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The synthesis of solid supports carrying base labile linkers to generate 3′-phosphate oligonucleotides

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ABSTRACT

Oligonucleotides carrying 3′-terminal phosphates and conjugates are important tools in molecular biology and diagnostic purposes. We described the preparation of solid supports carrying the base labile linker 4-((2-hydroxyethyl)sulfonyl)benzamide for the solid-phase synthesis of 3′-phosphorylated oligonucleotides. These supports are fully compatible with the phosphoramidite chemistry yielding the desired 3′-phosphate oligonucleotides in excellent yields. The use of mild deprotection conditions allows the generation of partially protected DNA fragments.

Oligonucleotide carrying 3′-phosphates are important intermediates for the synthesis of oligonucleotide conjugates as well as for oligonucleotides that cannot be extended by DNA polymerases.[1–3] In addition, the presence of the 3′-phosphate protects oligonucleotides from degradation by exonucleases. The synthesis of these compounds can be achieved using solid supports functionalized with base labile linkers that connect the solid support to the oligonucleotides. In this way, nucleoside phosphoramidites can be used to assemble the desired oligonucleotide sequences and, ammonia deprotection will generate the desired 3′-phosphate oligonucleotides. There are several base-labile linkers used for this purpose (Scheme 1), including ethylsulfoxide linker,[1–8] 2-nitrophenylethyl linker (NPE[9]), fluorenylmethyl linker (FM[10]), substituted 2-cyanoethyl[11] and Lönnberg's chemical phosphorylation supports carrying bis(carboxymethylamido) groups.[12,13] The hydroxyethylsulfoxide (ESE) linker can be prepared from 2,2′-sulfonyldiethanol and was originally described as DMT-containing phosphoramidite for the phosphorylation of the 5′-position of oligonucleotides.[1] When this reagent is used as hemisuccinate derivative for the functionalization of solid supports,[14,15] the resulting solid supports are between the most commonly used linkers for the synthesis of oligonucleotide 3′-phosphates. In this communication, we describe a novel preparation of the β-eliminating linker [4-((2-hydroxyethyl)sulfonyl)benzoic acid, ESB, **5**] that contains the hydroxyethylsulfoxide moiety attached to a benzoic acid derivative. The presence of the carboxylic acid function and the hydroxyethylsulfoxide moiety allows the functionalization of solid supports with the β-eliminating linker, generating 3′-phosphate oligonucleotides.

The synthesis of 4-((2-hydroxyethyl)sulfonyl)benzoic acid (**5**) is shown in Scheme 2. First, 4-mercaptobenzoic acid was esterified to yield methyl 4-mercapto benzoate (**2**) in 94 % yield. Next, methyl 4-mercapto benzoate (**2**) was reacted with 2-bromoethanol in the presence of cesium carbonate yielding methyl 4-((2-hydroxyethyl)thio)benzoate (**3**) in 91 % that was oxidized with *m*-chloroperoxybenzoic acid (m-CPBA) obtaining methyl 4-((2-hydroxyethyl)sulfonyl)benzoate (**4**) in 88 %. Hydrolysis of the methyl ester with lithium hydroxide yielded the desired compound **5**. The proposed route differs from a previously described protocol [16] in the use of the methyl ester **2** instead of the benzoic acid derivative **1** in the *S*-alkylation reaction resulting in more hydrophobic intermediates which can be easily purified on silica gel (see experimental conditions

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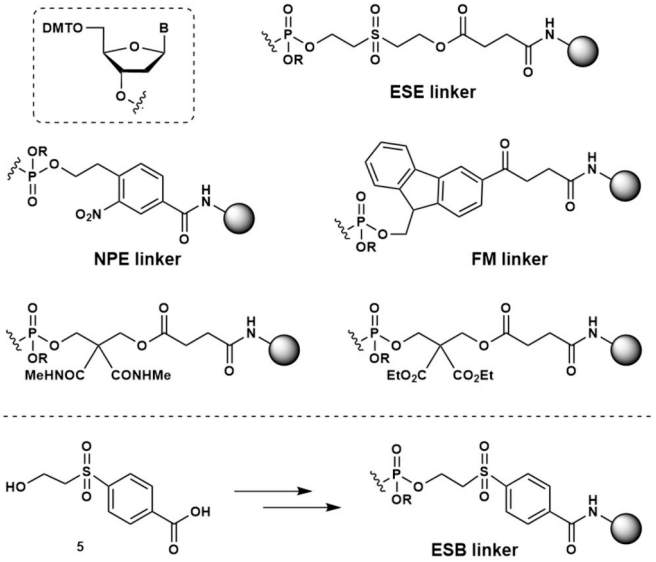


Scheme 1. Chemical structure of some of the linkers described for the synthesis of oligonucleotide 3′-phosphates as well as the structure of 4-((2-hydroxyethyl) sulfonyl)benzoic acid developed in this communication.

and spectroscopic characterization in the Supplementary Information section).

