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Efficient synthesis of antisense phosphorothioate oligonucleotides using a universal solid support

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Abstract—It is demonstrated that solid support containing a novel universal linker could be efficiently used to synthesize both phosphorothioate oligodeoxyribonucleotides and second-generation $2'$-O-methoxyethyloligoribonucleotides with high yield and quality as judged by ion-pair-liquid chromatography–electrospray mass spectroscopy, ^{31}P NMR and reversed phase HPLC. Analysis of oligonucleotides shows quality being superior to that produced with standard succinyl-linker solid supports, without contamination of materials resulting from linker or support backbone decomposition.
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1. Introduction

Synthesis of oligonucleotides and their analogs have undergone revolutionary changes in the last several years.[1–3] Currently, for small- as well as large-scale, the most widely used approach both by academic institutions and by pharmaceutical companies is the solid-supported synthesis utilizing β-cyanoethyl protected phosphoramidites of various nucleosides. The synthesis is performed in automated DNA/RNA synthesizer machines using controlled pore glass (CPG) or Amersham Biosciences' HL30 or PS200 polymeric solid support. The $3'$-nucleoside is linked to solid support generally through a succinyl-linker to give support-bound nucleoside. The synthesis consists of stepwise coupling of individual monomeric units followed by oxidation of resulting phosphite triester with iodine to give the phosphate triester. In case of phosphorothioate analogs sulfurization is performed and the reagent of choice is phenylacetyl disulfide (PADS)[4,5] or 3H-1,2-benzodithiole-3-one-1,2-dioxide (Beaucage reagent).[6,7] Thus, four solid supports are required for synthesis of oligodeoxyribonucleotides. The advancement of antisense therapeutics has resulted in use of RNA–DNA–RNA chimeric molecules wherein the wings consists of $2'$-O-alkyl RNA nucleotides ($2'$-O-methoxyethyl or 2-O-methyl).[8,9] This has necessitated the need to have a large number of pre-derivatized polymer supports. To obviate this need, several research groups have proposed the concept of a universal solid support. Gough et al. were the first to propose a universal support containing a linker, 3-anisoyl-$2'$-($3'$-O-

benzoyluridine-$5'$-O-succinyl, attached to CPG.[10] Since then several groups have reported various analogs of universal linkers.[11–26] These supports employ nucleosidic material, which does not get incorporated into oligonucleotide chains and hence goes to waste. Alternatively, some non-nucleoside-based universal supports have also been proposed, but cleavage of oligomers is either tedious or involves use of salts or other conditions. In addition, in our hands, evaluation of at least three of reported universal linker procedures when repeated and analyzed carefully by ion-pair-liquid chromatography–electrospray mass spectroscopy (a technique known to reveal small amounts (<1%) of adducts or linker molecules), showed the quality of oligonucleotides is not the same as compared to standard method and was contaminated with the pendent linker molecule. Also, there was considerable (ca. 10%) amount of oligonucleotide still attached to solid support making it an inefficient approach. These limitations have so far, outweighed the added convenience of one support material for every sequence, and pre-derivatized supports are still predominantly preferred.

Herein, we have redesigned the vicinal diol-based linker system so that the linker remains firmly bound to support during the deprotection and cleavage steps of DNA synthesis. Thus, any DNA molecule that is released from support is not contaminated with linker molecule.

2. Results and discussion

Early on we realized that a major drawback in design of various universal linker molecules is the presence of a hydroxyl group that was derivatized with a cleavable group

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such as succinyl or oxalyl. Instead, if the linker molecule possesses a non-hydroxyl functionality (e.g., carboxyl) that could be directly attached/coupled to amino-derivatized solid support to form an amide bond, then after oligomerization, the first step during ammonia deprotection is the unmasking of the protecting group on neighbouring hydroxyl group followed by intra-molecular attack on phosphate/phosphorothioate group similar to base-catalyzed hydrolysis of RNA leading to release of oligonucleotide. Thus, any oligonucleotide released from solid support has to be of good quality since the linker molecule will be still attached to solid support. In addition, if synthesis were to be efficient we have to demonstrate that there is no measurable amount of oligonucleotide still attached to solid support. Thus both quality and quantity (yield) issues could be addressed successfully if the molecule is designed appropriately.

