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PAPER

Synthesis of oligoribonucleotides with phosphonate-modified linkages†

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Solid phase synthesis of phosphonate-modified oligoribonucleotides using 2′-*O*-benzoyloxymethoxymethyl protected monomers is presented in both 3′→5′ and 5′→3′ directions. Hybridisation properties and enzymatic stability of oligoribonucleotides modified by regioisomeric 3′- and 5′-phosphonate linkages are evaluated. The introduction of the 5′-phosphonate units resulted in moderate destabilisation of the RNA/RNA duplexes (ΔT_m −1.8 °C/mod.), whereas the introduction of the 3′-phosphonate units resulted in considerable destabilisation of the duplexes (ΔT_m −5.7 °C/mod.). Molecular dynamics simulations have been used to explain this behaviour. Both types of phosphonate linkages exhibited remarkable resistance in the presence of ribonuclease A, phosphodiesterase I and phosphodiesterase II.

Introduction

The unique properties of RNA have stimulated the development of a variety of applications in diagnostics and therapeutics and in basic research where RNA can be used as a catalytic agent (ribozymes),[1] an affinity ligand (aptamers)[2] or an agent to induce gene silencing (RNA interference, micro RNA).[3,4] Because of this usefulness, the demand for modified RNA has increased dramatically in the past decade.

The chemical synthesis of RNA is a more difficult task than the synthesis of DNA because the 2′-hydroxy group of the ribose needs to be protected. The correct choice of a 2′-*O*-protecting group can considerably influence the length of the coupling time, the coupling yield and the quality of the final product. Thus, this protecting group must be stable throughout the cycles of solid phase synthesis, sterically-unhindered so as to not interfere with the chain growth and readily removable under conditions where the final RNA is completely stable.

The *tert*-butyldimethylsilyl (TBDMS) group[5] was the first 2′-protecting group used in commercial RNA synthesis. Since then, several protecting groups have been proposed in place of TBDMS, such as triisopropylsilyloxymethyl (TOM),[6] bis(2-acetoxyethyloxy)methyl (ACE),[7] *tert*-butyldithiomethyl (DTM),[8] 2-cyanoethoxymethyl (CEM),[9] 2-(4-toluylsulfonyl)ethoxymethyl (TEM),[10] pivaloyloxymethyl (POM),[11] 1,1-dioxo-1λ6-thiomorpholine-4-carbothioate (TC),[12] or acetal levulinyl ester (ALE).[13] The latter three groups are examples of a new trend in RNA synthesis – the use of a base-labile 2′-protecting group that allows for the one-pot deprotection of the 2′-hydroxyl and nucleobases and cleavage from the solid support.

Although there are several protecting groups available for synthesis in 3′→5′ direction, there is also a demand for 5′→3′ synthesis tailored, for example, to the synthesis of RNA with 5′-modified internucleotide linkages or for convenient introduction of ligands and labels at the 3′-end.

In our case, the need to develop new methodology for the synthesis of RNA in the reverse direction has originated from our intention to synthesise oligoribonucleotides that are modified by various nucleoside-5′-phosphonates. These compounds have been of interest to us for many years.[14]

Until now, only the TBDMS protecting group has been reported for RNA synthesis in the reverse direction using phosphoramidite condensation method.[15] Silyl protecting groups are removed by fluoride anions. However, this deprotection requires time-consuming desalting steps before the final purification of an oligoribonucleotide by HPLC. Moreover, 5′-phosphonate monomers are introduced into the oligonucleotide chain by the phosphotriester condensation method using pyridine as the coupling solvent. Thus during the phosphotriester coupling step, the use of the 2′-*O*-TBDMS group in the elongated unit might not be applicable because of possible migration of TBDMS to free 3′-hydroxy group under basic conditions.

Therefore, we report here a new RNA synthesis strategy based on the base-labile 2′-*O*-benzoyloxymethoxymethyl (BOMOM) group. Using this protecting group, we have synthesised two sets of oligoribonucleotides modified by regioisomeric 3′- and 5′-phosphonate linkages in both the 3′→5′ and 5′→3′ directions, respectively (Fig. 1). It is worth noting that only a few modifications of internucleotide linkages have been reported in the *ribo* series so far, and among these reports, phosphorothioate and

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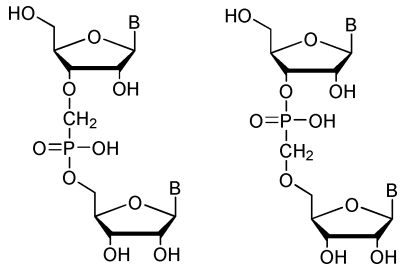


Fig. 1 Regioisomeric 3′-phosphonate (Bpc-B) and 5′-phosphonate (B-pcB) linkages.

boranophosphate oligoribonucleotides are the most widely used as research and diagnostic tools.[16]

Results and discussion

Synthesis of monomeric building blocks

First, we prepared appropriate 3′- and 5′-phosphoramidite building blocks as shown in Scheme 1.

