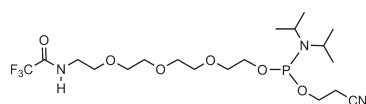


# Use of a MMT-protected hydrophilic amino linker in oligonucleotide synthesis

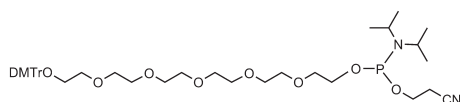
Sheena Aitken, David Hannah, Grant McGeoch and Catherine McKeen

## Introduction

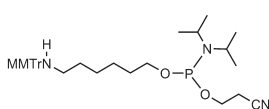
Recently we introduced a hydrophilic amino linker (**2182**) to alleviate the need for the use of a hydrophilic spacer (e.g. **2129**) in combination with an amino linker (e.g. **2123**, **2124**, **2133**) to improve the post-synthetic labelling efficiency of amino modified oligonucleotides in aqueous solution.<sup>1</sup>



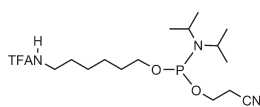
**2182**



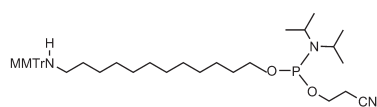
**2129**



**2123**



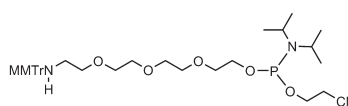
**2124**



**2133**

Although this has proved to be very successful in improving the post-labelling efficiency of amino modified oligonucleotides without the need of an additional spacer, the use of the TFA protecting group does not provide a significant difference in the retention time of the amino modified vs unmodified oligonucleotide by HPLC. This made purification difficult for longer oligonucleotides.

This problem has now been resolved by the introduction of the MMT protected hydrophilic aminolinker (**2193**).



**2193**

A series of oligonucleotides has been synthesised to optimise its use and test compatibility with various deprotection conditions.

## Experimental

### Oligonucleotide Synthesis

Anhydrous acetonitrile (**4050**) was flushed with argon and 10ml added to 1g of each of the standard amidites. 0.1M MMT amino-11-amidite (**2193**) was prepared by adding 1ml of anhydrous acetonitrile to 100µmol of amidite.

Prior to synthesis, anhydrous acetonitrile was placed on all unused amidite positions and all the amidite and activator lines primed using the ABI begin procedure.

The syntheses were carried out on an ABI 394 DNA/RNA automated synthesiser using a dT 1000Å SynBase™ 1µmol column (**2271**). The following reagents were also used: Activator, 0.25M ETT (**3140**); Cap A, THF/pyridine/ acetic anhydride (8:1:1) (**4110**); Cap B, 10% Methylimidazole in THF (**4120**); Oxidiser, 0.02M Iodine in THF/pyridine/water (7:2:1) (**4330**); Deblock, 3% TCA/DCM (**4140**).

### Cleavage and Deprotection

After synthesis the resin was pushed out of each column into the appropriately labelled tube and deprotected as outlined in Table 1. Upon completion, the tubes were vortexed and the contents of each tube loaded onto the top of a G25 column with the appropriately labelled tube sitting directly beneath and desalted to remove the deprotection solution.

### Quantification

Measuring the absorbance at 260nm and converting this value to OD/ml quantified the

<sup>1</sup> See *Synthesis of oligonucleotides modified with hydrophilic 5'-linker phosphoramidites for use in post-synthetic labelling in aqueous media* (TS2182/2187): <http://www.linktech.co.uk/documents/610/610.pdf>.

Table 1. Oligonucleotides synthesised

ID	Amino Mod.	Sequence	DMT ON/OFF	Amino Mod. Conc.	Coupling Time/s	Deprotection Conditions		
						Solution	Time/min	Temp./°C
1	-	TTT TTT	OFF	-	30	AMA	10	65
2	<b>2193</b>	TTT TTT	ON	0.1M	30	AMA	10	65
3	<b>2193</b>	TTT TTT	ON	0.1M	60	AMA	10	65
4	<b>2193</b>	TTT TTT	ON	0.1M	120	AMA	10	65
5	<b>2193</b>	TTT TTT	ON	0.1M	180	AMA	10	65
6	<b>2193</b>	TTT TTT	ON	0.1M	240	AMA	10	65
7	<b>2193</b>	TTT TTT	ON	0.1M	300	AMA	10	65
8	<b>2193</b>	TTT TTT	ON	0.1M	360	AMA	10	65
9	<b>2193</b>	TTT TTT	ON	0.1M	420	AMA	10	65
10	<b>2193</b>	TTT TTT	ON	0.1M	480	AMA	10	65
11	<b>2193</b>	TTT TTT	ON	0.1M	540	AMA	10	65
12	<b>2193</b>	TTT TTT	ON	0.1M	600	AMA	10	65
13	<b>2193</b>	TTT TTT	ON	0.1M	660	AMA	10	65
14	<b>2193</b>	TTT TTT	ON	0.1M	720	AMA	10	65
15	<b>2193</b>	TTT TTT	ON	0.1M	780	AMA	10	65
16	<b>2193</b>	TTT TTT	ON	0.1M	840	AMA	10	65
17	<b>2193</b>	TTT TTT	ON	0.1M	900	AMA	10	65
18	<b>2193</b>	TTT TTT	ON	0.1M	900	AMA	35	55
19	<b>2193</b>	TTT TTT	ON	0.1M	900	AMA	120	RT
20	<b>2193</b>	TTT TTT	ON	0.1M	900	Ammonium hydroxide	Overnight	55
21	<b>2193</b>	TTT TTT	ON	0.1M	900	i. AMA ii. $\text{NEt}_3$ / $\text{NEt}_3\cdot 3\text{HF}$ / NMP (1.5:2:3)	i. 10 ii. 150	i. 65 ii. 65
22	<b>2193</b>	TTT TTT	ON	0.1M	900	AMA	10	65

