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An astute synthesis of locked nucleic acid monomers

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Abstract

Novel attributes of Locked Nucleic Acid (LNA) makes it preferable over most of the other classes of modified nucleic acid analogues and therefore, it has been extensively explored in different synthetic oligonucleotide based therapeutics. In addition to five oligonucleotides of this class undergoing clinical trials, a healthy pipeline in pre-clinical studies validates the tenacity of LNA. Due to the increasing demand, an efficient biocatalytic methodology has recently been devised for the convergent synthesis of LNA monomers *via* selective enzymatic monoacetylation of diastereotopic hydroxymethyl functions of 3-*O*-benzyl-4-*C*-hydroxymethyl-1,2-*O*-isopropylidene-*α*-D-ribofuranose. This commentary article provides an insight into the different synthetic strategies followed for the synthesis of LNA monomers and their triumphs in clinical biotechnology.

Keywords: Locked nucleic acid, Nucleic acid therapeutics, Bio-catalysis, Novozyme®-435, Modified oligonucleotides, Linear synthesis, Convergent synthesis, Miravirsen

Since the acclamation of nucleic acid therapeutics, modification in the sugar moiety of nucleosides has continuously reflected its supremacy for developing drug candidates for the treatment of cancer and viral infections [1-3]. After the pioneering development in dideoxy- and acyclic- nucleos(t)ides [3], currently the most promising modification in the ribofuranose moiety has appeared through the inclusion of an extra methylene bridge between 2′-*O* & 4′-*C* atom and synthesis of oligonucleotides (ONs) involving the modified nucleosides, termed as locked nucleic acid (LNA) (Figure 1) [4,5]. Although, no significant potency was observed against cancer or viral infections by LNA monomers or its analogues [6]; there is hardly any synthetic oligonucleotide (ON) based therapeutic strategy which has not been allured by their unique features [4,5,7,8].

Seminal papers on LNA were independently instigated by Wengel [9,10] and Imanishi [11] groups. It is well known that the B-form DNA duplex possesses $C_{2'}$-*endo* (*S*-type) and the A-form RNA duplex has $C_{3'}$-*endo* (*N*-type) sugar puckering [12,13]. LNA is considered to be RNA mimic as the ancillary methylene bridge locks the sugar moiety into *N*-type sugar ring conformation (Figure 1). This conformational restriction results in preorganization of the backbone of LNA ONs, which leads to energetically favorable duplex formation *via* increased base stacking interactions according to standard Watson-Crick base pairing rules [14]. Generally, the melting temperature (T_m) of duplexes is raised by 2-8°C per LNA nucleotide incorporation when compared to the corresponding unmodified duplexes, depending on the sequence context and number of modifications [14-16]. This makes LNA the prime nucleotide modification candidate for the applications where high hybridization affinity is desirable.

LNA-modified ONs have been extensively utilized in different approaches to target the corresponding nucleic acid counterparts. These primarily include, (a) antigene approach to block transcription of a particular gene; (b) antisense approach to induce RNA degradation; (c) siRNA mediated RNA degradation; and (d) blocking of microRNA [7,8]. Since LNA possess high hybridization affinity and target selectivity, it is unsurprising that increasing success in cell-line based experiments has paved their way to five LNA-based modified ONs under active clinical trials (Table 1). One of the most advanced LNA-based drugs Miravirsen which has entered Phase II clinical study is being developed by Santaris Pharma A/S. Miravirsen is an inhibitor of miR-122, a liver specific

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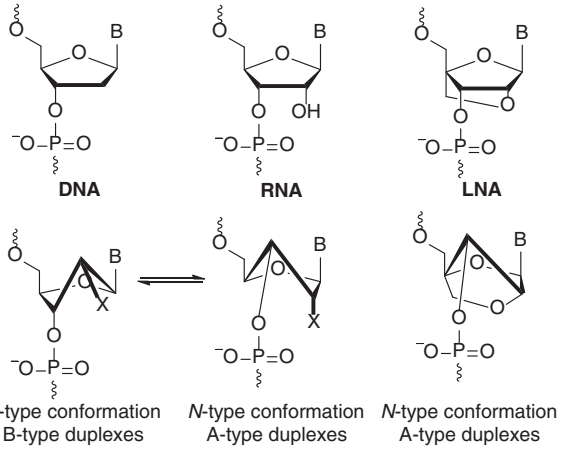