In the 3′-series, 2′-*O*-BOMOM (**3a–d**) and 3′-*O*-BOMOM (**5a–d**) derivatives were prepared by the reaction of 5′-*O*-DMTr nucleosides **1a–d** and benzoyloxymethoxymethyl chloride **10** (BOMOM-Cl)[17] in the presence of dibutyltindichloride, followed by the separation of the regioisomers on silica gel. By this method, the desired 2′-*O*-BOMOM derivatives **3a–d**, eluting as an TLC faster isomer, were obtained in 32–48% yield. In case of the guanosine derivative, we did not observe formation of 3′-*O*-BOMOM derivative **5b**.

In our preliminary experiments, we found the cyanoethyl (CE) protection of the phosphate groups incompatible with BOMOM group. During the basic deprotection step of a model oligoribonucleotide, we observed simultaneous cleavage of both groups which resulted in an undesired cleavage of internucleotide linkages. Therefore we decided to use methyl protection of the phosphate groups. Moreover, the phosphonate monomers are introduced into the oligonucleotide chain by the phosphotriester condensation method using 4-methoxy-1-oxido-2-pyridylmethyl group (MOP) as an intramolecular coupling catalyst.[18,19] Since both methyl and MOP groups are removed by treatment with benzenethiol, we obtained uniform phosphate/phosphonate deprotection protocol. Therefore, compounds **3a–d** were converted to the corresponding methyl phosphoramidites **7a–d** using methyl *N*,*N*,*N*′,*N*′-tetraisopropylphosphordiamidite.

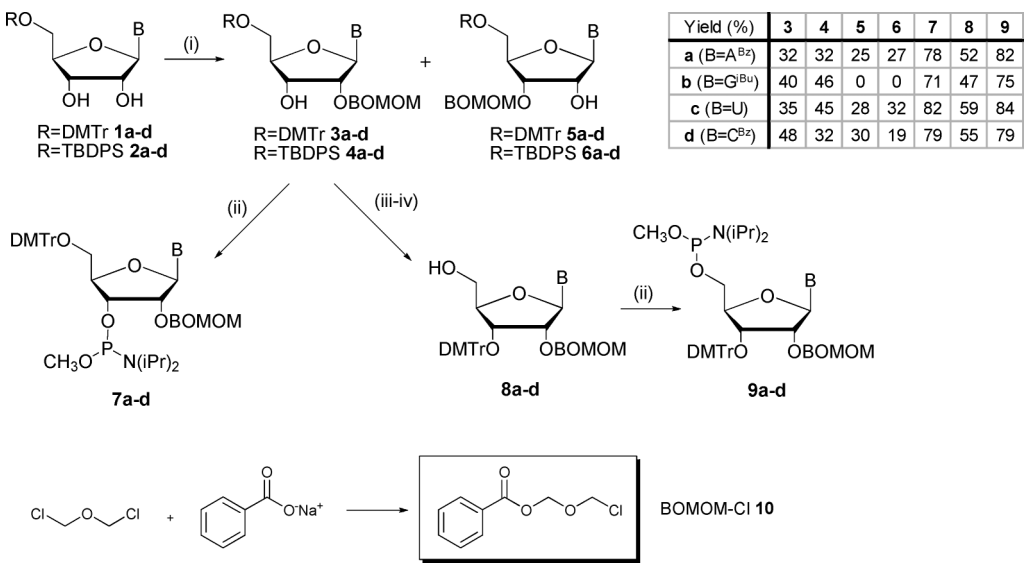
In the 5′-series, the synthetic path proceeded in a similar manner. 5′-*O*-TBDPS derivatives **2a–d** were protected with the BOMOM group, and after separation of the regioisomers on silica gel, the desired isomers **4a–d** were dimethoxytritylated with dimethoxytritylchloride in the presence of silver triflate. Once again, we did not observe formation of 3′-*O*-BOMOM derivative **6b** in the case of the guanosine derivative. Cleavage of the 5′-*O*-silyl group with TBAF (**8a–d**) followed by the phosphitylation reaction afforded 5′-phosphoramidites **9a–d** in good yields.

The stability of the BOMOM group in compounds **4** was examined in the presense of 3% DCA in DCM (DMTr deblocking conditions) and pyridine (phosphonate coupling conditions) by the NMR experiments. The BOMOM group was found completely stable under these conditions, neither migration, cleavage nor formation of the 2′,3′-*O*-cyclic formal was observed.

