loaded onto various solid supports.**

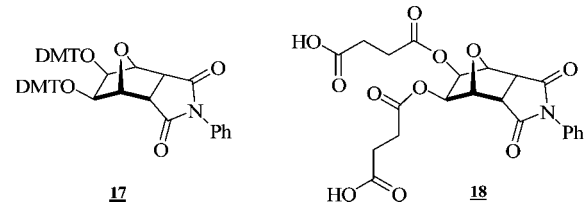


Figure 7. **Noncritical impurities formed during synthesis of UnyLinker succinate 12.**

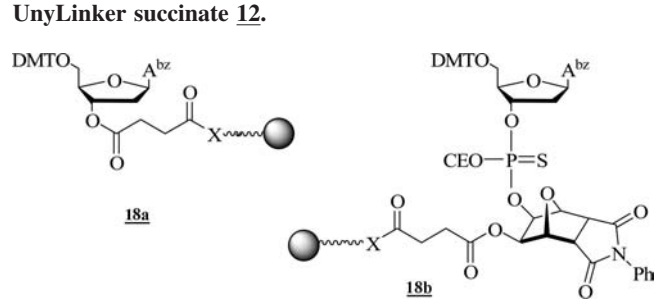
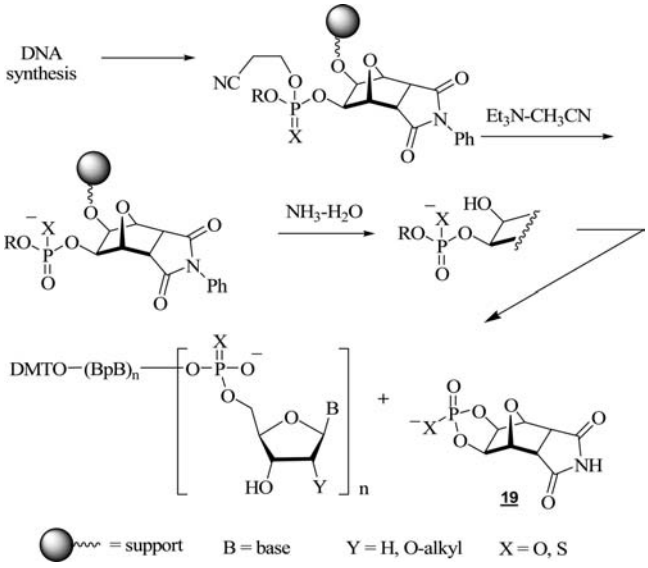


Figure 8. **Influence of 3′-substitution on depurination of dA nucleotide.**

(13) (a) Kappe, C. O.; Murphee, S. S.; Padwa, A. *Tetrahedron* **1997**, *53*, 14179. (b) Lautens, M.; Rovis, T. *Tetrahedron* **1998**, *54*, 1107.
(14) (a) Cooley, J. H.; Williams, R. V. *J. Chem. Educ.* **1997**, *74*, 582. (b) Keller, K. A.; Guo, J.; Punna, S.; Finn, M. G. *Tetrahedron Lett.* **2005**, *46*, 1181. (c) Rulisek, L.; Sebek, P.; Havlas, Z.; Hrabal, R.; Capek, P.; Svatos, A. *J. Org. Chem.* **2005**, *70*, 6295. (d) Sinner, F. M.; Buchmeiser, M. R. *Angew. Chem., Int. Ed.* **2000**, *39*, 1433.
(15) (a) Kolb, H. C.; Van Nieuwenhze, M. S.; Sharpless, K. B. *Chem. Rev.* **1994**, *94*, 2483. (b) Schroder, M. *Chem. Rev.* **1980**, *80*, 187. (c) Sharpless, K. B.; Akashi, K. *J. Am. Chem. Soc.* **1978**, *98*, 1986. (d) Akashi, K.; Palermo, R. E.; Sharpless, K. B. J. *Org. Chem.* **1978**, *43*, 2063. (e) Kobayashi, S.; Ishida, T.; Akiyama, R. *Org. Lett.* **2001**, *3*, 2649. (f) Kobayashi, S.; Endo, M.; Nagayama, S. *J. Am. Chem. Soc.* **1999**, *121*, 11229.
(16) (a) Horiuchi, C. A.; Dan, G.; Sakamoto, M.; Suda, K.; Usui, S.; Sakamoto, O.; Kitoh, S.; Watanabe, S.; Utsukihara, T.; Nozaki, S. *Synthesis* **2005**, 2861.
(17) (a) Fatialdi, A. J. *Chem. Rev.* **1980**, *80*, 85. and references cited therein. (b) Bhushan, V.; Rathore, R.; Chandrasekaran, S. *Synthesis* **1984**, 431. (c) Taylor, E. C.; Janini, T. E.; Elmer, O. C. *Org. Process Res. Dev.* **1998**, *2*, 147. (d) Branytska, O.; Neumann, R. *Synlett* **2005**, 2525. (e) Horiuchi, C. A.; Dan, G.; Sakamoto, M.; Suda, K.; Usui, S.; Sakamoto, O.; Kitoh, S.; Watanabe, S.; Utsukihara, T.; Nozaki, S. *Synthesis* **2005**, 2861.
(18) Milas, N. A.; Sussman, S. *J. Am. Chem. Soc.* **1936**, *58*, 1302.
(19) (a) Daniels, R.; Fischer, J. L. *J. Org. Chem.* **1963**, *28*, 320. (b) Freire, F.; Calderon, F.; Seco, J. M.; Fernandez-Mayoralas, A.; Quinoa, E.; Riguera, R. *J. Org. Chem.* **2007**, *72*, 2297 and references cited therein.
(20) (a) Alder, K.; Stein, G. *Ann.* **1935**, *515*, 185. (b) Alder, K.; Wirtz, H.; Koppelberg, H. *Ann.* **1956**, *601*, 138.
(21) (a) Criegee, R. *Ann.* **1936**, *522*, 75. (b) Criegee, R.; Marchand, B.; Wannowius, H. *Ann.* **1942**, *550*, 99.

Scheme 4. **Postulated mechanism for deprotection and release of oligonucleotide**



primary hydroxyl group; extended time (three times) was needed for complete removal. If removal of DMT group is not complete during oligomerization, it could be removed during subsequent acid-treatment cycles, leading to an increased (n − 1)-mer (deletionmers) in the final synthesized oligonucleotide. This prompted us to replace DMT with a more labile group. In connection with a different project aimed at reducing depurination, we were developing a DMT-like behaving but much easier to remove under acidic conditions viz. trimethylpixyl group as an alternative to the DMT group for oligonucleotide synthesis. This group is about 8 times faster to remove compared to DMT when treated under identical acidic conditions. Hence, we wanted to investigate if this pixyl group could minimize the time needed for complete removal. Treatment of diol **10** with 2,7-dimethyl-9-chloro-9-toluoylxanthene (pixyl chloride; made in our laboratory) in the presence of pyridine gave the monoprotected compound **13** in 73% isolated yield. Subsequent treatment with succinic anhydride in dichloromethane afforded the UnyLinker succinate **14** as its triethylammonium salt in almost quantitative yield (Scheme 3).

