

Synthesis of oligonucleotides modified with hydrophilic 5'-linker phosphoramidites for use in post-synthetic labelling in aqueous media

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Introduction

The use of modified linkers to incorporate labels such as dyes, haptens, enzymes and antibodies into oligonucleotides is widely known. Most commonly these are thiol^{1,2} or amino^{2,3} modified linkers. Although there are 3'-modified linkers, it is more common that these are added to the 5' end on an oligonucleotide using phosphoramidite chemistry (see **2123-2126** below).

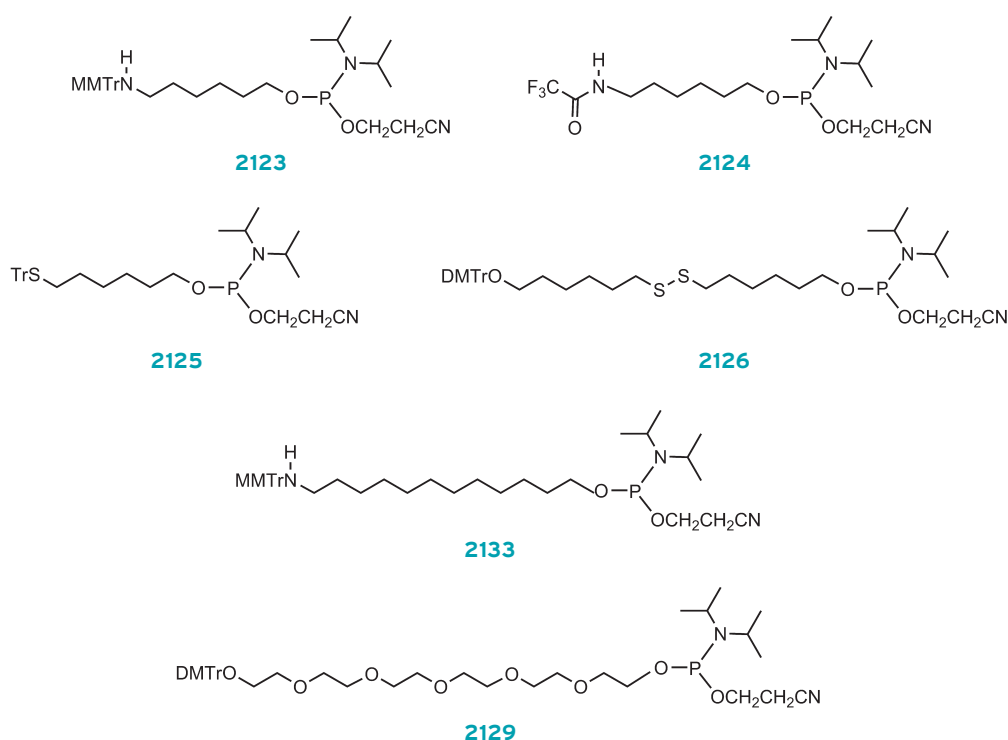
With the exception of the MMTr-protected amino linker (**2123**), where on-column labelling is possible, the use of these linkers requires the attachment of the desired label to be carried out post-synthetically and in aqueous solution. On the whole, solution-phase coupling with oligonucleotides modified with these linkers is sufficient. However, longer sequences, and those with significant secondary structure, often prove problematic and poor coupling of the label is often observed. To counteract this an amino modifier with a longer linker (**2133**)⁴ was developed which has been shown to improve the post-synthetic labelling reaction but in many cases, especially when coupling an enzyme or antibody to an oligonucleotide, there is a need to add a hydrophilic spacer such as spacer 18 (**2129**) to optimise the coupling reaction.

1 Chemical synthesis of oligonucleotides containing a free sulphhydryl group and subsequent attachment of thiol specific probes. B.A. Connolly and P. Rider, *Nucleic Acids Research*, **13**, 4485-4502, 1985.

2 The preparation and application of functionalised synthetic oligonucleotides: III. Use of H-phosphonate derivatives of protected amino-hexanol and mercapto-propanol or-hexanol. N.D. Sinha and R.M. Cook, *Nucleic Acids Research*, **16**, 2659-2670, 1988.

