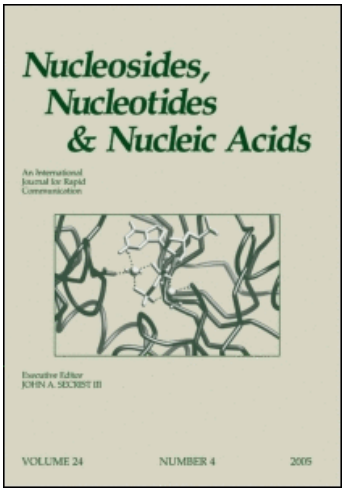


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# SYNTHESIS AND BIOLOGICAL EVALUATION OF LNA PHOSPHORAMIDATES

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*Santaris Pharma A/S, Hørsholm, Denmark*

□ *The synthesis of LNA phosphoramidates is presented. The LNA phosphoramidates were evaluated for their ability to inhibit cell proliferation of the human prostate cancer cell line 15PC3. A number of the LNA phosphoramidates showed cell proliferation inhibition determined by the MTS assay.*

**Keywords** LNA; phosphoramidates; proliferation



Nucleoside analogues are an important class of drugs that have found widespread use, in particular as anti-viral agents. Well-known examples include AZT, which revolutionized AIDS therapy, and the highly efficacios anti-cancer agent gemcitabine.[1]

The mode of action of the majority of nucleoside and nucleotide drugs is based on chain termination; i.e., they are analogues of naturally occurring deoxynucleotides required for the synthesis of viral DNA and hence compete with natural deoxynucleotides for incorporation into the growing viral DNA chain. However, unlike the natural deoxynucleotide substrates they often lack the 3′-hydroxyl group on the deoxyribose moiety. As a result, following their incorporation chain elongation is terminated. For nucleosides such as zidovudine (AZT) or didanosine (ddI) to be incorporated into viral DNA, they must be converted to the active 5′-triphosphates within the cell. Since therapeutically relevant nucleosides are synthetic analogues of the natural congeners they usually have poor affinity for kinases and monophosphates. Consequently they are often introduced chemically.[2] However, usually monophosphotylation is not adequate for a putative drug candidate since the phosphate is readily hydrolysed intracellularly. Moreover, the hydrophilicity of the monophosphate generally blocks spontaneous cellular

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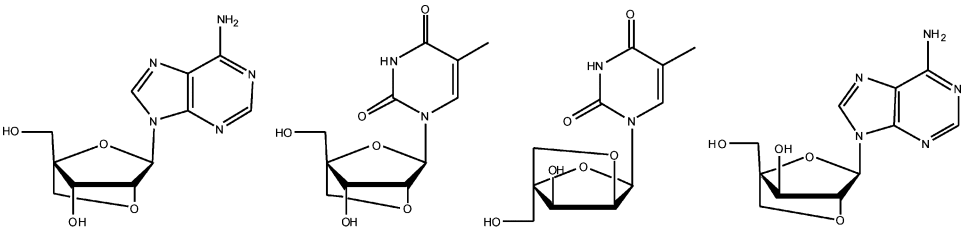


**FIGURE 1** The four LNA nucleosides: $\beta$-D-LNA-adenine (**1**); $\beta$-D-LNA-thymine (**2**); $\alpha$-L-LNA-thymine (**3**); $\beta$-D-xylo-LNA-adenine (**4**).

uptake of nucleotides. To solve this problem lipophilic monophosphate prodrugs such as simple phosphate esters (Adefovir dipivoxil), CycloSal,[3] SATE,[4] and phosphoramidates[5,6] have been prepared. Due to the many promising prospects of nucleotide prodrugs research within the field is continuously growing.

Recent years have seen the development of several LNA (locked nucleic acid) nucleosides but with a single exception[7] they have only been used as building blocks for oligonucleotides. LNA nucleosides as therapeutic agents in their own right remain an essentially unexplored area.

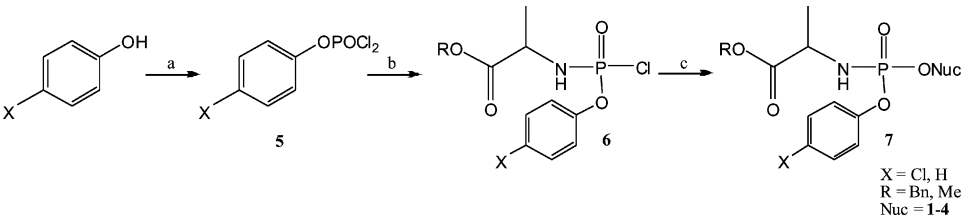
Herein we wish to report the synthesis of a series of lipophilic LNA prodrug analogues and their properties as inhibitors of cell proliferation.[8] The prodrug strategy was based on the phosphoramidate approach developed by McGuigan and co-workers.[9,10]



## RESULTS AND DISCUSSION

To explore the bicyclic chemical space present in LNA four phosphoramidates were investigated: two purine and two pyrimidine bicyclic nucleosides in both the $\alpha$-L and $\beta$-D configuration along with a $\beta$-D-xylo-adenosine derivative (Figure 1).[11,12]

The phosphoramidate moity was prepared according to the method by McGuigan and co-workers[6] and linked to the various bicyclic nucleosides as shown in Scheme 1.