Loading of UnyLinker to Solid Supports. Solid supports are generally functionalized with amino or hydroxyl groups. Depending on the functionality, loading conditions could vary slightly. Hence, a study was undertaken to evaluate the influence of activators, solvents, bases, and loading time. We found that HBTU and HATU were the best activators, MeCN was the best solvent, and ethyldiisopropylamine or triethylamine was the preferred base. Controlled pore glass (CPG), HL30, PS200, NittoPhase, OligoPrep, and few other polymeric supports were evaluated as solid supports (Figure 6). These supports were loaded in the range of 25–325 μmol/g as recommended by the manufacturers.

Advantages of UnyLinker 12 over Compound 4. Many of the hurdles in the design of molecule **4** were eliminated. First, there is a UV chromophore handle to monitor the progress of the reaction easily using TLC or HPLC. Second, the final product is a triethylammonium salt of succinic acid of the molecule. Stability of this compound should be similar to that of a nucleoside succinate. The molecule was stable to at least one year when stored at refrigerated temperatures. Third, the water solubility problem has been completely eliminated. Compound **12** is hydrophobic and easily soluble in many organic solvents. Finally, during synthesis of **11**, bis-DMT protected compound **17** is also formed (~5–10%) and is removed easily by selective extractions. However, even if this compound is present as a contaminant, it will not undergo further reaction with succinic anhydride and will be inert towards loading. Similarly, if compound **18** is formed (due to succinylation of any unreacted diol **10** that is carried over), it will get loaded to solid support but is incapable of chain elongation and will be innocuous (Figure 7). Thus, UnyLinker **12** is optimally designed for evaluating the synthesis of various oligonucleotides.

Synthesis of Oligonucleotides. Extensive evaluation of UnyLinker was carried out by synthesizing DNA and several modified oligonucleotides such as 2′-MOE, 2′-OMe, LNA, OMe/RNA mixed backbone, conjugates such as biotin, 5′-phosphate, 3′-phosphate, etc. Both phosphate and phosphorothioate diester backbones were synthesized. Syntheses of multiple oligonucleotides at various scales on different automated synthesizers were successfully carried out.

Small-Scale Synthesis of Phosphorothioate Oligonucleotides. 20-Mer 2′-*O*-methoxyethyl gapmer (0.2 μmol scale) P = S (5-10-5) [5′-[CTCTA]-GTTCCTCTCA-[ATGTC]] was synthesized on MerMade plate synthesizer. UnyLinker loaded on CPG (45 μmol/g loading) was used. Conditions used for oligonucleotide synthsis were 4,5-dicyanoimidazole (0.5 M solution in ACN) for coupling, Beaucage reagent (0.05 M solution in ACN) for thiolation, 3% DCA/toluene for detritylation. At the end of the synthesis, the solid support was incubated with concentrated aqueous ammonium hydroxide at 55 °C for 8 h. Crude oligonucleotides were analyzed by IP-LC−MS (Table 1).

Medium-Scale Synthesis of Phosphorothioate Oligonucleotides. Phosphorothioate oligonucleotide **4** (Table 6) (5-10-5 MOE gapmer) was chosen as an example; 1 mmol scale on Akta 100 synthesizer was performed; 10% DCA/toluene for detritylation, 0.2 M PADS for sulfurization, 1*H*-tetrazole for coupling, 2.0 equiv of phosphoramidites for coupling, triethylamine/MeCN treatment to remove the cyanoethyl group while the oligo is still bound to the support, etc. were used; crude weight was determined at end of synthesis; the support was then incubated in a known amount of ammonia, diluted with a known amount with water, and quantitated with RP HPLC. The crude oligonucleotide was analyzed by IP-LC−MS; both oligonucleotides were purified on a BioCad instrument; the DMT-on oligonucleotide was detritylated and lyophilized to constant weight (Table 2).

Evaluation of UnyLinker on OligoPrep. Application of UnyLinker on OligoPrep was evaluated at 1 mmol scale by synthesizing phosphorothioate oligonucleotide **4** (Table 6) and was compared to MOE meC-nucleoside-loaded OligoPrep and PS200 solid supports. Oligomerization conditions similar to the above synthesis were followed for OligoPrep except that twice the volume of deblock (2 × delivery time) was used

Table 1. **Comparison of phosphorothioate oligonucleotide on 0.2 μmol scale using MOE meC (controls #1, #2; repetition of same experiments) and UnyLinker (Syn #1, #2, #3; repetition of same experiments) loaded CPG solid supports[a]**

	mass	control #1 (%)	control #2 (%)	UnyLinker Syn #1 (%)	UnyLinker Syn #2 (%)	UnyLinker Syn #3 (%)
full length	1783.5	82.26	83.69	89.61	88.10	88.96
PO	1779.5	10.26	8.71	5.05	5.48	5.52
3′-TPT	1709.1	2.86	2.64	0.89	1.23	1.13
n-T	1703.4	1.05	1.1	1.21	0.84	0.81
n-MOE meC/MOE meU	1685.1	3.57	3.85	3.24	4.36	3.57
total		100	100	100	100	100

[a] TPT = 3′-terminal phosphorothioate monoester; PO = phosphate diester.

Table 2. **Comparison of phosphorothioate oligonucleotide on 1 mmol scale using MOE meC (0963-146)- and UnyLinker (0963-148)-loaded solid supports[a]**

Oligo **4**	0963-146	0963-148
n	94.87	95.59
PO 1789	2.71	1.7
n-dG	0.05	0.05
n-MOEG	0.05	0.26
n-MOE C/U	0.45	0.74
n-dA	0.03	0.06
n-MOEA	0.05	0.05
n-T/meC	0.15	0.2
−A	0.29	0.3
−A + H_2O	0.22	0.2
−A + MeOH	0.24	0.16
3′-TPT	0.14	0.04
CNET	0.33	0.31
n + 156	0.44	0.33
shortmer	1.54	1.64
full length	94.25	95.82
longmer	4.21	2.54

[a] TPT = 3′-terminal phosphorothioate monoester; PO = phosphate diester; CNET = cyanoethyl adduct on N^1 of thymine/uracil.

Table 3. **Yield comparison of UnyLinker and MOE meC nucleoside loaded on OligoPrep**

support	crude yield OD/μmol	crude yield mg/μmol	purified yield mg/μmol
PS200 MOE meC	113	7.3	3.8
OligoPrep MOE meC	116	8.0	4.2
OligoPrep UnyLinker	105	8.8	3.6
PS200 MOE meC	109	7.5	3.8
OligoPrep MOE meC	126	8.4	4.6
OligoPrep UnyLinker	123	8.7	3.4

to remove DMT group. The following tables (Tables 3 and 4) show the performance of UnyLinker on OligoPrep.