Next, compound **5** was coupled to amino-controlled pore glass (CPG) using dicyclohexylcarbodiimide (DCC) and 1-hydroxybenzotriazol (HOBt) as coupling agents.[10] In order to study the lability of the new supports, a DMT-Thymidine 2-cyanoethylphosphoramidite was coupled and the resulting DMT-*T*-support was treated with concentrated ammonia, 10 % piperidine/acetonitrile, dimethylamine (Me_2NH) aqueous solution/acetonitrile (1:3), 10 % triethylamine (Et_3N) in acetonitrile and 0.1 M 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) in acetonitrile. The percentage of DMT-T 3′-phosphate released from the support at different times was estimated by measuring the amount of DMT groups left on the support compared with the DMT group released during the treatment. From these experiments, the lability of the novel CPG support was found to be comparable to CPG functionalized with the ESE linker from commercial sources. The optimal conditions for the release were found to be: a) concentrated ammonia for 4 h at 55 °C, b) 0.1 M DBU in acetonitrile for 15 min at room temperature (rt), c) 10 % piperidine/acetonitrile for 2 h at rt, d) 40 % Me_2NH aqueous solution/ acetonitrile (1:3) at rt and e) 10 % Et_3N in acetonitrile for 2 h at rt. In all these cases, the release of the DMT-T 3′-phosphate was judged to be complete by the measure of the DMT cation remaining on the solid support.

The solid support functionalized with the ESB linker was used for the synthesis of several oligonucleotides (Table 1), which were assembled on an automatic DNA synthesizer using standard phosphoramidite synthesis cycles. In one case, the same oligonucleotide (sequence A) was prepared on commercially available CPG functionalized with the ESE linker for comparative purposes. After sequences assembly, the resulting solid supports were treated with concentrated ammonia and the resulting compounds were analyzed by analytical HPLC (Figure 1) and MALDI-TOF mass spectrometry, yielding the desired oligonucleotide 3′-phosphates with the same purity obtained using CPG functionalized with the ESE linker from commercial sources. In addition, we have

Table 1
Oligonucleotide prepared in this work.

#	Sequence (5′->3′)	Solid support	MS (expected)	MS (found)
A	TCACGTp	**ESB**	1846.3	1845.2
A	TCACGTp	**ESE**	1846.3	1845.2
B	TTTATTTp	**ESB**	2155.3	2151.6
C	TTTCTTTp	**ESB**	2131.3	2127.6
D	TTTGTTTp	**ESB**	2171.3	2167.5
E	TGTTGGp	**ESB**	1917.3	1916.1
F	CAGTTGGp	**ESB**	2519.4	2519.7
G	CAGUUGGp	**ESB**	2299.3	2299.0