oligos. This was achieved as described below.<sup>2</sup>

- Record the volume (mL) of the sample in the tube.
- Take 2.75ml of water and add this to a clean cuvette.
- Put this into the spectrophotometer and run a blank at 260nm.
- Take 50µl of the sample and add to the cuvette of water.
- Measure the absorbance at 260nm.
- Multiply the value by 55 then multiply by the volume (mL) recorded in step 1.
- This is the concentration of the oligo sample in terms of OD/ml.

HPLC samples were made up using Chromacol inserts in the Chromacol HPLC injection vial. The oligo sample (25µl) was placed into the insert and water (175µl) was added.

RP-HPLC was carried out using a Waters X-Bridge OST C18 2.5µm, 4.6x50mm column using buffer A: 0.1M TEAA and buffer B: 100% MeCN over a gradient of 0-50%B over 15min at a flow rate of 1ml/min.

<sup>2</sup> Note: if the measured absorbance value was greater than 1.5 then 5µl of sample in 45µl of water was used in step 4 and a dilution factor of 550 was used in step 6.

Each LCMS sample was diluted to a concentration of 1 OD/ml and filtered through a Millipore filter tube (0.22µm). LCMS was carried out on an Agilent LCMS system using a G6220 TOF system and 1200 series pumps. Buffer A: 190mM HFIP, 7mM TEA, 5% MeOH in water, buffer B: MeOH, source: Dual ESI (negative mode), column: Agilent Zorbax C18 2.1x30mm.

## Results & Discussion

### Oligonucleotide Synthesis

A series of amino-11 modified oligonucleotides using **2193** as shown in Table 1 were synthesised, where the coupling time was varied between 30-900s. All oligonucleotides were synthesised DMT-ON with the exception of the unmodified control T6 oligonucleotide 1.

After deprotection (see Table 1) the oligonucleotides were each passed through a G25 sephadex column to remove the deprotection solution. These were then quantified and analysed without purification. On average, the crude yield of the modified T6 oligonucleotides was 60 OD/ml.

### Analysis of results.

As expected, the difference in the retention time

Table 2. RP-HPLC analytical data

ID	Coupling Time/s	RT/min	% FL-DMT ON	RT/min	% FL-DMT-OFF and unmodified oligo
1	30	9.00	0.00	5.46	100.00
2	30	9.09	79.15	5.49	20.85
3	60	9.07	73.65	5.49	26.35
4	120	9.07	81.52	5.46	18.48
5	180	9.04	87.71	5.37	12.29
6	240	9.05	85.49	5.40	14.51
7	300	9.08	79.49	5.41	20.51
8	360	9.07	74.01	5.41	25.99
9	420	9.07	74.00	5.40	26.00
10	480	9.09	81.62	5.40	18.38
11	540	9.09	84.20	5.40	15.80
12	600	9.09	83.06	5.38	16.94
13	660	9.07	71.10	5.39	28.90
14	720	9.07	73.40	5.93	26.60
15	780	9.06	78.89	5.39	21.19
16	840	9.06	83.29	5.39	16.71
17	900	9.05	76.73	5.38	23.27
18	900	9.05	78.57	5.40	21.43
19	900	9.05	80.00	5.37	20.00
20	900	9.02	52.57	5.40	47.43
21	900	9.02	81.60	5.38	18.40
22	900	9.00	0.00	5.39	100.00

of the unmodified and the free amino-modified oligonucleotides is not sufficient to be able to distinguish between them with the RP-HPLC conditions used. However, the results (see Table 2) indicate that there is no significant difference in the coupling efficiency of the MMT-amino 11 CEP (**2193**) when coupled at 25s or 900s. The data also suggests that all deprotection conditions shown in Table 1 are compatible with this modification.

To confirm these results, the samples were further analysed by LCMS, which shows in all cases further detritylation had taken place in the time between HPLC and LCMS analysis (see Table 3).

The data indicates that even at 30s the coupling efficiency is greater than 99% and there is a marginal increase for coupling times of 2min or greater. This is shown in Figure 1. Also there are no degradation products observed in the LCMS as a result of the deprotection of the modified oligonucleotide using the conditions outlined in Table 1.

## Conclusions

MMT protected hydrophilic amino-11-modifier phosphoramidite (**2193**) is compatible with standard oligonucleotide synthesis and is stable to all deprotection conditions outlined in Table 1.