Large-Scale Synthesis of Phosphorothioate Oligonucleotides. Next, we scaled up the evaluation of UnyLinker to several hundred-fold (400–700 mmol scales) on OligoProcess synthesizer. Multiple oligonucleotides (Table 6; oligo sequences **1**−**8**) were performed using PS200 and NittoPhase solid supports loaded with UnyLinker at approximately 200 μmol/g. Optimized conditions similar to those of laboratory-scale Akta 100 syntheses were followed. Subsequently, the oligonucleotides were purified using C-18 reversed phase HPLC; appropriate fractions were collected, detritylated, precipitated, and lyophilized. An average of 2380 g of purified drug was obtained per 700 mmol synthesis. Extensive analysis was then performed on the purified active pharmaceutical ingredient (API). Consistently higher-quality oligonucleotides were obtained using UnyLinker loaded solid supports. Comparison of a phosphorothioate oligonucleotide synthesized on OligoProcess using UnyLinker- and MOE meC-loaded solid supports is shown in Table 5.

Synthesis of Chemically Modified Oligonucleotides. Apart from 2′-MOE, several other nucleoside and base-modified oligonucleotides were synthesized efficiently. These are shown in Table 7. Both fully thiolated and PS/PO backbone-containing oligonucleotides were also synthesized. Synthesis of 5′-phosphate monoester, biotin-conjugated oligonucleotides, LNA, 2′-α-fluoro, etc. demonstrates the versatility of this approach.

Improvement in Oligonucleotide Quality. Benzoyl group is routinely used to protect the exocyclic amino group of deoxyadenosine, 5-methyldeoxycytidine, 2′-*O*-methoxyethyladenosine, and 2′-*O*-methoxyethyl-5-methylcytidine. Even though it is accepted as a good protecting group, its stability during loading to solid support or upon storage of loaded support at room temperature for extended time is not ideal. A portion of this group falls off leading to unprotected amino group sites capable of reacting with amidites. A class of compounds termed (2*n* − 1)-mers are formed due to branching from these sites and have been characterized recently.[9d]

Another process-related impurity termed 3′-terminal phosphorothioate monoester (3′-TPT) is formed due to depurination of dA attached to solid support upon exposure to deblocking conditions. It has been reported that **18a** is more prone to depurination when compared to nucleosides having phosphate or phosphorothioate group attached at the 3′-end similar to compounds such as **18b**.[22] Use of **18a** for synthesis of Oligo **7** leads to formation of 3′-terminal phosphorothioate monoester (3′-TPT) (Figure 8).

On the other hand, high-quality oligonucleotide (Oligo **7**) was obtained by use of **15**. Very low levels (<0.1%) are still observed probably due to nonquantitative capping of solid support. Similar use of MOE meC- and MOE A-loaded solid supports leads to formation of (2*n* − 1)-mers. When UnyLinker-loaded support was used, these impurities were either eliminated or reduced substantially, leading to increased quality of oligonucleotide drugs (Figure 9). We have consistently seen between 1 and 3% increase in overall improvement in quality by using UnyLinker loaded solid supports (Table 8).

Evaluation of DMT vs Pixyl Group. Solid supports **15b** and **16** were compared for efficiency of removal of DMT and pixyl groups on secondary hydroxyl group of the UnyLinker molecule and its impact on (*n* − 1)-mer level in oligonucleotide

(22) (a) Septak, M. *Nucleic Acids Res.* **1996**, *24*, 3053. (b) Paul, C. H.; Royappa, A. T. *Nucleic Acids Res.* **1996**, *24*, 3048.

Table 4. **Quality comparison of UnyLinker and MOE meC nucleoside loaded on OligoPrep**

	UV purity (%)			mass purity (%)		
support	shortmer	full length	longer	full length + PO	$n-1$	depurination + TPT
PS200 MOE meC	4.0	91	4.9	96	1.3	1.7
OligoPrep MOE meC	7.4	89	3.8	96	1.1	1.9
OligoPrep UnyLinker	6.6	90	3.7	96	2.1	1.6
PS200 MOE meC	4.9	90	4.8	97	1.0	1.7
OligoPrep MOE meC	6.7	90	3.7	97	1.1	1.9
OligoPrep UnyLinker	6.7	90	3.8	96	2.0	1.6

Table 5. **Comparison of phosphorothioate oligonucleotides synthesized on OligoProcess using UnyLinker- and MOE meC-loaded solid supports[a]**

oligo **4**	SYN000031 (UnyLinker) (%)	SYN000032 (UnyLinker) (%)	SYN000014 (MOE meC succinate) (%)
n	93.5	94.2	94.9
PO	2.2	1.7	1.8
DMT-on total $n-1$	1.9	1.6	0.8
DMT-on *n*-(MOE meC + MOE meU) (#8)	1.5	1.2	–
DMT-on total depurination	0.16	0.11	0.3
DMT-on TPT	0.33	0.46	0.1
DMT-on CNET	0.10	0.06	0.2
DMT-on EPD	0.03	0.09	0.2
DMT-on 2′-OMe	0.87	0.68	0.7
other DMT-on	0.48	0.76	0.9
combined area % of two DMT-on peaks	76.6	78.8	70.2

[a] TPT = 3′-terminal phosphorothioate monoester; PO = phosphate diester; CNET = cyanoethyl adduct on N^1 of thymine/uracil.

Table 6. **Phosphorothioate oligonucleotides synthesized on GE Amersham Akta 100 and OligoProcess synthesizers**

oligo synthesized	sequence 5′–3′
oligo **1**	2′-*O*-methoxyethyl modified phosphorothioate 20-nucleotide gapmer, ISIS 345794
oligo **2**	[CTG]-AGTCTGTTT-[TCCATTCT]
oligo **3**	[GCTCC]-TTCCACTGAT-[CCTGC]
oligo **4**	[GCCTC]-AGTCTGCTTC-[GCACC]
oligo **5**	[TGGAA]-AGGCTTATAC-[CCCTC]
oligo **6**	[TCCCGC]-CTGTGACA-[TGCATT]
oligo **7**	d[GCCCAAGCTGGCATCCGTCA]
oligo **8**	[CAGC]-AGCAGAGTCTTCA-[TCAT]

synthesized. Phosphorothioate oligonucleotide **7** was synthesized on Akta 100 synthesizer using 10% dichloroacetic acid in toluene. Twice the normal volume and time was used for removal of these acid-labile groups on the UnyLinker molecule as compared to removal of the DMT group from the 5′-hydroxyl of oligonucleotide. Analysis of the crude oligonucleotides by IP-LC−MS showed no difference in level of total ($n-1$)-mer and *n*-dA (first base in sequence), indicating that no additional benefit was observed in using a more labile pixyl group as compared to the standard DMT group.