2.1. Synthesis of universal linker and loading to support

In our earlier initial communication, we proposed a novel compound **4** as an efficient and high quality yielding universal linker molecule.[27] Synthesis and loading of this molecule **4** is shown in Scheme 1. The synthesis starts with bis-hydroxylation of olefin **1** according to literature procedure. No significant changes were made to this procedure after several attempts were made to improve yield and product obtained was used further without any additional purification. Chemoselective protection of diol **2** with 4,4′-dimethoxytrityl chloride happened to be tricky. The molecule was highly soluble in water in spite of having a large lyphophilic DMT group (possibly due to presence of two carboxyl and one hydroxyl groups) and care should be taken to obtain reasonable yield (22%). The overall yield of two steps starting from olefin **1** was low ($< 10\%$) but due to inexpensive nature of starting materials, it did not discourage us from scaling up this molecule. We were able to scale up approximately 0.5 kg in a single batch. Treatment of the DMT compound **3** with acetic anhydride in pyridine gave the acetoxy protected cyclic anhydride **4** as colorless foam in almost quantitative yield. Loading of the universal linker molecule **4** to solid support was carried out in pyridine with amino-derivatized support to give the appropriate loading. The free carboxyl group generated

during ring opening during loading is capped by forming an amide bond with *n*-propyl amine using HATU and HOBt coupling condition. A loading of 90 μmol/g was obtained while using HL30 support (recommended loading level by manufacturer).

## 2.2. Synthesis of phosphorothioate oligodeoxyribonucleotide

To demonstrate this methodology, a 20-mer phosphorothioate oligodeoxyribonucleotide [PS-d(GTTCTCGCTGG TGAGTTTCA), ISIS 3521] was chosen as an example. Three syntheses were carried out (standard nucleoside containing succinate linked support as control and universal linker attached support in duplicate). The results are summarized in Table 1.

2.3. Investigation on yield of oligonucleotide

To determine if any oligonucleotide was still attached to support leading to loss of yield, the support after cleaving and washing thoroughly to remove the synthesized oligonucleotide, was incubated with aqueous methylamine at 55 °C for 14 h. The solution after filtration of solid support was analyzed by HPLC and also by UV measurement. No detectable level of oligonucleotide was observed. Subsequently, the support was dried thoroughly under high vacuum and then tested for presence of DMT group by the usual acid treatment (*p*-toluene sulphonic acid in CH_3CN). No orange color was observed. This clearly indicates that release of oligonucleotide is quantitative (Scheme 2).

2.4. Investigation on increased level of ($n-1$)-mer

The quality of an oligonucleotide could be measured and quantitated by various analytical methods. Depending upon the technique used, full or partial information could be obtained. Strong anion exchange chromatography, mass spectrometry, and ^{31}P NMR spectroscopy may be used for quantitative assessment of PO-content. Capillary gel electrophoresis (CGE) could be used for quantitation of deletion sequences [($n-1$)-mers]. For an analytical method to be good, it should have been demonstrated for its necessary accuracy, precision, linearity, range, selectivity and ruggedness for use in routine testing. In our laboratories, we use state-of-the-art ion-pair high performance liquid chromatography–mass spectroscopy (IP-LC–MS) technique as a specific, accurate, and sensitive means of quantitating oligonucleotides containing deletion sequences, depurinated sequences and 3′-terminal phosphorothioate monoester within a matrix of PS-oligonucleotides. Removal of deletionmers [($n-1$)-mers; internal and terminal] on preparative scale using chromatographic separation technology is difficult to achieve without significant yield loss. Incomplete detritylation, potential re-tritylation (due to reversible reaction), incomplete coupling followed by incomplete capping and some other unknown mechanisms are a potential source of formation of ($n-1$)-mers. The extent of ($n-1$)-mer formation is frequently used as a quality measure of the performance of detritylation conditions.
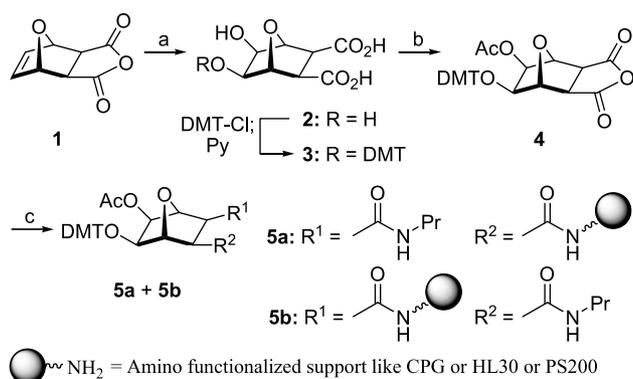