The recommended use is as follows:

### Dissolution

Use 0.1M amidite concentration in anhydrous

acetonitrile (100µmol/1ml, 250mg/3.75ml). Since the amidite is an oil it is recommended that this is prepared at least 10-15min prior to placing on the synthesiser and is vortexed immediately before use.

### Coupling

Activate with 0.25M ETT in acetonitrile and use a 2min coupling time.

### Cleavage & Deprotection

Treat the column with 20% DEA/MeCN prior to deprotection. In normal use, deprotect 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

If MMT is retained the following purification methods are feasible: cartridge; PAGE or RP-HPLC DMT-ON followed by detritylation; or RP-HPLC with on-column detritylation.

If MMT is removed prior to purification then RP-HPLC, IE-HPLC or PAGE are all suitable methods.

### Conjugation

The free amino group is available for conjugation using standard methods.

### Storage & Stability

Store dry in a freezer at -10 to -30°C. Stability in solution is 2-3 days.

Table 3. LCMS analytical data

ID	RT/ min	% Unmod / failures	Calc. MW	Obs. MW	RT/ min	% FL (-MMT)	Calc. MW	Obs. MW	RT/ min	% FL (+MMT)	Calc. MW	Obs. MW	Total % FL
1	6.55	100.00	1761.31	1762.33	N/A	0.00	N/A	N/A	N/A	0.00	N/A	N/A	0.00
2	6.59	0.10	1761.31	1762.32	7.04	99.80	2016.40	2017.42	8.83	0.10	2288.52	2290.53	99.90
3	6.58	0.10	1761.31	1762.32	7.04	99.80	2016.40	2017.41	8.86	0.10	2288.52	2290.53	99.90
4	6.50	0.00	1761.31	N/A	7.03	100.00	2016.40	2017.41	8.80	0.00	2288.52	N/A	100.00
5	6.50	0.00	1761.31	N/A	7.04	48.97	2016.40	2020.42	8.85	51.03	2288.52	2291.54	100.00
6	6.50	0.00	1761.31	N/A	7.03	99.90	2016.40	2017.41	8.86	0.10	2288.52	N/A	100.00
7	6.50	0.00	1761.31	N/A	7.04	100.00	2016.40	2017.41	8.80	0.00	2288.52	N/A	100.00
8	6.50	0.00	1761.31	N/A	7.03	100.00	2016.40	2017.42	8.80	0.00	2288.52	N/A	100.00
9	6.50	0.00	1761.31	N/A	7.03	100.00	2016.40	2039.39	8.80	0.00	2288.52	N/A	100.00
10	6.50	0.00	1761.31	N/A	7.03	99.9	2016.40	2017.42	8.86	0.10	2288.52	2290.53	100.00
11	6.50	0.00	1761.31	N/A	7.02	99.9	2016.40	2017.41	8.87	0.10	2288.52	2290.53	100.00
12	6.50	0.00	1761.31	N/A	7.03	100.00	2016.40	2017.42	8.80	0.00	2288.52	N/A	100.00
13	6.50	0.00	1761.31	N/A	7.02	100.00	2016.40	2017.42	8.80	0.00	2288.52	N/A	100.00
14	6.50	0.00	1761.31	N/A	7.03	100.00	2016.40	2017.42	8.80	0.00	2288.52	N/A	100.00
15	6.50	0.00	1761.31	N/A	7.03	100.00	2016.40	2017.41	8.80	0.00	2288.52	N/A	100.00
16	6.50	0.00	1761.31	N/A	7.03	100.00	2016.40	2017.41	8.80	0.00	2288.52	N/A	100.00
17	6.50	0.00	1761.31	N/A	7.03	38.03	2016.40	2039.39	8.85	61.97	2288.52	2290.54	100.00
18	6.50	0.00	1761.31	N/A	7.03	21.43	2016.40	2039.39	8.85	78.57	2288.52	2290.54	100.00
19	6.50	0.00	1761.31	N/A	7.04	99.9	2016.40	2017.41	8.79	0.10	2288.52	2290.53	100.00
20	6.50	0.00	1761.31	N/A	7.04	99.9	2016.40	2017.42	8.96	0.10	2288.52	2291.51	100.00
21	6.50	0.00	1761.31	N/A	7.03	61.8	2016.40	2017.41	8.85	38.2	2288.52	2290.54	100.00
22	6.50	0.00	1761.31	N/A	7.03	100.00	2016.40	2017.41	8.80	0.00	2288.52	N/A	100.00

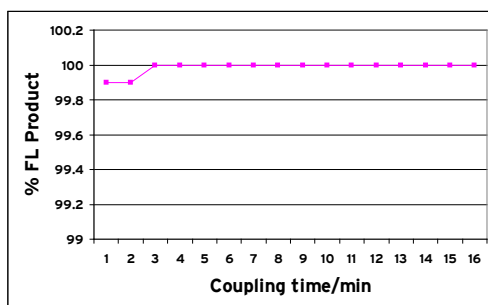


Figure 1. Effect of coupling time on coupling efficiency (data from Table 3)

### Further Information

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

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