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Synthesis of UNA and chimeric UNA/DNA, UNA/DNA/RNA oligonucleotides with unlocked nucleic acids (UNA)

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Introduction

As far back as 1995 the research group of Professor Jesper Wengel introduced the thymine UNA monomer as a modification in DNA oligonucleotides.[1] Along with many other acyclic nucleotide modifications, UNA was shown to induce decreased binding affinity towards a complementary strand. UNA has subsequently been studied as a constituent in gapmer antisense oligonucleotides, and compatibility with RNase H recognition and RNA cleavage has been reported.[2]

UNA (Unlocked Nucleic Acid) is an acyclic analogue of RNA in which the bond between the C2′ and C3′ atoms is not present (see Figure 1).

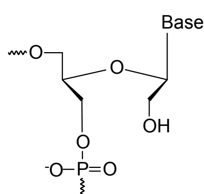


Figure 1. General structure of UNA.

Like LNA, UNA is an RNA analogue. However, whereas the additional methylene group linking the O2′ and C4′ atoms of LNA locks its furanose ring into a C3′-endo conformation, the lack of the C2′-C3′ bond in UNA makes this molecule very flexible. It can be helpful to think of LNA as 'locked RNA' and UNA as 'unlocked RNA'.

UNA enables fine tuning of duplex thermodynamic stabilities. Their antipodal structural characteristics make UNA and LNA complementary with respect to effect on binding affinity towards a DNA or RNA target: T_m is decreased by 5-10˚C per UNA monomer; T_m is increased by 3-10˚C per LNA monomer.

As the effect of UNA monomers is additive, a selected number of UNA monomers can be incorporated into DNA or RNA oligonucleotides to gradually decrease the thermodynamic stabilities of duplexes. It should however be noted that mismatch studies have shown that UNA monomers form Watson-Crick hydrogen bonds with complementary bases. Because of its acyclic ribose-like ring, UNA monomers can be applied to introduce local structural flexibility in a single or double stranded nucleic acid.

UNA monomers can be positioned strategically to induce either lack of discrimination of mismatches, i.e. universal base behavior, or increased discrimination of mismatches, i.e. improved hybridization specificity. UNA-modified RNA duplexes have been shown by CD spectroscopy to structurally mimic unmodified RNA duplexes.[3]

UNA monomers are very useful to improve siRNA mediated gene silencing in cell cultures or in vivo. UNA modified siRNAs exhibit a number of key characteristics:[4]

- One or two UNA monomers in the 3′-overhangs stabilise against degradation by nucleases;
- One UNA monomer in position 6 or 7 in the antisense strand alleviates miRNA-type off-target effects and leads to very potent gene silencing;
- UNA monomers in the antisense strand can be combined with other modifications.

1 **Synthesis and evaluation of oligodeoxynucleotides containing acyclic nucleosides: Introduction of three novel analogues and a summary**, P. Nielsen, L.H. Dreiøe and J.Wengel, *Bioorg. Med. Chem.*, **3**, 19-28, 1995.

2 **Efficient RNase H-Directed Cleavage of RNA Promoted by Antisense DNA or 2'F-ANA Constructs Containing Acyclic Nucleotide Inserts**, M.M. Mangos, K.-L. Min, E. Viazovkina, A. Galarneau, M.I. Elzagheid, M.A. Parniak, and M.J. Damha, *J. Amer. Chem. Soc.*, **125**, 654-661, 2003.

3 **UNA (unlocked nucleic acid): A flexible RNA mimic that allows engineering of nucleic acid duplex stability**, N. Langkjær, A. Pasternak and J. Wengel, *Bioorg. Med. Chem.*, **17**, 5420-5425, 2009.

4 **A large-scale chemical modification screen identifies design rules to generate siRNAs with high activity, high stability and low toxicity**, J.B. Bramsen, M.B. Laursen, A.F. Nielsen, T.B. Hansen, C. Bus, N. Langkjær, B.R. Babu, T. Højland, M. Abramov, A. Van Aerschot, D. Odadzic, R. Smicius, J. Haas, C. Andree, J. Barman, M. Wenska, P. Srivastava, C. Zhou, D. Honcharenko, S. Hess, E. Müller, G.V. Bobkov, S.N. Mikhailov, E. Fava, T.F. Meyer, J. Chattopadhyaya, M. Zerial, J.W. Engels, P. Herdewijn, J. Wengel and J. Kjems, *Nucleic Acids Research*, **37**, 2867-2881, 2009.