Mechanism of Deprotection. A reasonable pathway for cleavage and release of oligonucleotide from UnyLinker is proposed here. Treatment of support-bound, fully protected phosphorothioate triester with triethylamine in MeCN for 2 h removes all acrylonitrile formed due to β-elimination of cyanoethyl group, leading to a phosphorothioate diester backbone. Subsequent incubation with aqueous ammonium hydroxide cleaves the base-labile succinate ester linkage. The liberated vicinal hydroxyl group attacks the neighboring phosphorous center of the diester linkage, releasing the free 3′- hydroxyl oligonucleotide (Scheme 4). A cyclic phosphorothioate diester is formed having a characteristic downfield shift in ^{31}P NMR (78.2, 74.5 ppm) (Figure 10) as a pair of isomers.

Another interesting reaction happens at the other end of UnyLinker molecule during release of the oligonucleotide. Nucleophilic attack of ammonia on cyclic imide leads to diamide **20** which then undergoes facile intramolecular cyclization to form **19** (Scheme 5). The identity of this byproduct was confirmed by comparing it with an authentic sample prepared synthetically according to Scheme 6.

Synthesis of Cyclic Byproduct 19. Nucleoside 2′,3′-*O*,*O*-phosphorothioates are usually prepared via the reactions of 5′-*O*,*N*-protected ribonucleosides with thiophosphoryl chloride, cyclization of nucleoside 2′(3′)-phosphorothioate derivatives, or using salicyclic chlorophosphite as a phosphitylating agent.[23] However, recently, there was a report where 5′-protected ribonucleosides were reacted with diphenyl *H*-phosphonate in pyridine to afford the corresponding 2′,3′-cyclic *H*-phosphonate which upon sulfurization gave the nucleoside 2′,3′-*O*,*O*-cyclophosphorothioates.[24]

Following this protocol, treatment of diol **10** with commercially available diphenyl *H*-phosphonate in pyridine followed by sulfurization gave the cyclic phosphorothioate diester, **22**. Incubation in aqueous ammonium hydroxide at 55 °C for 12 h gave **19**, whose ^{31}P NMR (DMSO-d_6) showed two peaks corresponding to two isomers.

(23) (a) Gerlt, J. A.; Wan, W. H. Y. *Biochemistry* **1979**, *18*, 4630. (b) Eckstein, F.; Ginll, H. *Chem. Ber.* **1968**, *101*, 1670. (c) Holy, A.; Kois, P. *Collect. Czech. Chem. Commun.* **1980**, *45*, 2817. (d) Ludwig, J.; Eckstein, F. *J. Org. Chem.* **1989**, *54*, 631.

(24) Jankowska, J.; Wenska, M.; Popenda, M.; Stawinski, J.; Kraszewski, A. *Tetrahedron Lett.* **2000**, *41*, 2227.

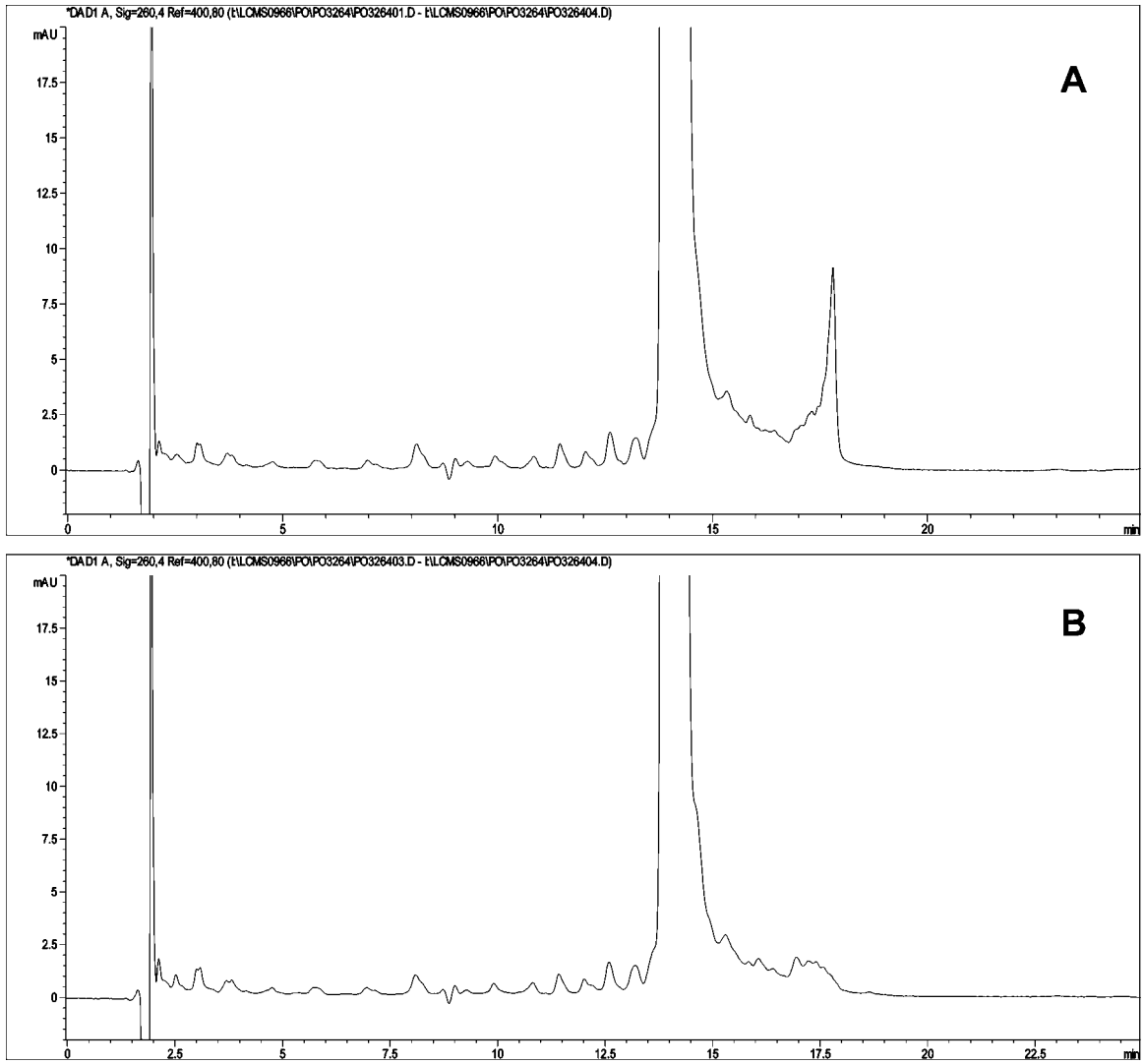


Figure 9. **Upper panel (A): HPLC−UV trace of the synthesized phosphorothioate oligonucleotide 4 using MOE meC nucleoside-loaded solid support. Lower panel (B): HPLC−UV trace of the synthesized phosphorothioate oligonucleotide using UnyLinker-loaded solid support.**