Scheme 1. Synthesis of universal linker loaded support: (a) (i) $OsO_4/H_2O_2/$ H_2O/acetone/*t*BuOH; 30 °C, 24 h; (ii) DMT chloride, pyridine; rt, 20 h; (b) (i) Ac_2O/NMI/Py; (c) (i) Py, rt; (ii) HATU/HOBT/MeCN/Py; (iii) *n*PrNH$_2$/MeCN; (iv) Ac_2O/NMI/Py.

Table 1. Comparison of oligonucleotides synthesized using standard succinate and universal linker supports

Support used for synthesis	Crude yield (mg/μmol)	Crude full length (%) (RP HPLC)	Purified full length (%) (IP-LC–MS)	(Depurinated species) (%) (IP-LC–MS)	P=S:P=O (^{31}P NMR)	P=S:P=O (IP-LC–MS)
dA succinate (**0849-149**)	6.2	70	82.4	5.1	99.66:0.34	99.59:0.41
Universal linker (**0849-150**)	5.9	77	86.6	2.2	99.62:0.38	99.64:0.36
Universal linker (**0849-151**)	6.4	77	87.2	2.3	99.60:0.40	99.61:0.39

Depurinated species include *n*-G, *n*-A/*n*-G+H_2O, *n*-A+H_2O, 3′-TPT.
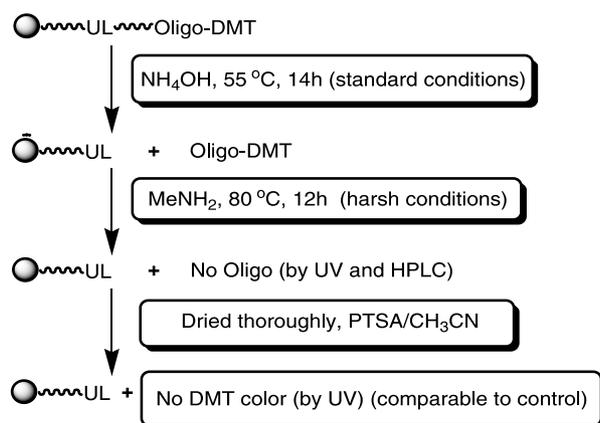
Scheme 2. Investigation of any oligonucleotide attached to support leading to yield loss.

Analyses of the synthesized phosphorothioate oligonucleotides by IP-LC–MS clearly indicate that removal of DMT group from the secondary hydroxyl group of the universal linker molecule is slow as shown by increased levels of dA (as compared to oligonucleotide synthesized using succinate loaded support) (Table 2). We reasoned that all other cycles and conditions being equal, the increased level of dA could come from inefficient detritylation during the first cycle. This kind of precise information would not have been possible with other kinds of techniques. In addition, we have shown earlier that increased depurination occurs when base-protected deoxyadenosine has an electron withdrawing group attached at 3′-position (succinate group) as compared to having a phosphate/thioate group. A majority of this 3′-terminal depurinated species undergoes elimination followed by fragmentation during ammonia incubation step leading to formation of 3′-terminal phosphorothioate monoester (3′-TPT).[28] This possibility is eliminated or substantially reduced while using universal linker molecule (Table 2). We also confirmed that increased levels of dA is not due to inefficient coupling as use of large excess of

Table 2. IP-LC–MS analysis of oligonucleotide synthesized using standard succinate and universal linker supports

Species	**0829-149** (%)	**0829-150** (%)	**0829-151** (%)
n [ISIS 3521]	84.6	84.8	85.1
P=O	7.0	5.5	4.9
n-dG	1.2	1.0	1.3
n-dA	**0.5**	**4.9**	**4.9**
T	2.0	1.4	1.6
n-dC	0.7	0.8	0.8
n-G	0.2	0.1	0.1
n-A/*n*-G+H_2O	0.7	0.7	0.5
n-A+H_2O	**2.2**	**0.5**	**0.5**
3′-TPT	**0.9**	**0.3**	**0.3**
Sum	100.0	100.0	100.0

phosphoramidite synthon (15 equiv), extended contact time (20 min), use of recycling during coupling and various other efforts did not lead to any measurable improvement.