BOMOM-Cl **10** was prepared by the alkylation of sodium benzoate with one equivalent of bis(chloromethyl)ether.

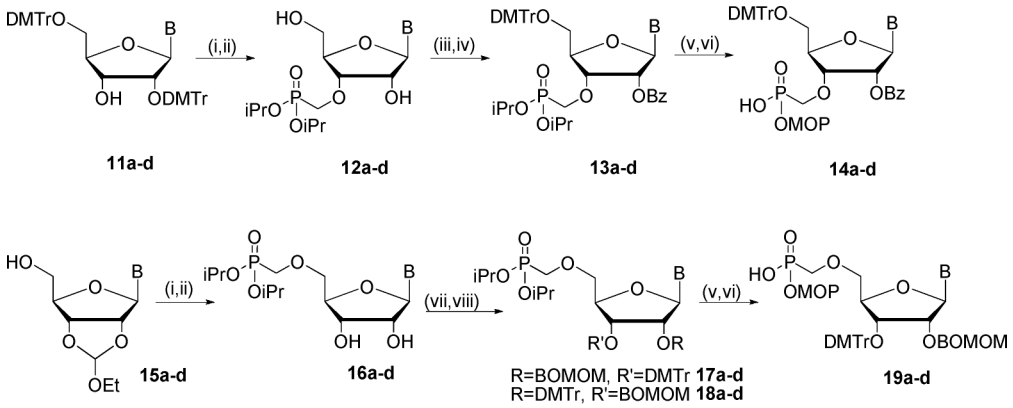
Next, we prepared the appropriate 3′- and 5′-phosphonate monomers according to the method shown in Scheme 2.

In the 3′-series, 2′,5′-bis-DMTr nucleosides **11a–d** were phosphonylated with diisopropyl tosyloxymethylphosphonate in the presence of sodium hydride. Upon acidic deprotection, these nucleosides provided 3′-phosphonates **12a–d**. 5′-Dimethoxytritylation of these compounds followed by benzoylation of the 2′-hydroxy group afforded fully protected phosphonates **13a–d**. Note that in the case of 3′-phosphonate monomers 2′-*O*-Bz group was used instead of 2′-*O*-BOMOM. The reason for this change is that all attempts to introduce BOMOM group selectively on



Yield (%)	3	4	5	6	7	8	9
a (B=A^{Bz})	32	32	25	27	78	52	82
b (B=G^{iBu})	40	46	0	0	71	47	75
c (B=U)	35	45	28	32	82	59	84
d (B=C^{Bz})	48	32	30	19	79	55	79

Scheme 1 Reagents and conditions: (i) BOMOM-Cl, Bu_2SnCl_2, DIPEA, DCE, 80 °C(ii) CH_3O-$P(NiPr_2)_2$, tetrazole, ACN, r.t. (iii) TBAF, THF, r.t. (iv) DMTr-Cl, silver triflate, py, r.t.



Yield (%)	12	13	14	16	17	18	19
a (B=A^{Bz})	72	70	62	64	36	24	62
b (B=G^{iBu})	67	63	46	71	59	0	58
c (B=U)	75	76	65	79	39	26	59
d (B=C^{Bz})	86	79	72	66	35	22	58

Scheme 2 Reagents and Conditions: (i) $TsOCH_2PO(OiPr)_2$, NaH, DMF, r.t. (ii) 80% AcOH, r.t. (iii) DMTr-Cl, py, r.t. (iv) BzCN, Et_3N, ACN, r.t. (v) Me_3SiBr, 2,6-lutidine, ACN, r.t. (vi) MOP, DCC, py, r.t. (vii) BOMOM-Cl, Bu_2SnCl_2, DIPEA, DCE, 80 °C(viii) DMTr-Cl, silver triflate, py, r.t.

2′-hydroxyl failed and the alkylation reactions in the presence of tin derivatives, DIPEA, NaH, DBU, Cs_2CO_3 and Ag_2O did not afford the desired product (data not shown). Therefore Bz group which is in this particular case fully compatible with the solid phase and deprotection cycle was used.

The deprotection of isopropyl ester groups with bromotrimethylsilane and the subsequent esterification of free phosphonic acid with 4-methoxy-1-oxido-2-pyridylmethanol (MOP-OH) in the presence of DCC afforded the appropriate phosphonomethyl monomers **14a–d**.

In the 5′-series, ethoxymethylidene derivatives **15a–d** were phosphonylated with diisopropyl tosyloxymethylphosphonate in the presence of sodium hydride. 5′-Phosphonates **16a–d** were obtained upon acidic deprotection of the ethoxymethylidene group. These compounds were protected with the BOMOM group. Because we did not observe any separation of regioisomers on silica gel, the mixture of BOMOM phosphonates was dimethoxytritylated with dimethoxytritylchloride in the presence of silver triflate to afford a mixture of regioisomers, **17a–d** and **18a–d**, which was then separated by preparative HPLC. The desired regioisomers **17a–d** were treated with bromotrimethylsilane and subsequently esterified with 4-methoxy-1-oxido-2-pyridylmethanol in the presence of DCC to afford appropriate phosphonomethyl monomers **19a–d**.