Synthesis of Trans Configured Molecule 26. Even though by design only the *cis*-diol-based UnyLinker molecule will liberate free 3′-hydroxy oligonucleotide, we wanted to find out, for therapeutic application purposes, that if **24** were to be present as a contaminant, would it lead to any other species or even give the right product or, as expected, have a fragment attached to the 3′-end of the oligonucleotide. The synthesis of trans-configured UnyLinker molecule was attempted according to Scheme 7. Treatment of olefin **9** with *m*-chloroperbenzoic acid gave the epoxide as a colorless solid in 85% yield. Ring opening of the epoxide with perchloric acid or many other acids did not afford the *trans*-diol **24**.[25] Epimerization with Raney nickel also failed to give the desired product. No further attempts were made to synthesize **24**. This probably indicates that in this bridge-head ring system, *trans*-diol is very difficult to be formed.

(25) (a) Fringuelli, F.; Germani, R.; Pizzo, F.; Savelli, G. *Synth. Commun.* **1989**, *19*, 1939. (b) Taylor, E. C.; Janini, T. E.; Elmer, O. C. *Org. Process. Res. Dev* **1998**, *2*, 147 and references cited therein. (c) Fringuelli, F.; Germani, R.; Pizzo, F.; Savelli, G. *Synth. Commun.* **1989**, *19*, 1939. (d) Swern, D.; Billen, G. N.; Scanland, J. T. *J. Am. Chem. Soc.* **1946**, *68*, 1504. (e) Owen, L. N.; Smith, P. N. *J. Chem. Soc.* **1952**, 4026.

Therapeutic Application of UnyLinker Chemistry. It is important to demonstrate the safe nature of any chemistry before being accepted for therapeutic applications. Initially, extensive testing (ICP−MS) of DMT-protected hydroxyl compound **11** as well as its succinate molecule **12** showed very low levels of osmium (<20 ppm). Subsequently, treatment of **11** with charcoal and extraction with solvent showed no detectable levels of osmium (<1 ppm). During the development of this process to eliminate residual osmium, an alternative approach was also successfully carried out. Amino-derivatized polymeric solid support (Wang resin) was capped with pentenoic anhydride under standard capping conditions. A solution of compound **11** containing a higher level of osmium tetroxide (320 ppm; intentionally spiked) was stirred with a 2-fold theoretical excess of this capped support overnight at room temperature. Filtration and concentration of solution afforded compound **11** containing no detectable levels (<1 ppm) of osmium (Scheme 8). This principle is similar to the dihydroxylation of an olefin except that no oxidizing agent is present and the cyclic osmium complex stays bound to the solid support and is removed after

Table 7. **Oligonucleotides synthesized on GE Amersham Akta 10/100 and other automated DNA/RNA synthesizers**