We offer the four UNA phosphoramidites (**2183-2186**) employing standard base protecting groups (Ac-C, Bz-A and iBu-G).

We have synthesised a series of UNA oligonucleotides to determine the optimum synthesis and deprotection conditions in which to use UNA monomers either as full UNA sequences or as UNA/DNA or UNA/DNA/RNA chimeras. For the latter tests we extended the comparison between the use of TC-RNA and TBDMS-RNA amidites when producing these chimeras.

Results & Discussion

Synthesis

The following oligonucleotides were synthesised and deprotected under varying conditions:

TTC GGC TTG TCC GTG GAA TCT CAC AGC TTA T

TTC GGC TTG TCC GTG G5A TCT C5C 5GC TT5 T

TTC 6GC TTG 6CC 6TG 6AA TCT CAC AGC TTA T

TT7 G7C TTG TCC GTG GAA TCT 7AC AG7 TTA T

8TC GGC T8G TCC GTG GAA 8CT CAC AGC T8A T

887 667 886 877 686 655 878 757 567 885 T

rATrG TrCT rUTT 8T7 T6T 5T

Where 5 = UNA-A, 6 = UNA-G, 7 = UNA-C and 8 = UNA-U.

All the sequences are derivatives of the full DNA sequence, which was selected to test the coupling of every base to every other base. The sequence does have the potential to self dimerise (in fact there are 18 potential self dimers as calculated using IDT's OligoAnalyzer 3.1).

In all cases the synthesis was carried out on an ABI 394 DNA/RNA synthesiser using standard 1μmol DNA (180s coupling for modifications, 30s for standard amidites) and RNA (300s and 30s) protocols. The DNA control was synthesised using the RNA cycle. The RNA/UNA/DNA oligonucleotides were synthesised in two parts using combinations of the cycles as shown in Table 1. Each of these oligos was synthesised twice, once using TBDMS-RNA chemistry and once using TC-RNA chemistry. In all cases the activator used for part 1 (UNA/DNA) was 0.25M ETT and for part 2 (RNA/DNA) 0.5M ETT.

For all oligonucleotides >98% coupling efficiencies were observed using the in built trityl monitor on the synthesiser, indicating there was no difference between either cycle in terms of UNA amidite coupling. Therefore 180s coupling is sufficient with 0.25M ETT for this chemistry.

Deprotection

The reported deprotection method for UNA[3] is AMA at 65˚C for 2h, however if used with sensitive modifications such as cyanine-type dyes would result in degradation of the modification. Furthermore, for a higher throughput, a faster deprotection method would be desirable. Therefore, the deprotection methods of choice were AMA at RT for 2h and AMA at 65˚C for 10min. Since the latter is standard during TBDMS-RNA chemistry deprotection this was incorporated into this test. The chemistry type vs. deprotection method is shown in Table 2.

The UNA/DNA/RNA oligonucleotides synthesised via TC-RNA chemistry were eluted from the resin with 0.1M TEAA. After deprotection the oligonucleotides were desalted by G25 and quantified. The crude oligonucleotides were then analysed by RP-HPLC on an Agilent Eclipse XBD-C18 5um, 4.6x150mm column and by MALDI-TOF mass spectrometry on a Bruker Ultraflex.

In general HPLC showed at least 80% purity, the lowest being the full DNA oligo (80%). In some cases there was a leading shoulder on the main peak but there was nothing in the MS to indicate that this is anything more than secondary structure. The highest purity was observed from the DNA/UNA chimeras and the full UNA oligonucleotides (94%). The results are summarised below in Table 3. As a comparison the retention time range of the analogous full RNA sequence is included. In all cases the MS was

Table 1. Synthesis cycles used for UNA/DNA/RNA chimera

	UNA: (TT 8T7 T6T 5T (Part 1)	RNA: rATrG TrCT rU (Part 2)
Synthesis Cycle (Modification coupling time)	DNA (180s)	DNA (180s)
	DNA (180s)	RNA (300s)
	RNA (300s)	DNA (180s)
	RNA (300s)	RNA (300s)

Table 2. Chemistry vs. deprotection method

Chemistry	Reagent	Time/min	Temp./˚C
DNA	AMA	120	RT
UNA/DNA	AMA	120	RT
UNA	AMA	120	RT
UNA/DNA/RNA (TBDMS)	1. AMA 2. NMP/$Et_3N.3HF/Et_3N$ (3:2:1.5)	10 150	65 65
UNA/DNA/RNA (TC)	EDA	120	RT
UNA/DNA/RNA (TC)	EDA/Toluene	120	RT

very clean showing the full-length sequence, the double charged species and, in most cases, the triple charged species.