**SCHEME 1** Reagents and conditions: (a) $POCl_3$, $Et_3N$, $Et_2O$, $-78°C$, 5–12 hours; (b) $Et_3N$, L-alanine methyl- or benzyl- ester hydrochloride, DCM, $-78°C$ to rt, 2–5 hours; (c) LNA nucleoside **1-4**, $^t$BuMgCl, Pyr/MeCN 2:1, 2 days (low to moderates yields).

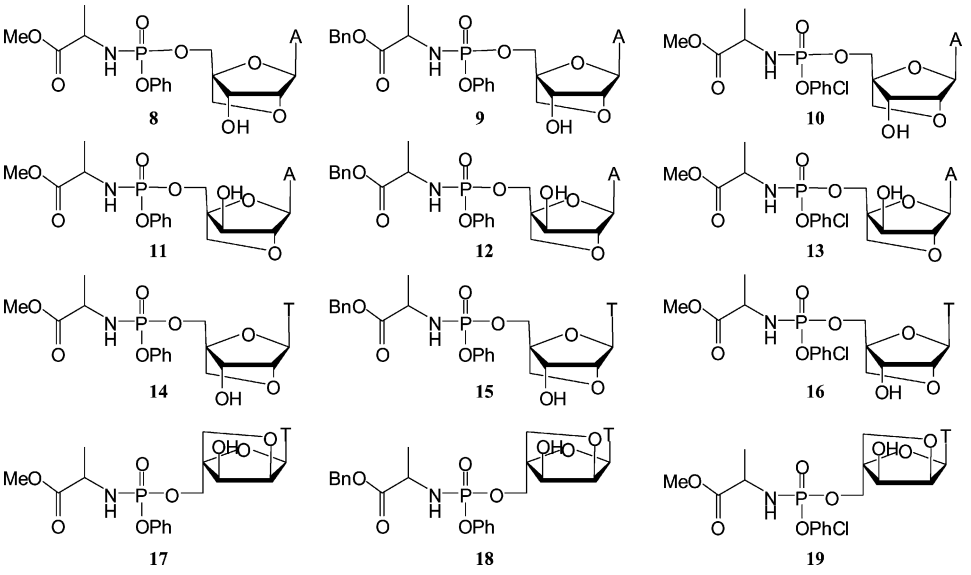



**FIGURE 2** LNA phosphoramidates: A, adenine; T, thymine; PhCl, *p*-chlorophenyl.



Synthesis of phosphochloridate species **6** was accomplished according to the procedures published by McGuigan and co-workers.[10] The aromatic moiety was treated with phosphorusoxychloride at −78°C to give phosphordichloridates **5** followed by treatment with L-alanine methyl or benzyl ester hydrochloride at −78°C to give phosphochloridates **6**.

The reactions were monitiored closely by LC-MS to prevent the formation of sideproducts. After concentration the residue was suspended in ether and filtered. The filtrate was concentrated in vacuo and stored as a 0.2 M solution in THF. The three selected phosphochloridates (X = H and R = Me; X = H and R = Bn; X = Cl and R = Me) have previously been reported to be among the most biologically active nucleoside derivatives within this class.[6]

The three different phosphochloridates were reacted under Grignard conditions[6] with four different LNA nucleosides (**1–4**, Figure 1) to give 12 LNA phosphoramidates as depicted in Figure 2.

The four LNA nucleosides **1–4**, LNA phosphoramidates **8–19**, and gemcitabine (as positive control) were tested for their ability to inhibit the proliferation of the human prostate cancer cell line 15PC3 as detected by the MTS assay. Only a few of these showed activity in the assay (Figure 3). The four parent LNA nucleosides **1–4** showed no inhibition in this assay. Compound **9** was the most potent compound and showed a clear dose–response relationship, with an $IC_{50}$ value below 100 $\mu$M after incubating for 72 hours. Gemcitabine (2′-deoxy-2′,2′-difluorocytidine hydrochloride) also showed a clear dose–response profile, with an $IC_{50}$ value below 0.1 $\mu$M after incubating for 72 hours. Compound **9** reached an