Figure 1 Structure and conformations of DNA, RNA and LNA; B = nucleobase.

microRNA that is required by Hepatitis C virus (HCV) for replication. The liver-expressed miR-122 protects HCV from degradation. Miravirsen is designed to recognize and sequester miR-122, making it unavailable for HCV. As a result, the replication of the virus is effectively inhibited and the level of HCV is profoundly reduced (Figure 2) [17]. If its phase III trial looks anything like its phase II, Miravirsen could be the first LNA-based drug to get FDA approval.

Two general strategies have been employed for the synthesis of LNA monomers; a linear strategy using commercially available RNA nucleosides as the starting material [11,19] and a convergent strategy where a common glycosyl donor is synthesized for coupling with different nucleobases [10,20,21]. Linear strategy was disclosed by Obika *et al.* [11] for the synthesis of LNA-U monomer **1a** with uridine (**2**) as the starting material (Scheme 1).

Following similar strategy, Koshkin *et al.* [19] synthesized LNA-A monomer taking adenosine as the starting material. Despite having some advantages, such as cheap and readily available RNA nucleosides as starting material and short synthetic route to LNA monomers, the linear approach suffers from poor yields. The two key reactions in the synthetic pathway, *i.e.* the introduction of the additional hydroxymethyl group at the *C*-4′-position of the protected RNA nucleoside **4** and the regioselective tosylation of the introduced 4′-*C*-hydroxymethyl group, generally proceeds with very low yields (Scheme 1).

Alternatively, in the quest to establish a general method for the synthesis of all LNA monomers *i.e.* LNA-U, **1a**; LNA-T, **1b**; LNA-A, **1c** and LNA-C, **1d**; the convergent strategy was explored by Koshkin *et al.* [10] using 3-*O*-benzyl-4-*C*-hydroxymethyl-1,2-*O*-isopropylidene-*α*-D-ribofuranose (**10**) as

Table 1 Current clinical trials of oligonucleotides modified with LNA [18]

S.no.	LNA modified oligonucleotide	Indication	Target	Clinical phase
1	Miravirsen (SPC-3649)	Hepatitis C virus (HCV)	MicroRNA-122	II
2	EZN-2968	Solid Tumours	Hypoxia-inducible factor-1 alpha (HIF-1*α*)	I
3	EZN-3042	Cancer	Survivin	I
4	EZN-4176	Cancer	Androgen receptor	I
5	SPC-4955	Hypercholesterolemia	Apolipoprotein B	I