oligo synthesized	sequence 5′–3′[a]
Oligo **9**	5′-PO-d[AATGCATGTCACAGGCGGGA]
Oligo **10**	$^{m}C_{ms}T_{ms}{}^{m}C_{mo}A_{mo}G_{mo}{}^{m}C_{mo}A_{mo}{}^{m}C_{mo}A_{mo}T_{mo}{}^{m}C_{mo}T_{mo}A_{mo}{}^{m}C_{mo}A_{mo}A_{mo}G_{ms}A_{ms}A_{ms}A$
Oligo **11**	$^{m}C_{ms}T_{ms}{}^{m}C_{ms}A_{ms}G_{ms}{}^{m}C_{ms}A_{ms}{}^{m}C_{ms}A_{ms}T_{ms}{}^{m}C_{ms}T_{ms}A_{ms}{}^{m}C_{ms}A_{ms}A_{ms}G_{ms}A_{ms}A_{ms}A$
Oligo **12**	$A_{ro}A_{eo}G_{ro}C_{eo}A_{ro}A_{eo}C_{ro}G_{eo}A_{ro}\text{-}G_{eo}A_{ro}A_{eo}G_{ro}C_{eo}G_{ro}A_{eo}U_{ro}A_{eo}A_{r}$
Oligo **13**	$5'\text{-}A_{es}{}^{m}C_{es}A_{es}{}^{m}C_{es}A_{es}A_{es}A_{es}T_{es}\text{-}T_{es}{}^{m}C_{es}G_{es}G_{es}T_{es}T_{es}{}^{m}C_{es}T_{es}A_{es}{}^{m}C_{es}A_{es}G_{es}G_{es}G_{e}$
Oligo **14**	$PO_3\text{-}U_{ro}U_{ro}A_{ro}U_{ro}C_{ro}G_{mo}C_{mo}U_{ro}U_{ro}C_{mo}U_{mo}C_{ro}G_{ro}\text{-}U_{ro}U_{ro}\text{-}G_{ro}C_{mo}U_{mo}U_{m}$
Oligo **15**	$5'\text{-}{}^{m}C_{es}G_{es}{}^{m}C_{es}G_{es}T_{es}A_{es}{}^{m}C_{es}{}^{m}C_{es}\text{-}A_{es}A_{es}A_{es}A_{es}G_{es}T_{es}A_{es}A_{es}T_{es}A_{es}A_{es}T_{es}G_{e}$
Oligo **16**	$^{m}C_{es}U_{fs}{}^{m}C_{es}A_{fs}G_{es}C_{fs}A_{es}C_{fs}A_{es}\text{-}U_{fs}{}^{m}C_{es}U_{fs}A_{es}C_{fs}A_{es}A_{fs}G_{es}A_{es}A_{e}$
Oligo **17**	$5'\text{-}T_{es}{}^{m}C_{es}A_{es}{}^{m}C_{es}A_{es}A_{es}G_{es}T_{es}T_{es}\text{-}A_{es}G_{es}G_{es}G_{es}T_{es}{}^{m}C_{es}T_{es}{}^{m}C_{es}A_{es}G_{es}G_{es}G_{es}A_{e}$
Oligo **18**	$5'\text{-}G_{es}{}^{m}C_{es}T_{es}{}^{m}C_{es}A_{es}T_{es}T_{es}T_{es}A_{es}\text{-}G_{es}T_{es}{}^{m}C_{es}T_{es}G_{es}{}^{m}C_{es}{}^{m}C_{es}T_{es}G_{es}A_{es}T_{e}$
Oligo **19**	$^{m}C_{ls}T_{ls}G_{ds}C_{ds}T_{ds}A_{ds}G_{ds}C_{ds}C_{ds}\text{-}T_{ds}C_{ds}T_{ds}G_{ds}G_{ds}A_{ds}T_{ds}T_{ds}T_{ds}G_{ls}A_{l}$
Oligo **20**	$^{m}C_{lo}T_{lo}G_{ds}C_{ds}T_{ds}A_{ds}G_{ds}C_{ds}C_{ds}\text{-}T_{ds}C_{ds}T_{ds}G_{ds}G_{ds}A_{ds}T_{ds}T_{ds}T_{do}G_{lo}A_{l}$
Oligo **21**	$5'\text{-}A_{es}{}^{m}C_{es}A_{es}T_{es}A_{es}{}^{m}C_{es}T_{es}{}^{m}C_{es}{}^{m}C_{es}\text{-}T_{es}T_{es}T_{es}{}^{m}C_{es}T_{es}{}^{m}C_{es}A_{es}G_{es}\text{-}A_{es}G_{es}T_{es}{}^{m}C_{es}{}^{m}C_{es}A_{e}$
Oligo **22**	$T_{ls}T_{ls}G_{ls}T_{ds}T_{ds}C_{ds}T_{ds}G_{ds}A_{ds}A_{ds}T_{ds}G_{ds}T_{ds}{}^{m}C_{ls}{}^{m}C_{ls}A_{l}$
Oligo **23**	$5'\text{-}A_{es}A_{es}{}^{m}C_{es}{}^{m}C_{es}T_{es}A_{es}T_{es}{}^{m}C_{es}{}^{m}C_{es}\text{-}T_{es}G_{es}A_{es}A_{es}T_{es}T_{es}A_{es}{}^{m}C_{es}T_{es}T_{es}G_{es}A_{es}A_{e}$
Oligo **24**	$5'\text{-}A_{ms}{}^{m}C_{ms}A_{ms}A_{ms}A_{ms}{}^{m}C_{ms}A_{ms}{}^{m}C_{ms}{}^{m}C_{ms}\text{-}A_{ms}T_{ms}T_{ms}G_{ms}T_{ms}{}^{m}C_{ms}A_{ms}{}^{m}C_{ms}\text{-}A_{ms}{}^{m}C_{ms}T_{ms}{}^{m}C_{ms}{}^{m}C_{ms}A_{m}$
Oligo **25**	5′-PO-d[GAGGGGTATAAGCCTTTCCA]
Oligo **26**	$G_{ls}{}^{m}C_{ls}T_{ls}{}^{m}C_{ls}A_{ds}T_{ds}A_{ds}{}^{m}C_{ds}\text{-}T_{ds}{}^{m}C_{ds}G_{ds}T_{ds}A_{ds}G_{ds}G_{ls}{}^{m}C_{ls}{}^{m}C_{ls}A_{l}$
Oligo **27**	$5'\text{-}G_{es}{}^{m}C_{es}A_{ds}{}^{m}C_{ds}T_{ds}T_{ds}T_{ds}G_{ds}T_{ds}\text{-}G_{ds}G_{ds}T_{ds}G_{ds}{}^{m}C_{ds}{}^{m}C_{ds}A_{ds}A_{ds}G_{ds}G_{es}{}^{m}C_{e}$
Oligo **28**	$5'\text{-}{}^{m}C_{es}{}^{m}C_{ds}A_{ds}G_{ds}{}^{m}C_{ds}A_{ds}T_{ds}{}^{m}C_{ds}\text{-}T_{ds}G_{ds}{}^{m}C_{ds}T_{ds}G_{es}{}^{m}C_{es}T_{es}T_{es}{}^{m}C_{e}$
Oligo **29**	$5'\text{-}{}^{m}C_{es}T_{ds}{}^{m}C_{es}A_{ds}G_{es}{}^{m}C_{ds}A_{es}{}^{m}C_{ds}\text{-}A_{es}T_{ds}{}^{m}C_{es}T_{ds}A_{es}{}^{m}C_{ds}A_{es}A_{ds}G_{es}A_{ds}A_{es}A_{e}$
Oligo **30**	5′-biotin-$G_{es}A_{es}A_{es}G_{es}T_{es}A_{ds}G_{ds}{}^{m}C_{ds}{}^{m}C_{ds}\text{-}A_{ds}{}^{m}C_{ds}{}^{m}C_{ds}A_{ds}A_{ds}{}^{m}C_{ds}T_{es}G_{es}T_{es}G_{es}{}^{m}C_{e}$
Oligo **31**	$5'\text{-}{}^{m}C_{es}T_{fs}C_{fs}A_{fs}G_{fs}C_{fs}A_{fs}C_{fs}A_{fs}\text{-}T_{fs}C_{fs}T_{fs}A_{fs}C_{fs}A_{fs}A_{fs}G_{fs}A_{fs}A_{es}A_{e}$
Oligo **32**	$5'\text{-}PO_3\text{-}A_{ro}U_{ro}A_{ro}G_{ro}A_{ro}C_{mo}U_{mo}U_{ro}\text{-}C_{ro}A_{mo}U_{mo}C_{ro}C_{ro}U_{ro}U_{ro}G_{ro}U_{mo}U_{mo}G_{m}$
Oligo **33**	$5'\text{-}U_{ro}A_{ro}C_{ro}G_{ro}C_{ro}A_{mo}A_{mo}A_{ro}C_{ro}C_{mo}\text{-}U_{mo}U_{ro}G_{ro}A_{ro}U_{ro}G_{ro}U_{mo}C_{mo}C_{m}$
Oligo **34**	$5'\text{-}PO_3\text{-}A_{ro}A_{ro}A_{ro}U_{ro}G_{ro}U_{mo}U_{mo}C_{ro}C_{ro}\text{-}A_{mo}G_{mo}C_{ro}C_{ro}C_{ro}A_{ro}G_{ro}G_{mo}G_{mo}C_{m}$
Oligo **35**	$5'\text{-}G_{ro}G_{eo}A_{ro}C_{eo}A_{ro}U_{eo}C_{ro}A_{eo}A_{ro}\text{-}G_{eo}G_{ro}U_{eo}U_{ro}U_{eo}G_{ro}C_{eo}G_{ro}U_{eo}A_{r}$
Oligo **36**	$5'\text{-}U_{ro}C_{ro}A_{ro}C_{ro}U_{ro}C_{ro}G_{ro}G_{ro}\text{-}C_{ro}U_{ro}G_{ro}G_{ro}A_{ro}U_{ro}G_{ro}G_{ro}A_{ro}G_{ro}U_{rs}T_{es}T$
Oligo **37**	5′-[CTCTA]-GTTCCTCTCA-[ATGTC](for plate synthesis comparison)

[a] ^{m}C = 5-methyl C; d = 2′-deoxy; e = 2′-*O*-methoxyethyl; m = 2′-*O*-methyl; r = 2′-OH; l = LNA; f = 2′-α-fluoro; o = PO; s = PS; PO_3 = phosphate monoester.