Synthesis and deprotection of modified oligoribonucleotides

Once all monomeric building blocks were prepared, various oligoribonucleotides were synthesised in both the 3′→5′ (**20, 21, 22, 24, 26, 28, 30, 32**) and the 5′→3′ directions (**20, 21, 23, 25, 27, 29, 31, 33**) (Table 1). Solid-phase synthesis was performed on a 1 μmol scale. Phosphoramidite units were incorporated using the phosphoramidite condensation method with a 3-minute coupling time and using 0.5 M ethylthiotetrazole (ETT) as the activator. Phosphonate units were incorporated using the phosphotriester condensation method with a 10-minute coupling time and using 0.3 M 2,4,6-triisopropylbenzenesulfonylchloride (TIPS) as the activator.

Upon completion of the synthesis, oligoribonucleotides anchored to the LCAA-CPG were first treated with a mixture of benzenethiol, TEA and DMF to remove the methyl and MOP protecting groups from the phosphate and phosphonate groups, respectively. Next, the oligoribonucleotides were treated with gaseous ammonia to remove the BOMOM and acyl protecting

Table 1 Hybridisation properties of phosphonate-modified oligoribonucleotides

3′-Phosphonate				5′-Phosphonate
T_m (°C) (ΔT_m/mod.)	Strands No.		Strands No.	T_m (°C) (ΔT_m/mod.)
37.1	20/21	5′-r(GCA UAU CAC)-3′/5′-r(GUG AUA UGC)-3′	20/21	37.1
18.1 (–6.3)	22/21	5′-r(GC<u>A</u> U<u>A</u>U C<u>A</u>C)-3′/5′-r(GUG AUA UGC)-3′	23/21	31.3 (–1.9)
26.2 (–5.5)	24/21	5′-r(GCA <u>UAU</u> CAC)-3′/5′ r(GUG AUA UGC)-3′	25/21	34.2 (–1.5)
24.2 (–6.5)	20/26	5′-r(GCA UAU CAC)-3′/5′-r(GUG <u>AUA</u> UGC)-3′	20/27	34.4 (–1.4)
23.3 (–4.6)	20/28	5′-r(GCA UAU CAC)-3′/5′-r(G<u>U</u>G A<u>U</u>A <u>U</u>GC)-3′	20/29	32.3 (–1.6)
no T_m	30/21	5′-r(<u>GCA</u> <u>UAU</u> <u>CAC</u>)-3′/5′-r(GUG AUA UGC)-3′	31/21	19.3 (–2.2)
no T_m	20/32	5′-r(GCA UAU CAC)-3′/5′-r(<u>GUG</u> <u>AUA</u> <u>UGC</u>)-3′	20/33	19.9 (–2.2)

Modified units are underlined. RNA/RNA duplexes (50 mM TRIS-HCl pH 7.2, 1 mM EDTA, 100 mM Na^+). Total strand concentration 4 μM. T_m ± 0.5 °C.

groups and to release the final product from the solid support. In the end, the RNA was washed from the column by TEAA buffer and purified using a Poly-Pak purification protocol (Glen-Research, DMTr ON mode). Using gaseous ammonia (0.7 MPa), we observed neither chain cleavage nor formation of guanosine formaldehyde adducts reported by Lavergne *et al.*[11]

The mechanism of BOMOM deprotection is very likely similar to that of POM,[11] consisting of the cleavage of the ester group, followed by the formation of 2′-*O*-formaldehydehemiacetal, which is completely stable and temporarily protects 2′-hydroxy group under basic conditions. This hemiacetal is hydrolysed under the conditions of the aqueous workup.

Concerning the coupling time and the stepwise yield, we did not observe any significant difference between the synthesis in the 3′→5′ or 5′→3′ direction. Nonamers **20** and **21** were isolated in 75–80% yield. The phosphonate-modified nonamers **22–33** were isolated in 55–71% yield. The phosphoramidite stepwise yield was 96–99%. The phosphonate stepwise yield was 93–96%.

Hybridisation properties of modified oligoribonucleotides

The use of modified oligonucleotides as therapeutic and diagnostic tools is based on the hybridisation of the oligonucleotide with a target sequence. Thus, determination of hybridisation properties is one of the basic experiments used to evaluate the examined modification.

The thermal stabilities of duplexes formed by phosphonate-modified oligoribonucleotide and complementary RNA strands have been evaluated by UV melting experiments. The obtained T_m values are summarised in Table 1.