2.5. Optimization on first detritylation step

There are at least two ways of removing the DMT group from the universal linker molecule efficiently viz., use of excess deblock solution and extending the contact time. After several experiments aimed at optimized detritylation condition, we found that efficient removal of DMT group could be achieved by using the same volume of acid solution but slowing down the delivery pump to double the contact time. Alternatively, the detritylation cycle could be repeated one more time (twice the deblock volume and time). With this optimized detritylation condition, we resynthesized the phosphorothioate oligonucleotide (ISIS 3521), purified and analyzed by ^{31}P NMR, RP-HPLC and IP-LC–MS. Comparable results were achieved between the two oligonucleotides synthesized (Table 3).

Table 3. IP-LC–MS analysis of oligonucleotide synthesized using optimized detritylation cycle for universal linker support (**0830-10**= using nucleoside-loaded support; **0830-11**=using universal linker loaded support)

Species	**0830-10** (%)	**0830-11** (%)
n [ISIS 3521]	85.8	90.0
P=O	6.8	6.0
n-dG	0.4	0.3
n-dA	**0.5**	**0.6**
T	0.9	0.5
n-dC	0.5	0.4
n-G	0.4	0.2
n-A/*n*-G+H_2O	1.0	0.7
n-A+H_2O	**2.4**	**0.7**
3′-TPT	**1.3**	**0.6**
Sum	100.0	100.0

2.6. Synthesis of 2′-*O*-methoxyethyl modified RNA chimera phosphorothioate oligonucleotides

Due to instability of wild-type DNA, phosphorothioate oligonucleotides, where one of the non-bridging oxygens of the internucleotide phosphate is formally replaced by a sulfur atom, are currently the modification of choice for design and development of therapeutic drugs. To further increase the therapeutic value of these phosphorothioate drugs, several nucleoside modifications have been investigated. Of these, 2′-*O*-methoxyethyl (MOE) modified oligoribonucleotide chimera has been selected in our laboratories (at Isis Pharmaceuticals) and multiple drugs are in various stages of human clinical trials against a variety of diseases.

Table 4. Comparison of oligonucleotides synthesized using succinate and universal linker supports

Support used for synthesis	Crude yield (mg/μmol)	Crude full length (%) (RP HPLC)	Purified full length (%) (IP-LC–MS)	$(n-1)$ (%) (IP-LC–MS)	P=S:P=O (IP-LC–MS)
MOE meC succninate (**0830-45**)	6.76	77	91.1	2.9	3.8
Universal linker (**0830-40**)	7.60	74	90.0	3.0	4.0
Universal linker (**0830-43**)	7.13	70	91.2	3.2	2.9

To demonstrate the applicability of universal linker molecule for synthesis of MOE oligonucleotides, a 20-mer phosphorothioate, [PS-MOE-(GCTCC)-d(TTCCAC-TGAT)-MOE-(CCTGC)-3′] (ISIS 113715) where deoxycytidine and MOE cytidine have 5-methyl substitution was chosen as an example. For control experiment, MOE meC succinate loaded PS200 Primer support (200 μmol/g) was used. Similar cycle conditions were employed for oligonucleotide synthesis like phosphorothioate oligodeoxyribonucleotide including optimized extended condition for the first cycle involving removal of DMT group from the universal linker molecule. The results are summarized in Table 4. In addition, a portion of 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) (Table 5). The level of n-MOE meC (first base attached to support) and n-MOE meU (since both deletionmers have same mass) compare well between three experiments indicating that overall quality of oligonucleotide obtained using universal linker attached support is good with no detectable levels of any 3′-modifications. The sample was analyzed by ^{31}P NMR, analytical RP-HPLC, and IP-LC–MS.