3 The synthesis of oligonucleotides containing a primary amino group at the 5'-terminus. B.A. Connolly, *Nucleic Acids Research*, **15**, 3131-3139, 1987.

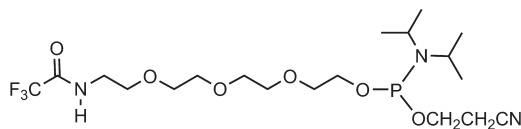
4 R.I. Hogrefe, H. Mackie, and M.L. Powell, *Amer. Biotechnologies News Ed.*, **6**, 47, 1988.



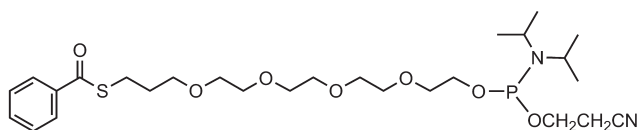
Although the use of the spacer improves the coupling reaction, it takes the label further away from the oligonucleotide. This can be problematic in some cases, e.g. molecular beacons where it is important that the quencher and the fluorophore are close enough to interact. In this case it is advisable to use a spacer at both the 5' and 3' end of the oligonucleotide. This clearly adds to the complexity and manufacturing cost of the oligonucleotide.

Recently, we have developed hydrophilic amino (**2182**) and thiol (**2187**) linkers to alleviate the need for the use of an additional hydrophilic spacer.

Comparisons have been carried out with existing products, utilising these new linkers in oligo synthesis and subsequent post-synthetic labelling in aqueous media.



2182



2187

Experimental

All oligonucleotides were synthesised on an Expedite 8909 automated synthesiser on a 1µmol scale using a 1000Å SynBase dT CPG 1µmol column (**2271**).

Synthesis conditions⁵ were as follows: standard amidites (Bz-dA (**2003**), Ac-dC (**2034**), iBu-dG (**2002**) and dT (**2001**)) and modifiers were used as 0.1M solutions in anhydrous acetonitrile (**4050**); coupling times were 30s for standard amidites and 15min for all modifiers. Liquid reagents used were: 0.25M ETT (**3140**) or 0.3M BTT (**3160**) (activators); THF/pyridine/acetic anhydride (8:1:1) (cap A) (**4110**); 10% methylimidazole in THF (cap B) (**4120**); 0.02M iodine in THF/pyridine/water (7:2:1) (oxidiser) (**4330**) and 3% TCA/DCM (deblock) (**4140**).

All amino-modified oligos, and those containing thiol **2187**, were synthesised DMT-ON, since there is no DMTr protection. All oligonucleotides modified with thiol **2126** were synthesised DMT-OFF, as were all unmodified oligos. All amino-modified oligos, and thiol-modified oligos **12** and **13**, were treated with 20% DEA/MeCN for 5min prior to cleavage and deprotection to prevent acrylonitrile adduct formation. Unless otherwise specified, cleavage and deprotection was carried out in concentrated aqueous ammonium hydroxide solution at 55°C for 4h followed by desalting using a G25 sephadex column.

RP-HPLC was carried out using an Agilent Eclipse XBD-C18 5µm, 4.6x150mm column using buffer A:- 0.1M TEAA and buffer B:- 100% MeCN over a gradient of 0-50%B [gradient 1] or 0-100%B [gradient 2] over 27.5minutes (1ml/min).

MALDI-TOF mass spectrometry was carried out on

a Bruker Ultraflex. This service was provided by Eurogentec SA.

Conjugation of amino-modified oligos with thioctic acid NHS ester:

In both cases the oligonucleotide was dried prior to the conjugation reaction and the conjugation buffer used was 0.1M sodium carbonate/bicarbonate buffer pH 9.75

(a) Method as described on page 111 of the Link Technologies 2010 Product Guide:

1. The oligo was dissolved in water (1ml).
2. The conjugation buffer (8ml) was added.
3. Thioctic acid solution (1ml) was then added (20mg/ml in DMF).
4. The reaction was vortexed then stirred overnight at room temperature.
5. The sample was desalted by G25.