The methylene group insertion into the internucleotide linkage caused in both cases a decrease in the melting temperature of the phosphonate-modified duplexes when compared to the unmodified duplex. The introduction of two or three 5′-phosphonate units in an alternate mode (**23, 25, 27, 29**) resulted in moderate destabilisation of the RNA/RNA duplexes (approximately −1.6 °C/mod.), whereas the introduction of 3′-phosphonate units (**22, 24, 26, 28**) resulted in considerable destabilisation of the duplexes (approximately −5.7 °C/mod.). Fully 5′-phosphonate-modified nonamers **31** and **33** still retained the hybridisation ability (ΔT_m −2.2 °C/mod.), whereas nonamers that were fully modified by 3′-phosphonate units **30** and **32** showed no hybridisation. Thus, it seems that the lengthening of the internucleotide linkage brought the additional degree of freedom into the conformation of the sugar-phosphate backbone, resulting in a decrease in the hybridisation ability. Surprisingly, there has been a remarkable difference between the hybridisation ability of oligoribonucleotides modified by regioisomeric 3′- and 5′-phosphonate linkages, which can be most clearly seen in case of fully modified nonamers **30**, **32** and **31, 33**.

In order to explain this unexpected behaviour, we performed molecular dynamics (MD) simulations at the atomic level with the explicit inclusion of water molecules into the simulated system. MD simulations uncovered substantial differences in the conformational preferences of modified internucleotide linkages. In the duplex, 5′-phosphonate linkages adopted a conformation that was strongly stabilised by a 2′OH-5″O hydrogen bond contact. In contrast, 3′-phosphonate linkages adopted various conformations without any specific stabilisation (Fig. 2).

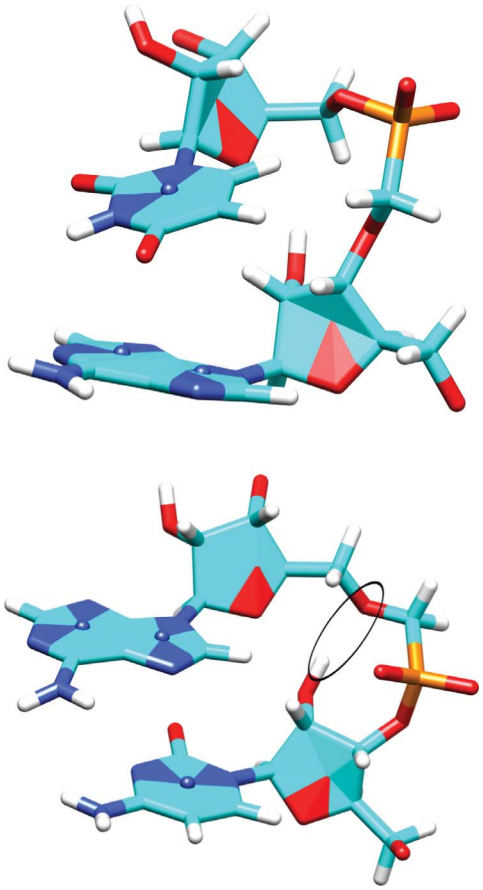


Fig. 2 Conformation of 3′-phosphonate (top) and 5′-phosphonate (bottom) linkages in the duplex.

Thus in the duplex, the strand modified by the 5′-phosphonate linkages was able to adopt a more stable and favourable conformation and thus exhibited significantly better hybridisation properties than the strand modified by 3′-phosphonate linkages. The NMR studies performed on 3′-phosphonate (Apc-A, **34**) and 5′-phosphonate (A-pcA, **35**) dimers (Fig. 1, B = A) showed agreement in the internucleotide linkage conformation of comparable torsion angles (ζ′, β, γ) of the Apc-A dimer **34** with those obtained by MD simulations. There were, however, significant differences in comparable torsion angles (ε, α′, γ) of A-pcA dimer **35**. Therefore, it seems that the 5′-phosphonate internucleotide linkage adopts a conformation involving the 2′OH-5″O hydrogen bond under the influence of the interaction between two strands in the RNA/RNA duplex.

The NMR studies on furanose ring conformation also revealed that both adenosine units in A-pcA dimer exhibited equal population of N and S conformers whereas 3′-end adenosine unit in Apc-A dimer exhibited population shifted to the S conformer (70% S). This result could also partially explain the lower binding affinity of the 3′-phosphonate-modified oligonucleotides to the RNA strand.

For more details about the MD and NMR studies, see the ESI.†

Nuclease stability of modified oligoribonucleotides

Synthesis of modified oligonucleotides is also closely connected to the search for modifications with enhanced nuclease stability. A

rapid degradation of phosphodiester oligonucleotides by nucleases in body fluids is one of the most important limitations to their *in vivo* use. The increase of the half-life of these compounds could thus bring remarkable benefits, including prolonged antiviral or diagnostic effects.