Table 5. IP-LC–MS analysis of oligonucleotides synthesized using MOE meC succinate and universal linker supports

Species	**0830-45** (%)	**0830-40** (%)	**0830-43** (%)
n [ISIS 113715]	93.2	92.9	94.0
P=O	3.8	4.0	2.9
n-dG	0.0	0.1	0.1
n-dA	0.1	0.1	0.1
n-T/n-5medC	0.3	0.4	0.4
n-MOE G	0.5	0.2	0.3
n-MOE meU/n-MOE meC	0.8	1.0	1.3
n-G	0.0	0.1	0.1
n-A/n-G+H_2O	0.8	0.7	0.6
n-A+H_2O	0.3	0.4	0.2
3′-TPT	0.2	0.1	0.0
Sum	100.0	100.0	100.0

Subsequently, multiple oligonucleotides (both deoxy and MOE) were synthesized at different scales, various supports and different synthesizers (Amersham Biosciences Akta 10 and Akta 100 DNA/RNA synthesizer); yield and quality were found to be equivalent or slightly better compared to succinate loaded supports.

2.7. Mechanism of release of oligonucleotide from support

A reasonable mechanism for release of oligonucleotide is depicted in Scheme 3. At the end of oligonucleotide synthesis, treatment of the support with triethylamine:aceto-nitrile removes the cyanoethyl group and generates the phosphate/phosphorothioate diester charged backbone. Subsequent treatment with concentrated aqueous ammonium hydroxide, removes the acetyl protection from the vicinal hydroxyl group. Intra-molecular attack on adjacent phosphate/phosphorothioate center followed by cyclization releases the 3′-hydroxyl oligonucleotide (Scheme 3).

2.8. Economic and quality impact of using universal linker solid support

There are both direct and indirect cost savings while using universal linker loaded solid support. While it is difficult to precisely quantify the savings, overall there is definitely substantial economic benefit by switching over to one inventory of this solid support instead of at least eight different (four deoxy and four MOE) supports. In addition, while using succinate loaded supports, in particular containing 5-methyl MOE cytidine nucleoside, we have observed instability of the benzoyl group as evidenced by formation of longer formation, possibly arising out of branching from the exocyclic amine group.[29] Currently, we don't know if this happens during loading protocol or upon storage of the support at room temperature. We do not observe this longer formation while using universal linker loaded support. Besides these benefits, one major advantage for therapeutic applications is that this universal linker loaded solid supports are no longer considered as starting materials but rather as raw materials. Eliminating a starting material in drug development is a considerable advantage towards cost reduction (both direct and indirect costs) and more than compensates the additional cost involved in performing one more cycle containing an amidite as compared to nucleoside-loaded supports.

3. Summary and conclusions

A novel, conformationally pre-organized non-nucleosidic universal solid support for oligonucleotide synthesis has been developed. The solid support featured two chemically equivalent hydroxy groups locked in *syn*-periplanar orientation and orthogonally protected with 4,4′-dimethoxytrityl and acetyl groups. The solid support was extensively tested in preparation of phosphorothioate analogs containing 2′-deoxy and 2′-O-methoxyethylnucleoside residues at the 3′-terminus. Upon completion of oligonucleotide chain assembly, the support-bound oligonucleotide material was treated with concentrated ammonium hydroxide, which removed the *O*-acetyl protection. The deprotected hydroxyl group then affected 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 solution. In addition, the solid support containing this universal linker molecule is stable at room
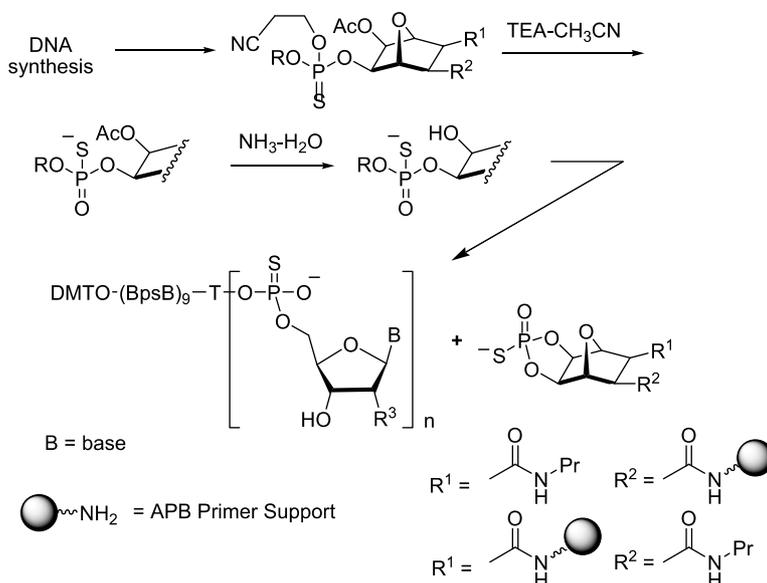
Scheme 3. Mechanism for release of oligonucleotide from support.