(b) More common method of NHS-ester conjugations.

1. The oligo was dissolved in 75µl of conjugation buffer.
2. Thioctic acid NHS solution (5mg in 120µl DMF) (30µl) was added.
3. The reaction was vortexed then stirred overnight at room temperature.
4. The sample was desalted by G25 treated with 0.1M TEAA.

6-Iodoacetamidofluorescein (6-IAF) labelling:

The thiol-modified oligonucleotide was freeze dried and 200µl of TCEP solution⁶ added. This was allowed to stand for 2h before being passed through a G25 column prepared with 0.1M Sodium Phosphate pH 7.4 and collected into a sample tube containing solid 6-IAF (1-2mg) and DMF (10µl) was added. The reaction was allowed to stand overnight then passed through a G25 column prepared with 0.1M TEAA.

⁵ Note that the other combinations of Cap mixtures (**4012** and **4122**) and oxidiser (**4132**) could also be used.

⁶ 87mM TCEP (25mg in 1ml of water).

Table 1. Amino-modified oligonucleotides

ID	Amino Mod.	Spacer	Sequence
1	-	-	TTC GGC TTG TCC GTG GAA TCT CAC AGC TTA T
2	2182	-	TTC GGC TTG TCC GTG GAA TCT CAC AGC TTA T
3	2182	-	TTC GGC TTG TCC GTG GAA TCT CAC AGC TTA T
4	2124	-	TTC GGC TTG TCC GTG GAA TCT CAC AGC TTA T
5	2124	-	TTC GGC TTG TCC GTG GAA TCT CAC AGC TTA T
6	2124	2129	TTC GGC TTG TCC GTG GAA TCT CAC AGC TTA T

Table 2. Analytical data for amino-modified oligonucleotides

ID	Amino Mod.	Spacer	HPLC on crude oligo				MS	
			% Unmod.	RT/min	% Amino Mod.	RT/min	Calc.	Obs.
2	2182	-	0	9.74	100	9.91	9704	9734
3	2182	-	0	9.74	100	9.86	9704	9743
4	2124	-	0	9.74	100	10.04	9628	9670
5	2124	-	0	9.74	100	9.82	9628	9671
6	2124	2129	0	9.74	100	10.19	9972	10003

Results & Discussion

Product design

Both **2182** and **2187** are based on multiple ethylene glycol spacer units. **2182** uses the common TFA protection on the amino group, whilst with **2187** the benzoyl protecting group was selected such that the free thiol is formed during standard oligonucleotide deprotection. Therefore there is no need for the use of silver salts as is required to remove the trityl group when using the S-Tr thiol modifier (**2125**). The lack of the disulphide bridge, as is present in the S-S thiol modifier (**2126**), means that there is no drop in this amidite's performance even after being on the synthesiser for 48h.

Oligonucleotide synthesis

All oligonucleotides⁷ were synthesised on an automated synthesiser on a 1µmol scale. The amino or thiol modification was incorporated at the 5'-end of the oligonucleotide and, where required, a spacer (**2129**) was incorporated between the modification and the oligonucleotide.

Amino Linkers

A comparison of the most commonly used commercially available amino linker (**2124**) with the new hydrophilic amino-11 linker (**2182**) was carried out. The sequences synthesised are shown in Table 1.

The RP-HPLC of the crude oligonucleotides

showed only one major peak in all cases and only the desired amino-modified product was observed in the MS. Mixed injections of the product and the unmodified oligonucleotide (**1**) showed that the main peak was the desired product and there was no evidence of any unlabelled oligonucleotide. This indicates that there is no difference in the coupling efficiency between the two amino linker phosphoramidites. The results are summarised in Table 2.

To test the post-synthetic labelling coupling efficiency of the amino-modified oligonucleotides, these were then coupled to thioctic acid NHS ester (**2166**). The crude conjugation product was analysed by RP-HPLC and MALDI MS. The oligonucleotides to be labelled with thioctic acid were split into two portions and each portion labelled using a different method. The first method is the one outlined in the Link Technologies Ltd product guide and the other is based on the more common method of post-synthetic labelling oligonucleotides with NHS esters. In both cases MS showed only the labelled product although HPLC of samples using the latter method showed that the reaction was about 75% complete. The presence of DMF in the sample using the first method resulted in the sample being eluted from the column within the first few minutes. The samples need to therefore be dried prior to purification or HPLC analysis. The analytical data is summarised in Table 3.