In connection with this, the nuclease stability of phosphonate dimers **34** and **35** and nonamers **26–29** has been examined in the presence of ribonuclease A (EC 3.1.27.5), ribonuclease T2 (EC 3.1.27.1), phosphodiesterase I (EC 3.1.4.1) and phosphodiesterase II (EC 3.1.16.1). Ribonuclease A is an endoribonuclease that preferentialy cleaves the 3′-end of uridine and cytidine residues, leaving a 3′-phosphorylated product, *via* a 2′,3′-cyclic monophosphate. Ribonuclease T2 is also an endoribonuclease which preferentialy cleaves the 3′-end of adenosine residues by the same mechanism. Phosphodiesterase I and II are exonucleases. Phosphodiesterase I cleaves from the 3′-end, releasing 5′-nucleotide units. Phosphodiesterase II cleaves from the 5′-end, releasing 3′-nucleotide units. The course of the cleavage reaction has been monitored by LC-MS.

Phosphodiester-linked ApA dimer was rapidly degraded by ribonuclease T2, phosphodiesterase I and II under experimental conditions (with a half-life less than one minute). In contrast, both types of phosphonate-modified dimers, **34** and **35**, exhibited exceptional nuclease resistance and no cleavage of internucleotide linkages was observed within two hours of incubation with these nucleases. Similar results were observed during the cleavage of modified nonamers **26–29** by ribonuclease A and phosphodiesterase I and II. Neither 3′-phosphonate (**28**) nor 5′-phosphonate (**27**) linkages were cleaved by ribonuclease A. We observed only complete cleavage of phosphodiester linkages. In the case of phosphodiesterase I and II, we observed an interesting cleavage pattern. The one-by-one cleavage of units occurred until the modified linkage was met. After that, the modified unit was omitted and the phosphonate dimer was cleaved, but neither the 3′-phosphonate nor 5′-phosphonate linkages were cleaved. Moreover, after this unexpected cleavage step, further cleavage did not continue in most cases (**26–29**). The cleavage profiles are enclosed in the ESI.†

Conclusions

In conclusion, we present here a new base-labile 2′-protecting group usable for the solid phase synthesis of oligoribonucleotides in both the 3′→5′ and 5′→3′ directions. To the best of our knowledge, benzoyloxymethoxymethyl is the first non-silyl protecting group used in the solid phase synthesis of oligoribonucleotides in the reverse direction. Moreover, post-synthetic deprotection of oligoribonucleotides using gaseous ammonia allows for a simple on-column deprotection protocol.

Utilising this novel methology, we prepared oligoribonucleotides modified by regioisomeric 3′- and 5′-phosphonate linkages in good yields and purity. Both linkages exhibited extraordinary stability against cleavage by nucleases. The 5′-phosphonate linkage proved to have sufficient hybridisation potential to be used in biochemical applications, such as siRNA or miRNA. Another application of phosphonate linkages could occur at specific sites of modified oligonucleotides, which require conformational flexibility such as loops or hairpins, or nuclease stability.

Experimental

Synthesis of monomers - general methods

Method A - Preparation of nucleosides 3, 4, 5 and 6. Dibutyltin dichloride (1.1 mmol) was added to a solution of 5′-protected derivative **1** or **2** (1.0 mmol) and DIPEA (2.2 mmol) in DCE (10 mL). The mixture was stirred 1 h at r. t. After this, the reaction mixture was warmed to 85 °C, and benzoyloxymethoxymethyl chloride **10** (1.1 mmol) was added. The reaction mixture was heated 3 h at 85 °C to afford a mixture of 2′-*O*-BOMOM and 3′-*O*-BOMOM derivatives. The regioisomers were separated by silica gel chromatography (elution with gradient of 0–50% acetone in toluene, guanosine derivatives used 0–100% acetone in toluene). The faster TLC eluting isomer was the 2′-*O*-BOMOM derivative (**3, 4**) and the slower TLC eluting isomer was the 3′-*O*-BOMOM derivative (**5, 6**).

Method B – Preparation of nucleoside 8. BOMOM derivatives **4** or **6** (1.0 mmol) in pyridine (10 mL) was added to a 30 min pre-stirred mixture of silver triflate (2.0 mmol) and dimethoxytrityl chloride (2.0 mmol) in pyridine (10 mL). The reaction mixture was stirred 16 h at r.t. The reaction was quenched by the addition of methanol (5 mL), the silver chloride was filtered off, and the mixture was concentrated under reduced pressure. The product was purified by chromatography on silica gel (elution with a gradient of 0–50% ethyl acetate in toluene).