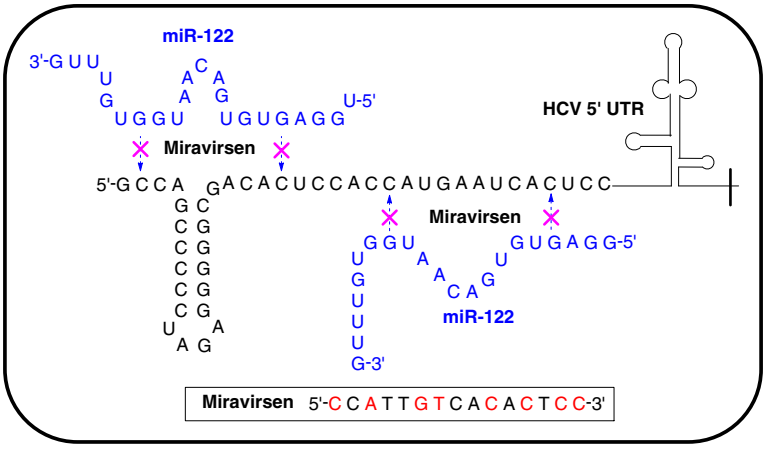
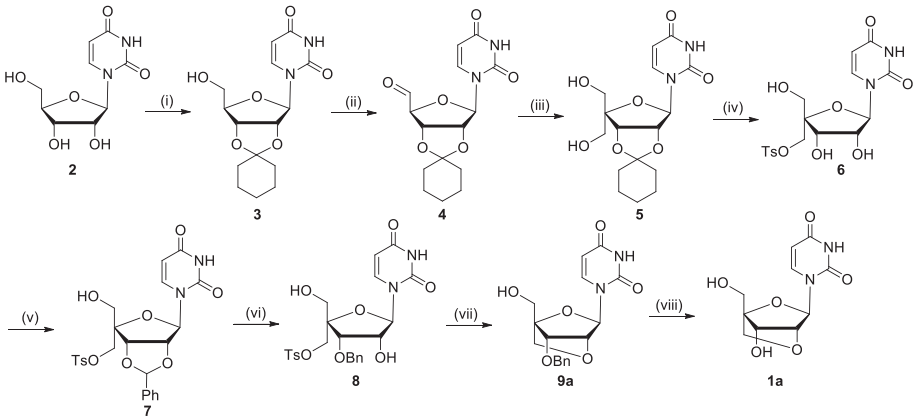
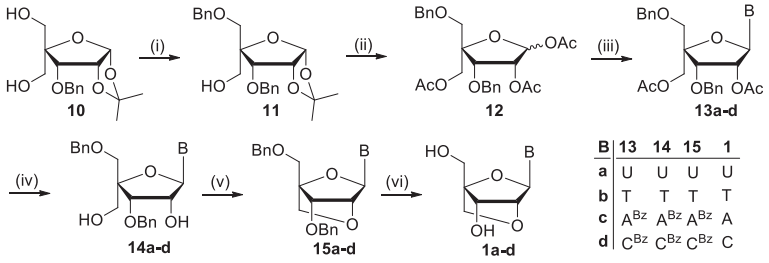


Figure 2 Mechanism of action of chemically modified drug Miravirsen. (LNA monomers in oligonucleotide are shown red).



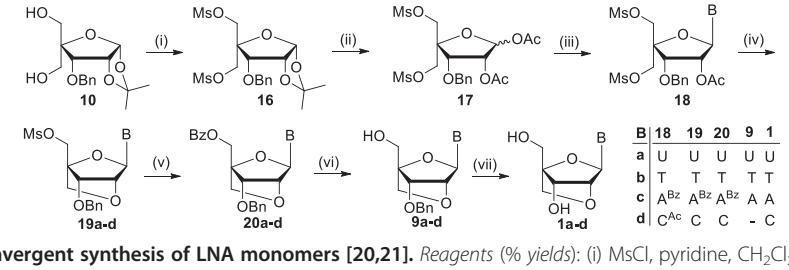
Scheme 1 Linear synthesis of LNA-U monomer [11]. *Reagents* (% *yields*): (i) Cyclohexanone, PTSA (quantitative); (ii) 2-iodobenzoic acid, CH_3CN (76%); (iii) (a) 37% HCHO, 2N NaOH, 1,4-dioxane; (b) $NaBH_4$ (38%); (iv) (a) TsCl, Pyridine; (b) TFA, water (34%); (v) PhCHO, $ZnCl_2$, (80%); (vi) $NaBH_3CN$, $TiCl_4$, CH_3CN (75%); (vii) NaHMDS, THF (61%); (viii) 10% Pd-C, H_2, MeOH (quantitative).

a starting material, which can be synthesized easily from D-glucose [22,23]. Regioselective 5-*O*-benzylation of **10** followed by acetolysis afforded the furanose **12** in 55% yield, a key intermediate for coupling reactions with a variety of nucleobases. The Vorbrüggen coupling with silylated nucleobases afforded the nucleosides **13a**-**d**, which on deacetylation led to the formation of benzylated nucleosides **14a**-**d**. The tosylation of the primary hydroxyl group in benzylated nucleosides **14a**-**d** followed by *in situ* base-induced intramolecular ring closure afforded the 2′-*O*,4′-*C*-linked locked nucleoside derivatives **15a**-**d**. Debenzylation on dibenzylated nucleosides **15a**-**d** efficiently yielded the LNA monomers **1a**-**d** (Scheme 2).