Table 8. **IP-LC−MS analysis of Oligo 4 synthesized using MOE meC-loaded and UnyLinker-loaded solid supports at 1 mm scale on Akta 100 synthesizer[a]**

sequence: oligo **4**	using MOE meC-loaded support (%)	using support **15e** (%)
n	94.87	95.59
P = O	2.71	1.7
n-dG	0.05	0.05
n-MOE G	0.05	0.26
n-MOE meC/MOE meU	0.45	0.74
n-dA	0.03	0.06
n-MOE A	0.05	0.05
n-T/5-meC	0.15	0.2
−A	0.29	0.3
−A + H_2O	0.22	0.2
−A + MeOH	0.24	0.16
3′-TPT	0.14	0.04
CNET	0.33	0.31
N + 156	0.44	0.33
early eluting shortmer	1.54	1.64
full length	94.25	95.82
late eluting longmer	4.21	2.54

[a] TPT = 3′-terminal phosphorothioate monoester; PO = phosphate diester; CNET = cyanoethyl adduct on N^1 of thymine/uracil.

filtration. This concept may have additional industrial applications such as in water purification, etc.

Several hundreds of oligonucleotides have been synthesized in our laboratories so far using UnyLinker loaded onto various solid supports. Phosphorothioate oligonucleotides manufactured for therapeutic applications undergo rigorous analytical tests and show no trace of osmium. Multitudes of modified oligonucleotides have been evaluated for pharmacological, toxicological, pharmacokinetic, and other biological evaluations in various animal models. Many phosphorothioate 2′-*O*-methoxyethyl-modified oligonucleotide drugs manufactured using UnyLinker-loaded solid supports are being evaluated in the human clinic. These evidences clearly indicate the safe nature of using this chemistry.

Economical Impact of Using UnyLinker Chemistry. There are several direct and indirect savings to using this UnyLinker-loaded solid supports. Even though it may be difficult to calculate the indirect cost, it is obvious that any labor involved in QC testing of several different nucleoside loaded supports can be eliminated. Warehousing, stability testing, and

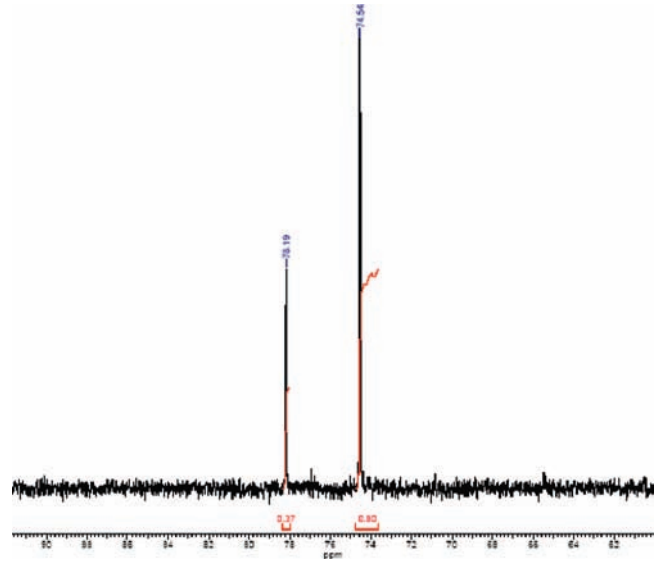


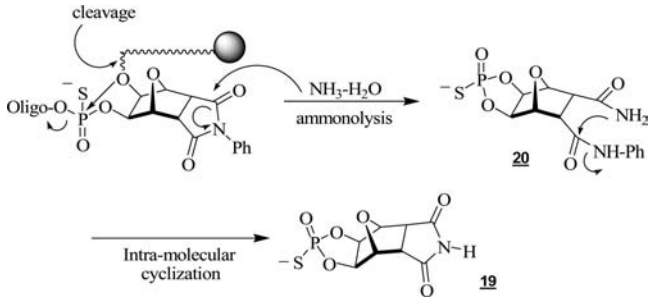
Figure 10. **^{31}P NMR ($CDCl_3$) of 19 from oligonucleotide synthesis.**

Table 9. **Synthesis parameters of cycle used on Akta 100 DNA/RNA synthesizer[a]**

step	reagent	volume (mL)	time (min)
detritylation	10% dichloroacetic acid/toluene	72 (144)	3 (6)
coupling	phosphoramidite (0.2 M) and 1H-tetrazole (0.45 M) or DCI (0.7 M) + NMI (0.1 M) in MeCN	8.8, 8.8	3
sulfurization	PADS (0.2M) in 3-picoline/MeCN (1:1, v/v) or pyridine/MeCN (1:1, v/v)	44	3
capping	Ac_2O/pyridine/MeCN,NMI/MeCN	30, 30	2.5

[a] Volume and time in parentheses indicate the conditions used for detritylation of UnyLinker molecule.

Scheme 5. **Mechanism depicting formation of cyclic by-product**



various documentations related to it that are involved in routine GMP manufacturing of drugs can be avoided. Labor involved in these indirect operations could be a significant cost-saving factor. On the direct cost savings, as in any other field, cost and quantity are directly related. Synthesis of this molecule has been outsourced and scaled up to several kilogram quantities, and loading has been done on 10−20-kg batch sizes. At this scale it costs us few cents per micromole to synthesize and load onto polymeric supports.

Conclusions

In summary, we have designed a linker molecule that has all the features of being termed a universal linker. Various oligonucleotide modifications have been synthesized to demonstrate its utility. Large-scale syntheses of phosphorothioate oligonucleotides for therapeutic applications have been carried out, leading to improved quality in addition to eliminating one crucial starting material in the supply chain. A mechanism for cleavage and release of oligonucleotides is proposed and supported by characterization of the byproduct. Here at Isis Pharmaceuticals, Inc. we have switched over to this UnyLinker-loaded solid support, both for research (plates and small-scale syntheses) and large-scale (OligoProcess) routine manufacture of antisense drugs.[26]

Experimental Section

Oligonucleotide Synthesis. Oligonucleotide syntheses were performed either on an Akta 10/100 or OligoProcess or MerMade 396-plate synthesizer by the phosphoramidite-coupling method. Syntheses on Akta 10 and 100 (closely resembling the production-scale synthesizer) and OligoProcess DNA/RNA synthesizers were performed using a stainless steel fixed-bed synthesis column. Appropriate amounts of solid supports were packed in the column for synthesis. Dichloroacetic acid (10%) in toluene was used for deblocking of the dimethoxytrityl (DMT) groups from the 5′-hydroxyl group of the nucleotide. Extended detritylation conditions (twice the column volume and contact time as the normal cycle) were used to remove the DMT group from the secondary hydroxyl group of the UnyLinker molecule.