To cleave the 5′-*O*-TBDPS group, fully protected nucleoside (1.0 mmol) was dissolved in 0.5 M TBAF in THF (10 mL), and the reaction mixture was stirred 2 h at r.t. After that, the mixture was diluted with toluene and concentrated under reduced pressure. The product was purified by chromatography on silica gel (elution with a gradient of 0–50% acetone in toluene, guanosine derivatives used 0–100% acetone in toluene).

Method C – Preparation of phosphoramidites 7 and 9. Tetrazole (1.1 mmol) was added to a solution of derivative **3, 8** (1.0 mmol) and methyl *N*,*N*,*N*′,*N*′-tetraisopropylphosphordiamidite (2.0 mmol) in DCM (10 mL). The reaction mixture was stirred 2 h at r.t. After that, TEA (1 mmol) was added and the reaction mixture was evaporated. The product was purified by chromatography on silica gel (elution with a gradient of 0–50% ethyl acetate in toluene, guanosine derivatives used 0–50% acetone in toluene). In addition, the guanosine derivative were dissolved in DCM and precipitated from hexane. Lastly, phosphoramidites were freeze-dried from benzene.

Method D – Preparation of phosphonates 12 and 16. Sodium hydride (3.0 mmol) was added at 0 °C to a stirring solution of protected nucleoside **11** or **15** (1.0 mmol) and diisopropyl tosyloxymethylphosphonate (2.0 mmol) in DMF (10 mL). The reaction mixture was left to warm up gradually to r.t. and then stirred 16 h at r.t. The reaction was quenched by the addition of glacial acetic acid (1 mL) at 0 °C, and the mixture was concentrated under reduced pressure. After that, the nucleoside-phosphonate was treated with 80% acetic acid (10 mL) for 16 h at r.t. and then concentrated under reduced presure. The product was purified by chromatography on silica gel (elution with a gradient of 0–10% ethanol in chloroform).

Method E – Preparation of phosphonate 13. Dimethoxytrityl chloride (1.1 mmol) was added to a stirring solution of phosphonate **12** (1.0 mmol) in pyridine (10 mL), and the mixture was stirred 16 h at r.t. The reaction was quenched by the addition of triethylamine (1 mL) and methanol (5 mL), and the mixture was concentrated under reduced pressure. The residue was dissolved in chloroform (50 mL) and extracted with a saturated solution of sodium hydrogencarbonate (3 × 20 mL). The organic layer was dried over anhydrous sodium sulfate and evaporated. After that, benzoyl cyanide (1.5 mmol) was added to a stirring solution of 5′-the dimethoxytrityl phosphonate derivative (1.0 mmol) and triethylamine (0.2 mmol) in acetonitrile (10 mL), and the mixture was stirred 16 h at r.t. The reaction was quenched by the addition of methanol (5 mL), and the mixture was concentrated under reduced pressure. The product was purified by chromatography on silica gel (elution with a gradient of 0–50% ethyl acetate in toluene).

Method F – Preparation of MOP phosphonates 14 and 19. Bromotrimethylsilane (4.0 mmol) was added to a solution of diisopropyl phosphonate **13** or **17** (1.0 mmol) and 2,6-lutidine (8.0 mmol) in acetonitrile (10 mL). The reaction mixture was stirred 16 h at r.t. and then concentrated under reduced presure. The residue was treated with 2 M TEAB (5 mL) and methanol (5 mL). The solution was evaporated, and the residue was dissolved in chloroform (50 mL) and extracted with 0.2 M TEAB (3 × 20 mL). The organic layer was dried over anhydrous sodium sulfate and evaporated. The crude nucleoside phosphonic acid was used for the further steps without purification. DCC (5.0 mmol) was added to a solution of nucleoside phosphonic acid (1.0 mmol) and 4-methoxy-1-oxido-2-pyridylmethanol (3.0 mmol) in pyridine (6 mL), and the reaction mixture was stirred for 3 days at r.t. After that, the reaction mixture was diluted with water (4 mL) to achieve a 60% solution of pyridine in water. The solution was then heated 16 h at 50 °C to cleave off one ester group before being concentrated under reduced pressure. The product was purified by chromatography on silica gel (elution with a gradient of 0–100% of a mixture ethyl acetate/ethanol/acetone/water 4 : 1 : 1 : 1 in ethyl acetate) and lyophilised from dioxane.