The results show that the UNA chemistry is compatible with AMA deprotection at both 65˚C for 10 minutes and AMA at room temperature for 2 hours. This is also compatible with both TBDMS and TC chemistries.

It is worth noting that in UNA/DNA/RNA chimeras no strand cleavage is observed despite the O2'-acyl protecting group on the UNA monomers. This can be explained by the flexible structure of the UNA leaving no structural preorganisation of the deprotected 2'-OH group for nucleophilic attack on the O3'-phosphate moiety.

Table 3. Summary of analytical data

Chemistry	Purity in RP-HPLC	Observation in RP-HPLC	Retention Time of Main Peak/min
DNA	>80%	Single peak	8.60-9.19
UNA/DNA (5=uA)	>82%	Small leading shoulder on main peak	9.20-9.21
UNA/DNA (6=uG)	>82%	Small leading shoulder on main peak	9.20-9.21
UNA/DNA (7=uC)	>94%	Small leading shoulder on main peak	9.09-9.15
UNA/DNA (8=uU)	>93%	Small leading shoulder on main peak	8.85-8-87
UNA	>94%	Single peak	8.79-8.82
UNA/DNA/RNA	>88%	Single peak	8.96-9.07
RNA	-	-	8.85-8.96

Experimental

Preparation of Amidites

Anhydrous acetonitrile (**4050**) was flushed with argon and used to dilute 1g of each of the standard amidites, TBDMS-RNA amidites and UNA amidites to 0.1M (see Table 4). Each of the TC-RNA amidites (1g) was dissolved in anhydrous toluene to 0.1M. These were shaken vigorously to ensure complete dissolution.

Setting up the Synthesiser

Anhydrous acetonitrile was placed on all unused amidite positions and all the amidite and activator lines primed using the 'ABI begin procedure' on the synthesiser. The amidite bottles were placed on the synthesiser utilising the 'change bottle' feature but not using the 'auto dilute' feature. Once all the amidites were loaded a second 'ABI begin procedure' was carried out. The desired sequence was entered manually using the 'edit sequence' feature.

Cleavage and Deprotection

DNA, UNA and UNA/DNA.

After synthesis the columns were dried using the 'reverse flush' function on the synthesiser with only the top of the column connected to the column position. The resin was then pushed out of each column into an appropriately labelled tube and AMA (500µl) added. The tubes were vortexed and allowed to stand for 2h at room temperature. After this time the tubes were vortexed and the contents of each tube loaded onto the top of a G25 column with an appropriately labelled tube sitting directly beneath and desalted to remove the deprotection solution.

UNA/DNA/RNA-TBDMS

After synthesis, the columns were washed with 20% DEA/acetonitrile to remove the cyanoethyl protecting groups then thoroughly washed with MeCN (removing the cyanoethyl groups reduces the risk of any isomerisation or chain cleavage as a result of the interaction of the 2'-OH group with the 3'-phosphate during deprotection). The columns were dried using the 'reverse flush' function on the synthesiser with only the top of the column connected to the column position. The resin was then pushed out of each column into an appropriately labelled tube and AMA (500µl) added. The tubes were vortexed and heated at 65˚C for 10min. After this time the tubes were vortexed and the contents of each tube loaded onto the top of a G25 column with an appropriately labelled tube sitting directly

Table 4. Dilution of UNA amidites

Item No.	Mol. Wt.	Unit Wt.	Dilution (0.1M)/ml 250mg	500mg	1g
2183	980.07	331.23	2.55	5.10	10.20
2184	893.97	307.20	2.80	5.59	11.19
2185	962.05	347.23	2.60	5.20	10.39
2186	852.92	308.18	2.93	5.86	11.72

beneath and desalted to remove the deprotection solution.