temperature for extended period of time (at least 1 year) as shown by consistent good quality of oligonucleotides produced with it. Extended detritylation condition to remove the DMT group further indicates that this group is very stable.

4. Experimental

4.1. Materials and methods

Anhydrous acetonitrile (water content $< 0.001\%$) was purchased from Burdick and Jackson (Muskegon, MI). 5′-*O*-Dimethoxytrityl-3′-*N,N*-diisopropylaminoe-3′-*O*-(2-cyanoethyl) phosphoramidites (T, dA^{bz}, dC^{bz}, dG^{ibu}) were purchased from Amersham Pharmacia Biotech, Milwaukee, WI. Toluene was purchased from Gallade, Escondido, CA. Dichloroacetic acid was purchased from Clariant Life Sciences. All other reagents and dry solvents were purchased from Aldrich and used without further purification. Primer support HL30 and PS200 was obtained from Amersham Biosciences, Uppsala, Sweden. 1*H*-Tetrazole was purchased from American International Chemical, Natick, MA. Phenylacetyl disulfide (PADS) was purchased from Acharya Chemicals, Dombivli, India. ^{31}P NMR spectra were recorded on a Unity-200 spectrometer (Varian, Palo Alto, CA) operating at 80.950 MHz. Capillary gel electrophoresis was performed on a eCAP ssDNA 100 Gel Capillary (47 cm) on a P/ACE System 5000 using Tris/borate/7 M urea buffer (all Beckman), running voltage 14.1 kV, temperature 40 °C. Thin-layer chromatography was performed on silica gel 60F-254 (Merck) plates and compounds were detected under shorter-wavelength UV light.

4.1.1. Preparation of universal linker 1α,2α,3α, 4α,5α,6α)-5,6-dihydroxy-7-7-oxabicyclo[2.2.1]hetane-2,3-dicarboxylic acid (2). The title compound was synthesized by bis-hydroxylation of (3α*R*,4*S*,7*R*,7α*S*)-rel-3α,4,7,7α-tetrahydro-4,7-epoxyisobenzofuran-1,3-dione, **1** with hydrogen peroxide in presence of osmium tetroxide

(OsO₄) as described in literature and used without any additional purification.[30]

4.1.2. (1α,2α,3α,4α,5α,6α)-5-Hydroxy-6-(4,4′-dimethoxy-trityloxy)-7-oxabicyclo[2.2.1]heptane-2,3-dicarboxylic acid (3). 4,4′-Dimethoxytrityl chloride (38.75 g, 114.5 mmol) was added in aliquots to a solution of compound **2** (16.95 g, 77.5 mmol) in anhydrous pyridine (200 mL) over a period of 6 h. The reaction mixture was stirred overnight at room temperature. The solvent was evaporated, and the residue taken up in ethyl acetate (1 L) and 1 M aqueous triethylammonium acetate (100 mL). The organic solution was washed with 1 M aqueous triethylammonium acetate (120 mL), diluted with methanol (100 mL), dried over sodium sulfate, and evaporated. The residue was dissolved in ethyl acetate (250 mL) and treated with ether (750 mL). A colorless amorphous solid, which precipitates was collected, washed with ether, and dried to give pure product as a free acid (27.5 g, 56%). ^{1}H NMR (pyridine-d_5): δ 7.87 (2H, m); 7.72 (2H, m); 7.66 (2H, m); 7.34 (2H, m); 7.25 (1H, m); 6.96 (4H, m); 5.35 (1H, d, $J=1.2$ Hz); 4.39 (1H, d, $J=6.4$ Hz); 4.23 (1H, d, $J=6.4$ Hz); 3.85 (1H, d, $J=1.2$ Hz); 3.68 (3H, s); 3.65 (3H, s); 3.26 (1H, d, $J=9.2$ Hz); 3.05 (1H, d, $J=9.2$ Hz). ^{13}C NMR (100.573 MHz, DMSO-d_6): δ 172.1, 171.9, 158.5, 145.7, 136.7, 136.2, 130.0, 129.9, 129.1, 128.1, 127.9, 127.8, 126.9, 87.4, 83.6, 82.1, 76.3, 74.5, 55.3, 46.8, 46.5. HRESMS: calcd for $C_{29}H_{27}O_9$ (M⁻), 519.1655; found, 519.1663.