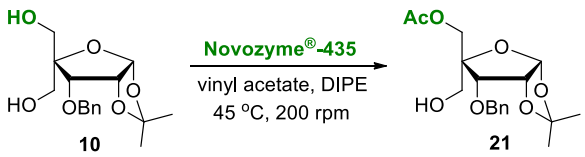
B	13	14	15	1
a	U	U	U	U
b	T	T	T	T
c	A^{Bz}	A^{Bz}	A^{Bz}	A
d	C^{Bz}	C^{Bz}	C^{Bz}	C

Scheme 2 Convergent synthesis of LNA monomers [10]. *Reagents* (% *yields*): (i) BnBr, NaH, DMF (71%); (ii) (a) Ac_2O, Pyridine; (b) 80% AcOH; (c) Ac_2O, Pyridine (77%); (iii) nucleobase, *N*,*O*-*bis*(trimethylsilyl)acetamide, TMS-triflate, CH_3CN or 1,2-dichloroethane (**13a**, 75%; **13b**, 76%; **13c**, 52%; **13d**, 74%); (iv) NaOMe, MeOH (**14a**, 95%; **14b**, 97%; **14c**, 73%; **14d**, 54%); (v) (a) TsCl, Pyridine; (b) NaH, DMF (**15a**, 30%; **15b**, 42%; **15c**, 44%; **15d**, 51%); (vi) 20% $Pd(OH)_2$-C, H_2, EtOH (**1a**, 78%; **1b**, 98%); BCl_3, DCM (**1c**, 84%); 10% Pd-C, 1,4-cyclohexadiene, MeOH (**1d**, 36%).



B	18	19	20	9	1
a	U	U	U	U	U
b	T	T	T	T	T
c	A^{Bz}	A^{Bz}	A^{Bz}	A	A
d	C^{Ac}	C	C	-	C

Scheme 3 Improved convergent synthesis of LNA monomers [20,21]. *Reagents* (% *yields*): (i) MsCl, pyridine, CH_2Cl_2 (98%); (ii) Ac_2O, AcOH, conc. H_2SO_4 (97%); (iii) nucleobase, *N*,*O*-*bis*(trimethylsilyl)acetamide, TMS-triflate, CH_3CN or 1,2-dichloroethane (**18a**, 90%; **18b**, 88%; **18c**, 68%; **18d**, 82%); (iv) aq. NaOH, THF or dioxane (**19a**, 97%; **19b**, 94%; **19c**, 78%; **19d**, 87%); (v) NaOBz, DMF (**20a**, 97%; **20b**, 86%; **20c**, 88%; **20d**, 93%); (vi) aq. NaOH, THF (**9a**, 95%; **9b**, 91%); NH_4OH, MeOH (**9c**, 86%); (a) 20% $Pd(OH)_2$-C, HCO_2NH_4, MeOH; (b) NH_4OH (**1d**, 77%); (vii) 20% $Pd(OH)_2$-C, 88% HCOOH, THF/MeOH (9:1) (**1a**, 91%); 20% $Pd(OH)_2$-C, HCO_2NH_4, MeOH or EtOH (**1b**, 83%; **1c**, 91%).



Scheme 4 Novozyme®-435 mediated monoacetylation of diol **10** [24].

Although, using the convergent strategy (Scheme 2), synthesis of LNA monomers with all natural nucleobases was standardized, regioselective benzylation of dihydroxy furanose derivative **10** remained unanswered [10]. Hence, in order to avoid the regioselective transformation on the furanose diol **10**, an alternate convergent synthesis was optimized by Koshkin *et al.* [20] (Scheme 3). Permesylation of furanose diol **10** afforded the dimesylated derivative **16**, which on acetolysis followed by acetylation, afforded an anomeric mixture of 1,2-di-*O*-acetyl-3-*O*-benzyl-4-*C*-methanesulfonyloxymethyl-5-*O*-methanesulfonyl-D-ribofuranose (**17**). The glycosyl donor **17** was used as a common intermediate for coupling reactions with different nucleobases to afford the LNA monomers **1a-d** [20,21] as shown in Scheme 3.