The samples were then completely dried and NMP/Et_3N.3HF/Et_3N (3:2:1.5) (500µl) added then heated at 65˚C for 2.5h. The samples were then allowed to cool to room temperature then the tubes were vortexed and the contents of each tube loaded onto the top of a G25 column with an appropriately labelled tube sitting directly beneath and desalted to remove the deprotection solution.

UNA/DNA/RNA-TC

After synthesis the columns were washed with 20% DEA/acetonitrile to remove the cyanoethyl protecting groups then thoroughly washed with acetonitrile. The columns were dried using the 'reverse flush' function on the synthesiser with only the top of the column connected to the column position. The columns were then placed on a vacuum manifold and the following steps were carried out using the appropriate deprotection solution (see Table 2 for details).

1. Place the columns on a vacuum manifold with a 3ml syringe barrel on the top of each column.
2. Turn the vacuum on to ensure the resin is dry.
3. Turn the vacuum off and close all the taps to the column positions.
4. Add the deprotection solution (500µl) to the syringe barrel.
5. Turn the vacuum on.
6. One column at a time, open the tap to allow the solution to come through the column but close the tap before the solution comes through the tap.
7. Turn off the vacuum.
8. Leave for 2h.
9. Turn the vacuum on and open the taps to the columns to remove the deprotection solution.
10. Turn the vacuum off.
11. Add 2.5ml of dry acetonitrile to the syringe barrel.
12. Turn the vacuum on to remove the acetonitrile.
13. Repeat the acetonitrile wash.
14. Dry the resin under vacuum for 2min.
15. Turn off the vacuum.
16. Place an appropriately labelled tube (RNase free) under each of the columns.
17. Add 1ml of 0.1M TEAA, pH 7.0 prepared with DEPC treated RNase free water (or other suitable buffer).
18. Turn on the vacuum to elute the product into the tube.
19. Desalt the oligo by G25.

Conclusions & Summary Use Protocol

Dissolution

UNA phosphoramidites are dissolved in dry acetonitrile to a concentration of 0.1M.

Coupling

A coupling time of 3-5min is recommended for up to 1µmol synthesis. Use 0.25M ETT as activator.

Cleavage & Deprotection

UNA is amenable to cleavage and deprotection methods as used for standard DNA chemistry, e.g. AMA at RT for 2h, or at 65˚C for 10min - 4h depending on the nature of the other modifications in the oligo. Typically this would be AMA, at 65˚C for 35min. With reference to the synthesis of UNA-RNA chimeras, this is also compatible with both TBDMS-RNA and TC-RNA chemistry deprotections.

Purification

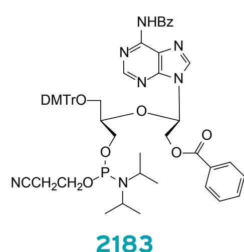
No change is required to the purification method as for standard DNA or RNA.

Storage & Stability

All phosphoramidites should be stored at -10 to -30°C. They are stable in solution for 2-3 days.

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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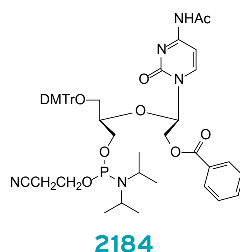

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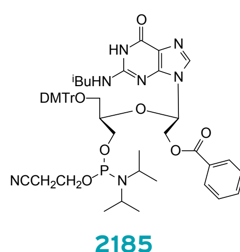

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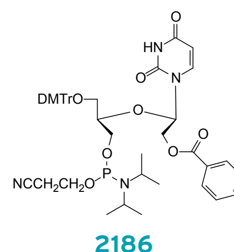

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Ordering Unlocked Nucleic Acids (UNA)

Product	Pack Size	Cat. No.
Bz-A-UNA-CE Phosphoramidite	250mg	2183-B250
	500mg	2183-B500
	1g	2183-C001
Ac-C-UNA-CE Phosphoramidite	250mg	2184-B250
	500mg	2184-B500
	1g	2184-C001

Product	Pack Size	Cat. No.
iBu-G-UNA-CE Phosphoramidite	250mg	2185-B250
	500mg	2185-B500
	1g	2185-C001
U-UNA-CE Phosphoramidite	250mg	2186-B250
	500mg	2186-B500
	1g	2186-C001

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