4.1.3. (1α,2α,3α,4α,5α,6α)-5-Acetoxy-6-(4,4′-dimethoxy-trityloxy)-7-oxabicyclo[2.2.1]heptane 2,3-dicarban-hydride (4). Compound **3** (1.57 g, 3.0 mmol) was treated with acetic anhydride (3.0 g) and pyridine (15 mL) for 3 h at room temperature. The mixture was concentrated and co-evaporated with pyridine (5×15 mL) to give the title compound as colorless foam. Due to instability nature of molecule (being an anhydride), further purification was not attempted and was used as such in the next step. ^{1}H NMR (pyridine-d_5): δ 7.73 (2H, m); 7.60–7.55 (overlaps with a solvent peak, m); 7.40 (2H, m); 7.30 (1H, m); 7.00 (4H, m);

5.71 (1H, d, $J = 6.4$ Hz); 5.21 (1H, s); 4.52 (1H, d, $J = 6.4$ Hz); 3.91 (1H, d, $J = 7.2$ Hz); 3.74 (3H, s); 3.73 (3H, s); 3.67 (1H, s); 3.63 (1H, d, $J = 7.2$ Hz); 2.26 (3H, s). ^{13}C NMR (100.573 MHz, DMSO-d_6): δ 176.0, 173.3, 158.5, 145.4, 136.6, 136.2, 130.1, 129.9, 129.1, 128.3, 127.8, 127.8, 126.9, 87.4, 83.6, 82.1, 76.3, 74.5, 55.3, 46.8, 46.5, 20.9. IP-LC–MS: calcd for $C_{30}H_{27}O_9$ (M$^-$), 531.161; found, 531.204.

4.2. Loading of universal linker molecule (4) to HL30 solid support

Amino-derivatized HL30 primer support (4.0 g, 0.51 mmol) was gently shaken with compound **4** (1.39 g, 2.55 mmol) in pyridine (17 mL) for 4 h. The suspension was filtered, and solid support was washed with pyridine (3 × 20 mL). The solid support was additionally washed with ethyl acetate, dried, and capped by treating with a mixture of Ac$_2$O–pyridine–N-methylimidazole–THF (10/10/10/70, v/v/v/v) for 3 h at room temperature. Finally, the support was washed with acetonitrile (CH$_3$CN), ethyl acetate and dried. Loading of **5** (94 ± 0.4 μmol/g) was determined by the standard DMT assay.

4.3. Capping of solid support

The solid support from previous step (1.0 g) was treated with 0.2 M HATU and 0.15 M HOBT in CH$_3$CN–pyridine (4/1, 6 mL) for 5 min. The liquid phase was removed, and solid support was treated with 0.5 M n-propylamine in CH$_3$CN (5 mL) for 15 min. The solid support was then washed with CH$_3$CN (5 × 10 mL) and capped with a mixture of Ac$_2$O–pyridine–N-methylimidazole–THF (10/10/10/70, v/v/v/v) for 8 h at room temperature. Finally, the solid support **5** was washed with CH$_3$CN (50 mL), ethyl acetate (50 mL) and dried. Loading of **5** (90 ± 0.4 μmol/g) was determined by standard DMT assay.

4.4. Loading of universal linker molecule (4) to PS200 and controlled pore glass solid supports

Loading of universal linker molecule (**4**) to amino-derivatized supports like PS200 and controlled pore glass (CPG) were similar to the above described protocol for HL30 except that reagents were adjusted accordingly to obtain loadings of 200 ± 5 and 45 ± 5 μmol/g, respectively.