Post-synthetic labelling of the amino-modified oligonucleotides with thioctic acid in all cases proved very efficient and comparable between the hydrophilic amino-11 linker (**2182**), the amino C6 linker (**2124**), and with both **2124** and the spacer **2129** between the oligonucleotide and the linker.

⁷ All the synthesised sequences are derivatives of the full DNA sequence (ID 1 in Table 1), which was selected since every base couples to every other base. However, the sequence does have the potential to self dimerise. In fact there are 18 potential self dimers as calculated using IDT OligoAnalyzer 3.1 (<http://eu.idtdna.com/analyzer/Applications/OligoAnalyzer>).

Table 3. Analytical data for post-synthetically labelled amino-modified oligonucleotides

ID	Amino Oligo ID	Label	HPLC on crude oligo				MS	
			% Unlab.	RT/min	% Lab.	RT/min	Calc.	Obs.
7	3	2166	25.3	9.8	74.8	12.3	9892	9919
8	5	2166	21.5	9.7	78.5	12.5	9816	9846
9	6	2166	15.3	9.8	84.7	13.2	10160	10178

Table 4. Thiol-modified oligonucleotides

ID	Thiol Mod.	Spacer	Sequence
10	2187	-	TTC GGC TTG TCC GTG GAA TCT CAC AGC TTA T
11	2187	-	TTC GGC TTG TCC GTG GAA TCT CAC AGC TTA T
12	2187	-	TTC GGC TTG TCC GTG GAA TCT CAC AGC TTA T
13	2187	-	TTC GGC TTG TCC GTG GAA TCT CAC AGC TTA T
14	2126	-	TTC GGC TTG TCC GTG GAA TCT CAC AGC TTA T
15	2126	-	TTC GGC TTG TCC GTG GAA TCT CAC AGC TTA T
16	2126	2129	TTC GGC TTG TCC GTG GAA TCT CAC AGC TTA T

Table 5. Analytical data for thiol-modified oligonucleotides

ID	Thiol Mod.	Spacer	HPLC on crude oligo plus TCEP						MS	
			% Unmod.	RT/min	% Thiol Mod.	RT/min	% Capped Prod.	RT/min	Calc.	Obs.
10	2187	-	23.3	9.0	0	-	67.2	11.0	9779	9850
11	2187	-	20.2	9.1	4.3	10.3	75.5	11.0	9779	9850
12	2187	-	22.1	9.6	0	-	75.3	11.2	9779	9862
13	2187	-	27.9	9.5	0	-	69.5	11.2	9779	9854
14	2126	-	22.7	9.2	77.2	10.9	0	-	9777	9796
15	2126	-	19.5	9.1	80.4	10.9	0	-	9777	9802
16	2126	2129	23.7	9.1, 9.6	73.4	11.9, 14.0	0	-	10122	10149

Thiol Linkers

A comparison of the most commonly used commercially available thiol linker (2126) with the new hydrophilic linker (2187) was carried out. The sequences synthesised are shown in Table 4.

The HPLC of the crude oligonucleotides modified with the S-S thiol (2126) showed three peaks, corresponding to unmodified oligonucleotide where the modification has not completely coupled, the oligonucleotide modified with the free thiol (i.e. the disulphide bridge has cleaved during the deprotection) and the protected thiol modified oligonucleotide (HO-C6-S-S-C6-O-oligo). In some cases the dimer of the free thiol was also observed where two thiol oligonucleotides form a disulphide bridge.

To simplify the HPLC trace, 10µl of 87mM TCEP/H₂O solution was added to each of the HPLC samples for all thiol modified oligonucleotides. In this way only the free thiol derivative is

observed in the chromatograms. The results are summarised in Table 5.