It seems easy to synthesize LNA monomers following the convergent strategy which utilizes the furanose diol **10** as the starting material. However, the use of **10** was found to be complicated due to the presence of two diastereotopic hydroxymethyl groups (Scheme 2 and Scheme 3). Therefore, we focused our attention towards lipase mediated diastereoselective protection of one of the hydroxymethyl groups in the crucial intermediate **10** with a base labile group such as acetyl, that can be hydrolyzed *insitu* concomitantly with 2′-*O*,4′-*C*-cyclization towards the end of the synthesis to get the LNA monomers [24]. Screening of different lipases in organic solvents for the diasteroeselective acetylation of one of the two hydroxyl groups in dihydroxy compound 3-*O*-benzyl-4-*C*-hydroxymethyl-1,2-*O*-isopropylidene-*α*-D-ribofuranose (**10**) revealed that *Candida antarctica* lipase-B (Novozyme®-435) in diisopropyl ether (DIPE) in the presence of vinyl acetate as acetyl donor carries out the selective 5′-*O*-monoacetylation (Scheme 4).

In a successful biocatalytic transformation reaction, a solution of the compound **10** and vinyl acetate in DIPE was incubated with Novozyme®-435 (10% w/w of **10**) at 45°C and 200 rpm in an incubator shaker. The progress of the reaction was monitored on analytical TLC. On completion, reaction was quenched by filtering off the enzyme and solvent was removed under reduced pressure. The crude product thus obtained was washed with hexane to afford monoacetylated compound 5-*O*-acetyl-3-*O*-benzyl-4-*C*-hydroxymethyl-1,2-*O*-isopropylidene-*α*-D-ribofuranose (**21**) in quantitative yield. Using the optimized conditions, Novozyme®-435 was utilised for ten recycles of selective acetylation of compound **10** and was found to be equally regioselective for each cycle (Figure 3).

The synthesis of LNA monomers **1a-d** was successfully achieved from enzyme-mediated monoacetylated compound **21**. Tosylation of **21** afforded compound **22** which on subsequent acetolysis gave an anomeric mixture **23** in 95% overall yield. Aiming for the convergent synthesis of LNA monomers, the mixture **23** was used as common glycosyl donor for the Vorbrüggen's coupling reaction with uracil, thymine, 6-*N*-benzoyladenine and cytosine to yield the corresponding 2′,5′-di-*O*-acetyl-3′-*O*-benzyl-4′-*C*-*p*-toluenesulfonyloxymethyl-ribonucleosides **24a-d** in 71-89% yields. Subsequently, deacetylation and concomitant intramolecular cyclization under alkaline conditions afforded the 3′-*O*-benzyl-2′-*O*,4′-*C*-methylene-ribonucleosides **9a-d** in 89-95% yields. Deprotection of the 3′-*O*-

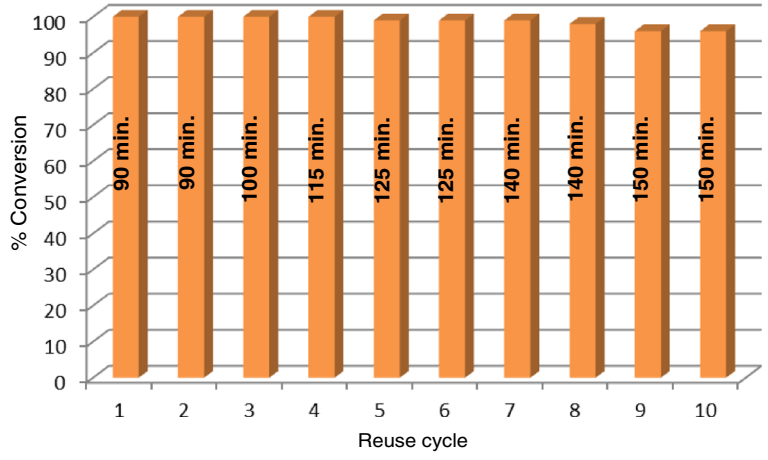
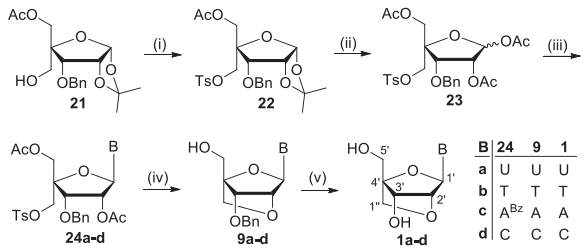