4.5. Oligonucleotide synthesis

All syntheses were performed on a Amersham Biosciences OligoPilot II DNA/RNA synthesizer at approximately 160 μmol scale in a 6.33 mL fixed column using β-cyanoethyl phosphoramidite synthons (1.75 equiv, 0.2 M in CH$_3$CN). 1H-Tetrazole (0.45 M in CH$_3$CN) was used as activator and phenylacetyl disulfide (PADS) (0.2 M in 3-picoline/CH$_3$CN 1:1, v/v) as sulfur transfer reagent.[31] 3% Dichloroacetic acid in toluene was used for removal of acid-labile dimethoxytrityl group[32,33] Capping reagents were made to the recommended Amersham Biosciences receipe: Cap A: N-methylimidazole–CH$_3$CN (1/4 v/v), Cap B: acetic anhydride–pyridine–CH$_3$CN (2/3/5, v/v/v). Solid supports loaded with universal linker molecule at recommended loading levels were used. Amidite and tetrazole

solutions were prepared using anhydrous CH$_3$CN (ca. 10 ppm) and were dried further by addition of activated 4 Å molecular sieves (∼50 g/L). Details of synthesis cycle are given in Table 6. At the end of each synthesis, the support was thoroughly dried to determine the crude weight yield, treated with a solution of triethylamine–CH$_3$CN (1/1, v/v) at room temperature for 2 h to remove the β-cyanoethyl protecting groups,[34] then treated with 30% aqueous ammonium hydroxide solution for 12 h at 55 °C to effect release from support and base deprotection. Yield (expressed in mg of oligonucleotide/μmol of support),[35] ^{31}P NMR and analytical RP-HPLC (full length determination) data were collected for each synthesis. 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). The final product after lyophilization was obtained as a colorless hygroscopic solid (yield: 0.56–0.62 g).

Table 6. Synthesis parameters of cycle used on pharmacia OligoPilot II synthesizer

Step	Reagent	Volume (mL)	Time (min)
Detritylation	3% Dichloroacetic acid/toluene	72	1.5
Coupling	Phosphoramidite (0.2 M), 1H-tetrazole (0.45 m) in acetonitrile	10, 15	5
Sulfurization	PADS (0.2 M) in 3-picoline–CH$_3$CN (1/1, v/v)	36	3
Capping	Ac$_2$O/pyridine/CH$_3$CN, NMI/CH$_3$CN	24, 24	2

4.6. 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 Auto-sampler). For analysis an acetonitrile (A)/0.1 M triethylammonium acetate gradient was used: 5–35% A from 0 to 10 min, then 35–40% A from 10 to 20 min, then 40–95% 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 and was evaporated in vacuum, redissolved in water and the DMT group was removed as described below.

4.7. 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.

4.8. MS sample preparation

HPLC-purified and dedimethoxytritylated oligonucleotide was dissolved in 50 μL water, ammonium acetate (10 M,

5 µL) and ethanol were added and vortexed. The mixture was cooled in dry ice for 20 min and after centrifugation the precipitate was isolated. This procedure was repeated two more times to convert the oligonucleotide to the ammonium form.

4.9. IP-HPLC–MS analysis

HPLC with UV and MS detection was performed using an HPLC system consisting of a binary pump, a degasser, a column oven and a variable wavelength UV detector. After passing through the UV detector, the column eluate was introduced directly into a single quadrupole, electrospray mass spectrometer. Samples were separated using a C_{18} column (3 µm, 2×150 mm) and eluted at 0.2 mL/min with a gradient of CH_3CN in 5 mM tributylammonium acetate (TBAA) as the ion-pairing agent.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.tet.2006.02.040. Supplementary material contains copies of ^{31}P (D_2O) NMR, reversed-phase HPLC and IP-LC–MS analysis of phosphorothioate oligonucleotides (ISIS 3521 and ISIS 113715) made using standard nucleoside succinate loaded support and universal linker loaded support.

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35. Experience has taught us that yields expressed in terms of weight/µmol of support are more reliable than those expressed in terms of optical density/µmol.