The coupling reaction of the Bz-S derivative was at least as good as the thiol C6 S-S derivative however, MS showed that the predominant product using the Bz-S derivative had a mass of between 70-80 Da higher than expected. It is not uncommon for crude oligonucleotides to show a higher MW in the MALDI due to salt adducts, however this is generally in the region of +20 rather than +70.

Initially this was thought to be due to the fact that the thiol had reacted with the cyanoethyl groups during deprotection and was now blocked by acrylamide (+53Da) therefore oligonucleotides 12 and 13 were synthesised as before but this time they were treated with 20% DEA/MeCN prior to cleavage and deprotection. These oligonucleotides were treated with TCEP and underwent a conjugation reaction with 6-IAF. HPLC analysis suggested that the reaction had

worked, but MS showed that the main component of the mixture was in fact the capped thiol adduct.

To ensure that the problem was not coming from the deprotection the following conditions were then attempted:

- AMA, room temperature, 2h
- AMA, 10min, 65°C
- 0.5M NaOH in MeOH/H₂O 4:1

However there was no difference in the outcome and the main product in all cases was the capped thiol oligonucleotide.

Changing the activator however to either 0.4M tetrazole or 0.3M BTT resulted in the desired product and no capped product was observed. Two poly T₁₀ oligonucleotides (**17**) and (**18**) were synthesised using tetrazole and BTT respectively and deprotected in AMA for 10min at 65°C. In both cases the main peak in the HPLC is the dimerised oligonucleotide T₁₀-S-S-T₁₀ that was reduced to

the free thiol with the addition of TCEP solution⁸ to the HPLC sample. The MS showed the correct product but as the sodium salt, the predominant peak being N+5Na⁺ but with salt peaks ranging from N+4Na⁺ to N+11Na⁺ for both oligonucleotides. The results are summarised in Table 6.

Therefore the use of either tetrazole or BTT to couple the Bz-S TEG phosphoramidite (**2187**) results in the correctly modified thiol oligonucleotide. It is thought that the Bz-S undergoes a transthioesterification-type reaction with the ETT during the coupling reaction. This would result in a product (**A**) with a mass of +69 in relation to the calculated mass of the thiol modified oligonucleotide (see Scheme 1).

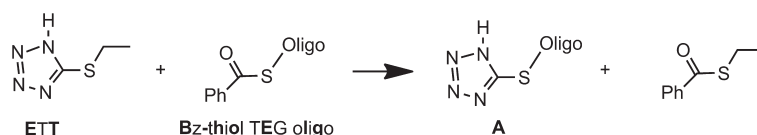
Although in principle the use of BTT could result in the same capped thiol modified oligonucleotide, it is thought that the larger benzoyl group may be preventing this from taking place.

⁸ 10µl of 87mM TCEP (25mg in 1ml of water) was added to show that the main peak is reduced.

Table 6. Analytical data for thiol-modified oligonucleotides using alternative activators

ID	Thiol Mod.	Activator	Seq.	HPLC on crude oligo plus TCEP						MS	
				% Unmod.	RT/min	% Thiol Mod.	RT/min	% T ₁₀ -S-ST ₁₀	RT/min	Calc.	Obs.
17	2187	0.4M Tetrazole	T ₁₀	8.9	10.1	32.4	12.1	58.8	12.6	3281	3390
18	2187	0.3M BTT	T ₁₀	6.13	10.1	14.0	12.2	79.8	12.6	3281	3390

Scheme 1. Oligo transthioesterification in presence of ETT



To test the post-synthetic labelling coupling efficiency of the thiol-modified oligonucleotides, these were then coupled to 6-iodoacetamido fluorescein (6-IAF). Note for the conjugated products HPLC analysis was carried out using the best method for the oligonucleotide conjugate combination [gradient 1 or 2 as detailed in the Experimental section]. Oligonucleotides **10** and **14** were used as controls and for mixed injections during HPLC analysis. The analytical data is summarised in Table 7.

In all cases the thiol-modified oligonucleotides couple well post-synthetically with 6-IAF. Reactions of the thiol TEG modified oligonucleotides (**20**, **21**) proved as efficient as both the thiol C6 (**22**) and the thiol C6-HEG (**23**) oligonucleotides.