4,5-Dicyanoimidazole (0.7 M) in the presence of *N*-methylimidazole (0.1 M)[27,28] or 1*H*-tetrazole (0.45 M) in MeCN was used as activator during the coupling step. During the coupling step 1.75 equiv of amidites (both deoxy and 2′-*O*-methoxyethylribonucleosides) and a ratio of 1:1 (v/v) of amidite to activator solution were used. Amidite and activator solutions were prepared using low-water MeCN (water content <30 ppm) and were dried further by addition of activated 4Å molecular sieves (~50 g/L). Phosphorothioate linkages were introduced by sulfurization with a 0.2 M solution of phenylacetyl disulfide in MeCN in 3-picoline or pyridine (1:1 v/v) for a contact time of 2–3 min.[6] Phosphate diester linkages were incorporated via oxidation of phosphite triesters using a solution of iodine/tetrahydrofuran/pyridine/water for 2 min. Capping reagents were made to the manufacturer's receipe: Cap A: *N*-methylimidazole/MeCN (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 9.

At the end of synthesis, the support-bound DMT-on oligonucleotide was treated with a solution of triethylamine and MeCN (1:1, v/v) for 2 h to remove acrylonitrile formed by deprotection of cyanoethyl group from phosphorothioate triester.[9a] Subsequently, the solid support containing oligonucleotide was incubated with concentrated aqueous ammonium hydroxide at 55 °C for 13 h to complete cleavage from support, elimination of UnyLinker molecule to liberate 3′-hydroxy group of oligonucleotide and deprotection of base-protecting groups. Removal of 2′-*O*-silyl group was effected using a solution of *N*-methylpyrrolidinone/triethylamine/TEA·3HF (1.5:0.75:1.0, v/v/v) at 65 °C for 3 h. The purification of RNA oligonucleotides was carried out by anion-exchange chromatography and desalted to afford the oligonucleotide.

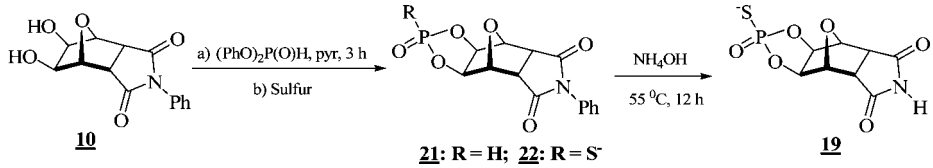
HPLC Analysis and Purification of Oligonucleotides. Analysis and purification of oligonucleotides by reversed phase high performance liquid chromatography (RP-HPLC) was

(26) Other pharmaceutical companies such as Eli Lilly, Oncogenex, iCo Therapeutics, Johnson & Johnson, Australian Therapeutics Limited (ATL) have started using oligonucleotide drugs manufactured using UnyLinker chemistry.

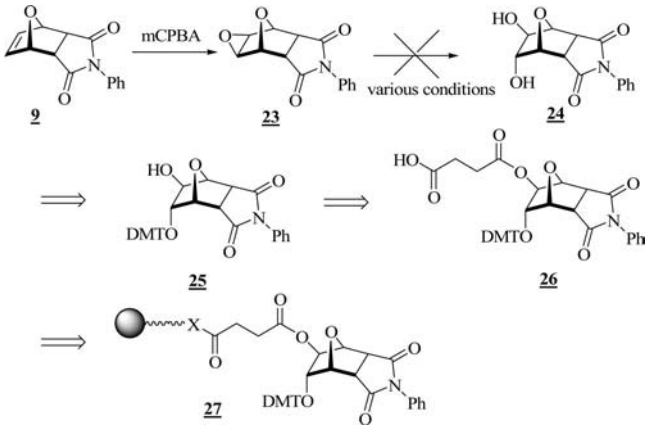
(27) (a) Wang, Z.; Siwkowski, A.; Lima, W. F.; Olsen, P.; Ravikumar, V. T. *Bioorg. Med. Chem.* **2006**, *14*, 5049. (b) Vargeese, C.; Carter, J.; Yegge, J.; Krivjansky, S.; Settle, A.; Kropp, E.; Peterson, K.; Pieken, W. *Nucleic Acids Res.* **1998**, *26*, 1046.

(28) (a) Hayakawa, Y.; Iwase, T.; Nurminen, E. J.; Tsukamoto, M.; Kataoka, M. *Tetrahedron* **2005**, *61*, 2203. (b) Eleuteri, A.; Capaldi, D. C.; Krotz, A. H.; Cole, D. L.; Ravikumar, V. T. *Org. Process Res. Dev* **2000**, *4*, 182 and references cited therein for many other activators.

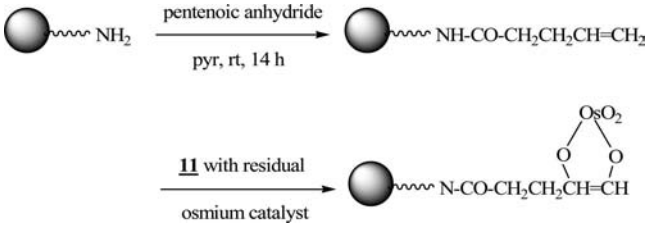
Scheme 6. **Synthesis of cyclic phosphorothioate diester 19**



Scheme 7. **Attempted synthesis of trans-UnyLinker-loaded support 27**



Scheme 8. **Complete removal of residual osmium from UnyLinker 11**



performed on a Novapak C_{18} column (3.9 mm × 300 mm) using a HPLC system (600E System Controller, 996 Photodiode Array Detector, 717 Autosampler). For analysis an MeCN (A)/0.1 M triethylammonium acetate gradient was used: 5% to 35% A from 0 to 10 min, then 35% to 40% A from 10 to 20 min, then 40% to 95% A from 20 to 25 min, flow rate = 1.0 mL/min/50% A from 8 to 9 min, 9 to 26 min at 50% flow rate = 1.0 mL/min, t_R(DMT-off) 10–11 min, t_R(DMT-on) 14–16 min. The DMT-on fraction was collected, evaporated in vacuum, and 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 min at room temperature sodium acetate (2.5 M, 20 μL) was added, followed by cold ethanol (1.2 mL). The mixture was vortexed and cooled in dry ice for 20 min. The precipitate was spun down with a centrifuge, the supernatant was discarded, and the precipitate was rinsed with ethanol and dried under vacuum.

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Supporting Information Available

General methods and experimental details related to UnyLinker molecule, loading conditions to various solid supports, oligonucleotide synthesis; copies of ^{1}H NMR, ^{13}C NMR of various compounds; IP-UV−HPLC−MS, tables containing characterization of synthesized oligonucleotides; tables containing crystallographic data. This material is available free of charge via the Internet at http://pubs.acs.org.

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