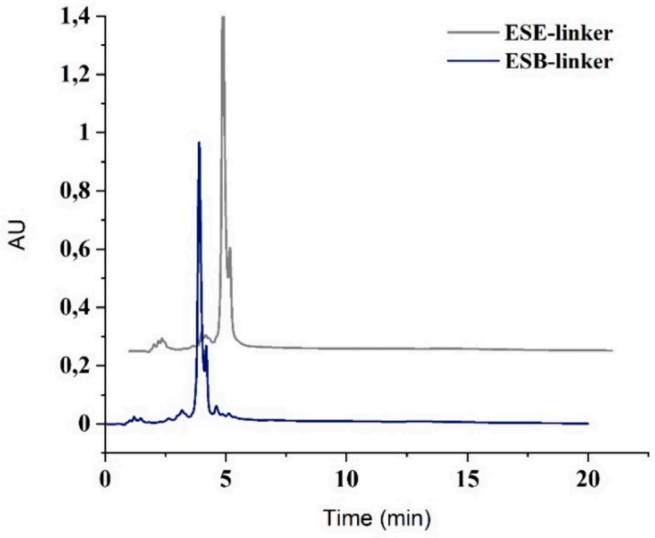
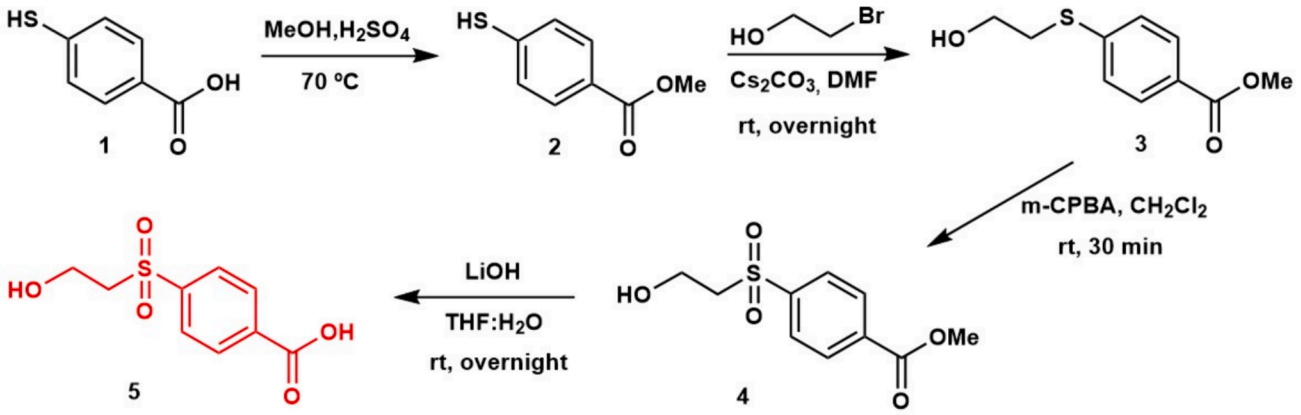


Fig 1. HPLC profiles of the 5′-TCACGTp-3′ oligonucleotide sequence prepared using the solid support functionalized with the ESE linker (grey) and the solid support functionalized with the ESB linker (blue).



Scheme 2. Synthesis of 4-((2-hydroxyethyl)sulfonyl)benzoic acid (**5**).

Table 2
Synthesis of partially-protected oligonucleotides using CPG solid supports functionalized with the ESB linker.

Sequence (5′->3′)	Formula	$MS_{expected}$	MS_{found}, DBU	MS_{found}, Et_3N	MS_{found}, Me_2NH
$TTTA^{Bz}TTTp$	$C_{77}H_{96}N_{17}O_{49}P_7$	2260.4	2308.5 (M + $2Na^+$)	2361.2 (M + Et_3N) + 2414.2 and 2467.2 acrylonitrile adducts (M + Et_3N + 53)	2308.6 (M + $2Na^+$)
$TTTC^{Bz}TTTp$	$C_{76}H_{96}N_{15}O_{50}P_7$	2236.4	2284.7 (M + $2Na^+$)	2336.7 (M + Et_3N) + 2390.8 and 2444.0 acrylonitrile adducts (M + Et_3N + 53)	2285.1 (M + $2Na^+$) 2180.3 (loss of the benzoyl group)
$TTTG^{Ibu}TTTp$	$C_{74}H_{98}N_{17}O_{50}P_7$	2242.4	2290.2 (M + $2Na^+$)	2343.3 (M + Et_3N) + 2396.6 and 2449.4 acrylonitrile adducts (M + Et_3N + 53)	2290.2 (M + $2Na^+$)
$TG^{Ibu}TTG^{Ibu}G^{Ibu}p$	$C_{72}H_{95}N_{21}O_{43}P_6$	2128.5	2179.1 (M + $2Na^+$)	2179.0 (M + $2Na^+$) + 2232.0 and 2285.0 acrylonitrile adducts (M + $2Na^+$+53)	2179.1 (M + $2Na^+$)
$C^{Bz}A^{Bz}TG^{Ibu}TTG^{Ibu}G^{Ibu}p$	$C_{105}H_{127}N_{29}O_{56}P_8$	2939.1	2990.8 (M + $2Na^+$)	2991.0 (M + Et_3N) 3043.0 and 3094.0 acrylonitrile adducts (M + Et_3N + 53)	Not determined