Conclusions

The use of the hydrophilic amino-11-modifier (**2182**) or the Bz-S TEG (**2187**) phosphoramidites

proved to be an efficient means of incorporating a hydrophilic linker into an oligonucleotide without the need to use an additional hydrophilic spacer such as HEG (**2129**).

While there is no change to standard oligonucleotide synthesis methods when using these modifications, it is important **not to use ETT with the Bz-S TEG phosphoramidite** during the coupling reaction to avoid the formation of the capped thiol modified oligonucleotide (**A**). However the amidite couples well using 0.3M BTT or 0.45M tetrazole.

Recommended protocols are given below.

Dissolution

Use a 0.1M amidite concentration in anhydrous acetonitrile (see dilution Table 8). Since the amidites are oils it is recommended that the solution is prepared at least 10-15min prior to placing on the synthesiser and is vortexed immediately before use.

Table 7. Analytical data for post-synthetically labelled thiol-modified oligonucleotides

ID	Thiol Oligo ID	Label	HPLC Grad.	HPLC on crude oligo			MS		Comments	
				% Unlab.	RT/min	% Lab.	RT/min	Calc.		Obs.
19	11	6-IAF	1	100	11.6	0	~12.5	10168	9877	This was carried out with a capped thiol oligo although a small product peak (10187) was observed in the MS
20	17	6-IAF	2	50.2	10.6	49.8	11.2	3669	3702	The change in HPLC gradient used for analysis was due to the fact that there is little difference between the RT of the dimerised oligo and the labelled product. No unlabelled oligo is observed in the MS.
21	18	6-IAF	2	24.4	10.7	75.6	11.2	3669	3703	The change in HPLC gradient used for analysis was due to the fact that there is little difference between the RT of the dimerised oligo and the labelled product. A very small unlabelled oligo peak is observed in the MS.
22	15	6-IAF	1	18.5	9.92	81.5	11.6	10033	10044	Only the product is observed in the MS. The conjugation reaction was carried out twice on this sample since the initial coupling efficiency was only around 4%
23	16	6-IAF	1	34.7	10.1	65.3	12.4	10377	10388, 9970	Both the labelled and the unlabelled oligos are observed in the MS. The conjugation reaction was carried out twice on this sample since the initial coupling efficiency was only around 8%

Coupling

2182 - Use any activator, e.g. 0.25M ETT or 0.3M BTT in acetonitrile.

2187 - Use 0.3M BTT in acetonitrile. **Do not use ETT.**

A 15min coupling time is used for both products.

Cleavage & Deprotection

Treat the column with 20% DEA/MeCN prior to deprotection.

For deprotection in AMA, the temperature and time will depend on the other modifications and protecting group chemistry of the other amidites. Typically this would be AMA, 10min at 65°C.

Purification

Use standard methods such as those detailed above.

Conjugation

Conjugate using methods appropriate for the label you are using. When using **2187** treat the modified oligonucleotide with TCEP or DTT immediately prior to conjugation since it will predominantly be in the dimerised form (oligo-S-S-oligo).

Storage & Stability

Both modifiers are stored dry in a freezer at -10 to -30°C. Stability in solution is 2-3 days.

Testing of the amino product (**2182**) has shown that it is stable in the freezer for over a year. Tests on the thiol product are ongoing.

Table 8. Phosphoramidite dilutions

Item No.	Mol. Wt.	Unit Wt.	Dilution (0.1M)/ml			Dilution (0.067M)/ml		
			250mg	500mg	1g	250mg	500mg	1g
2182	852.92	308.18	2.93	5.86	11.72	4.37	8.75	17.50
2187	572.70	331.34	4.37	8.73	17.46	6.52	13.03	26.06

Ordering Information

Product	Pack Size	Cat. No.
Hydrophilic	100µmol	2182-F100
Amino 11 CE	250mg	2182-B250
Phosphoramidite		
Hydrophilic	100µmol	2187-F100
S-Bz TEG-CE	250mg	2187-B250
Phosphoramidite		

Further Information

For up to date ordering and protocol information please see www.linktech.co.uk, e-mail us at sales@linktech.co.uk or call +44(0)1698 849911.

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