Method G – Preparation of phosphonates 17 and 18. Dibutyltin dichloride (1.1 mmol) was added to a solution of 5′-phophonomethyl derivative **16** (1.0 mmol) and DIPEA (2.2 mmol) in DCE (10 mL). The mixture was stirred 1 h at r. t. After that, the reaction mixture was warmed up to 85 °C, and benzoyloxymethoxymethyl chloride **10** (1.1 mmol) was added. The reaction mixture was heated 3 h at 85 °C to afford a mixture of 2′-*O*-BOMOM and 3′-*O*-BOMOM derivatives. The regioisomers were purified by chromatography on silica gel (elution with a gradient of 0–50% acetone in toluene, guanosine derivatives used 0–100% acetone in toluene) as an unseparable mixture of regioisomers and used for further steps without separation. The mixture of BOMOM derivatives (1.0 mmol) in pyridine (10 mL) was added to a 30 min pre-stirred mixture of silver triflate (2.0 mmol) and dimethoxytrityl chloride (2.0 mmol) in pyridine (10 mL). The reaction mixture was stirred 16 h at r.t. The reaction was quenched by the addition of methanol (5 mL), the silver chloride was filtered off and the mixture was concentrated under reduced pressure. The regioisomers were purified by chromatography on silica gel (elution with a gradient of 0–50% acetone in toluene, guanosine derivatives used 0–100% acetone in toluene) as an unseparable mixture of regioisomers. Next, the mixture was separated by preparative HPLC (elution with a gradient of 50–100% methanol in water). The faster reverse phase eluting isomer was 3′-*O*-BOMOM derivative (**18**) and the slower reverse phase eluting isomer was 2′-*O*-BOMOM derivative (**17**).

Table 2 Solid phase synthesis protocol

Phosphoramidite condensation method		
1. Detritylation	3% DCA in DCM	120 s
2. Condensation	0.1 M phosphoramidite in ACN 0.5 M ETT in ACN	180 s
3. Capping	Ac_2O/2,6-lutidine/THF 1 : 1 : 8 6.5% DMAP in THF	60 s
4. Oxidation	10% tBuOOH in DCM	90 s
Phosphotriester condensation method		
1. Detritylation	3% DCA in DCM	120 s
2. Condensation	0.1 M phosphonate in pyridine 0.3 M TIPS-Cl in ACN	600 s
3. Capping	Ac_2O/2,6-lutidine/THF 1 : 1 : 8 6.5% DMAP in THF	60 s

Synthesis and purification of oligoribonucleotides

Oligonucleotides **20–33** were synthesised using the protocol shown in Table 2. Synthesis was performed on a 1 μmol scale in the 3′→5′ direction on [2(3)-*O*-benzoyl-5-*O*-dimethoxytrityl-1-(4-*N*-benzoylcytosine-1-yl)-β-D-ribofuranos-3(2)-*O*-succinoyl]LCAA-CPG **36** and in the 5′→3′ directions on [2-*O*-benzoyloxymethoxymethyl-3-*O*-dimethoxytrityl-1-(2-*N*-isobutyrylguanin-9-yl)-β-D-ribofuranos-5-*O*-succinoyl]LCAA-CPG **37** using GenSyn V02 DNA/RNA synthesiser.

Deprotection of nonamers was achieved in two steps. First, the column with oligoribonucleotide anchored to the LCAA-CPG was treated with a benzenethiol/TEA/DMF (1 : 1.4 : 2 v : v : v) mixture for 6 h to remove the methyl and MOP protecting groups from the phosphate and phosphonate groups, respectively. Then, the column was washed with DMF, ACN and dried. Second, the column was inserted into the pressure vessel and treated with gaseous ammonia (0.7 MPa) for 8 h to the remove BOMOM and acyl protecting groups,and to release the final product from the solid support. Finally, the deprotected RNA was washed off of the column by a 0.1 M TEAA buffer and purified using a Poly-Pak purification protocol (GlenResearch, DMTr ON mode).

T_m experiments

T_m experiments were performed on a CARY 100 Bio UV Spectrophotometer (Varian Inc.) equipped with a Peltier temperature controller and thermal analysis software. The samples were prepared by mixing complementary strands of RNA together to give a 4 μM final concentration in 50 mM TRIS/HCl pH 7.2, 1 mM EDTA and 100 mM Na^+. A heating-cooling cycle in the range 5–60 °C with a gradient of 0.2 °C min^{-1} was applied. T_m values were determined from the maxima of the first derivative plots of absorbance *versus* temperature ($T_m \pm 0.5$ °C).

Nuclease stability experiments

Dimers **34** and **35** and nonamers **26–29** (25 μM) were incubated 2 h at r.t. in 50 mM TRIS/HCl pH 7.6, 10 mM Mg^{2+} with the appropriate nuclease (0.3 unit; ribonuclease A, EC 3.1.27.5; ribonuclease T2, EC 3.1.27.1; phosphodiesterase I, EC 3.1.4.1; phosphodiesterase II, EC 3.1.16.1). The reaction mixture was then analysed by LC-MS.

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