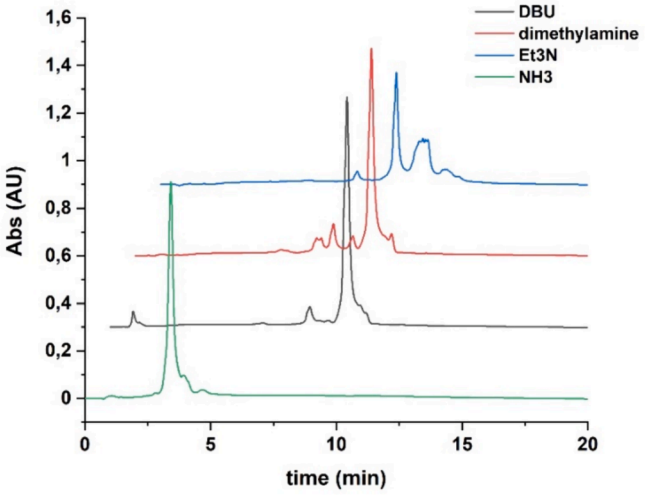


Fig 2. HPLC profiles of 5′-TGTTGG-3′ oligonucleotide E sequences prepared on a solid support functionalized with the ESB linker and treated with ammonium solution (green) and three different amines: DBU (black), Me_2NH (red) and Et_3N (blue). The profiles were obtained using a 0 to 50 %B gradient in 20 min.

prepared a short RNA sequence (sequence G) on the ESE linker support using cyanoethyl phosphoramidites protected with the *tert*-butyldimethylsilyl (TBDMS) at the 2′-position. After ammonia treatment, TBDMS groups were removed with triethylamine.HF in *N*-methylpyrrolidone and the resulting DMT-oligonucleotide was purified on RNA cartridge purification (Glen Research) following the instructions of the manufacturer giving the expected 3′-phosphate RNA oligonucleotide that was characterized by MALDI-Tof (Table 1, Figure S20).

Since the release of the oligonucleotides can be achieved under mild conditions using secondary and tertiary amines, we studied alternative methods to obtain partially-protected oligonucleotide derivatives. To this end, solid supports B-F were treated at room temperature with: i) 0.1 M DBU in acetonitrile for 15 min, ii) 40 % Me_2NH aqueous solution/acetonitrile (1:3) and iii) 10 % Et_3N in acetonitrile for 2 h (Table 2). As the 2-cyanoethyl of the phosphate group is removed by β-elimination generating acrylonitrile, we added 7 mg of thymine to the triethylamine and DBU solutions to prevent alkylation of thymidine by acrylonitrile.[17,18] In all the cases, the deprotection solutions were concentrated and desalted with Sephadex G-25 prior HPLC analysis. Figure 2 shows the analytical HPLC of the treatment of the solid support carrying sequence E with ammonia, DBU, Et_3N and Me_2NH. The best conditions for the release of the partially-protected oligonucleotides from the ESB linker were found to be 0.1 M DBU in acetonitrile (Table 2). This is the shortest treatment (15 min) and also generates the cleanest compounds. The second optimal deprotection conditions were obtained with the use of Me_2NH solutions, which gave similar results to the DBU treatment, except for a partial release (around 30 %) of the benzoyl group of 2′-deoxycytidine (Table 2). Finally, the treatment with Et_3N solutions generated acrylonitrile adducts, as shown by the more hydrophobic impurities in the HPLC chromatograms and the presence of peaks at M + 53 and M + 106 (Table 2). These impurities are most probably generated during to the long treatment time (2 h) and cannot be avoided even with the use of thymine as a scavenger for acrylonitrile (see Figure S19).

In conclusion, we have described a novel route for the synthesis of a linker molecule for the preparation of oligonucleotide 3′-phosphates that is easy to prepare and compatible with the solid-phase phosphoramidite methodology. In addition, we have showed that under mild conditions it is possible to generate oligonucleotide 3′-phosphates carrying the benzoyl and isobutyryl groups in the nucleobases, opening up the possibility of using these linkers for the rapid preparation of protected DNA fragments.[19] To achieve this goal, phosphoramidites carrying a phosphate-protecting group different from the 2-cyanoethyl group are required.[20] Work in this direction is underway.

CRediT authorship contribution statement

Kwazi Masuku: Writing – original draft, Methodology, Investigation, Data curation. **Luis Miguel Menéndez-Méndez:** Writing – review & editing, Investigation, Data curation. **Sikabwe Noki:** Writing – review & editing, Investigation, Data curation. **Beatriz G. de la Torre:** Data curation, Writing – review & editing, Supervision, Methodology. **Fernando Albericio:** Writing – review & editing, Supervision, Methodology, Funding acquisition, Conceptualization. **Susana Fernández:** Writing – review & editing, Supervision, Methodology. **Miguel Ferrero:** Writing – review & editing, Supervision, Methodology. **Anna Aviñó:** Writing – review & editing, Supervision, Methodology. **Ramon Eritja:** Writing – review & editing, Writing – original draft, Supervision, Methodology, Funding acquisition, Conceptualization. **Carme Fàbrega:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Methodology, Investigation, Funding acquisition, Data curation.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bmcl.2024.129819.

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