Figure 3 Study of recyclability of lipase Novozyme®-435 for acetylation reaction on diol 10. (All these reactions were performed on 100 mg batch size w.r.t. **10**).



Scheme 5 Chemo-enzymatic convergent synthesis of 2'-*O*,4'-*C*-methylene-ribonucleosides (LNA monomers) 1a-d [24]. *Reagents & conditions* (% *yields*): (i) TsCl, pyridine, CH_2Cl_2, 0°C to rt (98%); (ii) Ac_2O, AcOH, H_2SO_4 (100:10:0.1), 0°C to rt (97%); (iii) nucleobase, *N*,*O*-*bis*(trimethylsilyl) acetamide, TMS-triflate, acetonitrile or 1,2-dichloroethane, 80°C (24a, 89%; 24b, 88%; 24c, 71%; 24d, 78%); (iv) aq. NaOH, 1,4-dioxane, rt (+NH_4OH for 9c) (9a, 95%; 9b, 93%; 9c, 89%; 9d, 94%); (v) 20% $Pd(OH)_2$-C, HCOOH, THF:MeOH (9:1), reflux (1a, 91%); 20% $Pd(OH)_2$-C, HCO_2NH_4, MeOH or EtOH, reflux (1b, 83%; 1c, 91%; 1d, 81%).

benzyl group in nucleosides **9a-d** afforded the LNA monomers, *i.e.* 2′-*O*,4′-*C*-methyleneuridine (**1a**), 2′-*O*,4′-*C*-methylenethymidine (**1b**), 2′-*O*,4′-*C*-methyleneadenosine (**1c**) and 2′-*O*,4′-*C*-methylenecytidine (**1d**) in 81-91% yields (Scheme 5).

Starting from the diol **10**, the overall yields for developed chemo-enzymatic convergent synthesis of LNA monomers (Scheme 5) have been compared with the literature convergent methodology (Scheme 3). The results revealed that the developed biocatalytic methodology is more efficient in all cases with remarkable improvement for LNA-A (Table 2).

Conclusion

Unprecedented success of Locked Nucleic acid (LNA) in oligonucleotide based therapeutics demands a cost efficient, convenient and environment friendly synthetic route for LNA monomers. Therefore, Novozyme®-435 mediated selective protection of 3-*O*-benzyl-4-*C*-hydroxymethyl-1,2-*O*-isopropylidene-*α*-D-ribofuranose has been highlighted which lead to relatively efficient and environment friendly synthesis of LNA monomers in comparison to the earlier reports.

Table 2 Overall yields of LNA monomers for the reported classical chemical and chemo-enzymatic convergent synthesis

LNA monomer	Overall yield (%) (Scheme 3)*	Overall yield (%) (Scheme 5)*
U; **1a**	69.6	72.4
T; **1b**	51.1	63.9
A; **1c**	34.7	54.1
C; **1d**	48.6	55.9

*The overall yields have been calculated from dihydroxy compound **10**.

Abbreviations

LNA: Locked nucleic acid; ON: Oligonucleotide; HCV: Hepatitis C virus; DIPE: Diisopropyl ether.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

VKS, PR, VKM and AKP wrote the manuscript, with VKS being the main contributor. All the authors read and approved the final manuscript.

Authors' information

VKS, PR and VKM are research fellows under the supervision of AKP at the Department of Chemistry, University of Delhi, India.

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