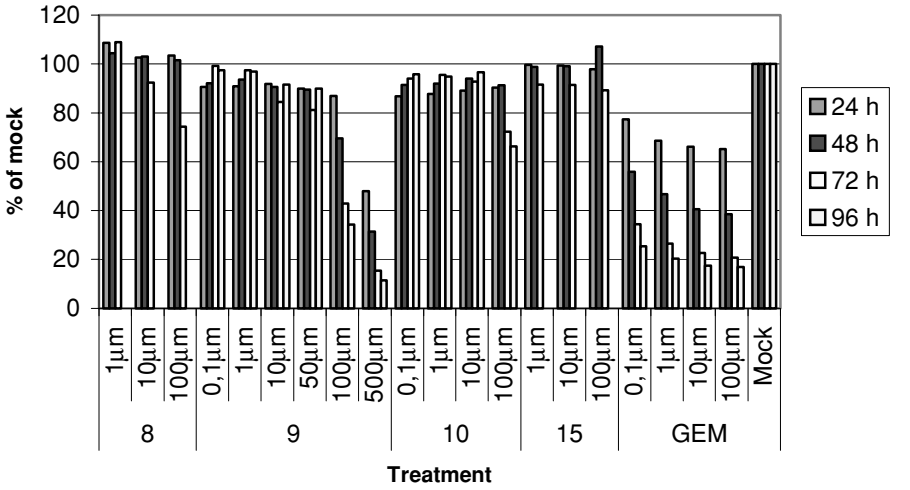



**FIGURE 3** MTS assay of selected LNA phosphoramidates and gemcitabine (GEM, positive control) normalized to Mock (blank).



inhibition level comparable to gemcitabine but at approximately 100 times the concentration. The different dose- and time-response profiles between **9** and gemcitabine could be a punitive indication that the mechanisms of action are different.

When the four active compounds (**8**, **9**, **10**, and **15**) were compared it was clear that the adenine-containing nucleoside was more potent than the thymine nucleoside (compound **9** versus **15**) Moreover, the benzylester alanine amino acid susbtituent gave more potent compounds than the corresponding methylesters (compound **8** versus **9**).

Interestingly, none of the isomers with unnatural stereochemistry ($\alpha$-L-LNA and xylo-LNA) showed any activity. The MTS assay did not give any mechanistic insight into the mode of action of the phosphoramidate derivatives.

In conclusion, we have developed a route for the synthesis of LNA phosphoramidates. Twelve phosphoramidates based on four LNA nucleosides and three phosphoramidite building blocks resulted in the identification of one compound with a significant effect on the growth of 15PC3 cancer cells. Based on the obtained data further work with LNA-based bicyclic nucleoside prodrugs is under way and will be reported in due course.

## EXPERIMENTAL

### General Procedure for the Synthesis of LNA Phosphoramidates

(1*R*,3*R*,4*R*,7*S*)Phenyl-[benzyloxy-L-alaninyl]-[(-3-(adenine-9-yl)-7-hydroxy-2,5-dioxabicyclo[2:2:1]heptane-1-yl)methyl] phosphate (**9**):(1*S*,3*R*,4*R*,7*S*)-3-(adenine-9-yl)-7-hydroxy-1-hydroxymethyl-2,5-dioxabicyclo[2:2:1]heptane

(125 mg, 0.45 mmol) was concentrated from anhydrous pyridine twice, dried under vacuum over $P_2O_5$ for 2 hours, and dissolved in a mixture of pyridine and acetonitrile (2:1; 5 mL). To the solution was added $^t$BuMgCl (1 M in THF, 0.5 mL, 0.5 mmol) and the solution was stirred at room temperate under argon for 15 minutes. Phenyl-[benzyloxy-L-alaninyl]phosphorchloridate (0.2 M in THF, 4.5 mL, 0.9 mmol) was added and the solution was stirred under argon for 24 hours. After this time $^t$BuMgCl (1 M in THF, 0.5 mL, 0.5 mmol) was added followed by the phenyl-[benzyloxy-L-alaninyl]phosphorchloridate (0.2 M in THF, 4.5 mL, 0.9 mmol). After an additional 24 hours the solution was concentrated in vacuo and the residue redissolved in $CH_2Cl_2$ (25 mL). The $CH_2Cl_2$ solution was washed with aqueous HCl (1 N, 25 mL), brine (2 × 25 mL), dried ($MgSO_4$), filtered, and concentrated in vacuo to give a yellow gum. Purification by dry column vacuum chromatography (DCVC)[13], eluted with MeOH in $CH_2Cl_2$ 0–10%, v/v gave phosphoramidate **9** (13 mg) as a colorless solid.

LC-MS (ES; M+H), found 597.2 (calculated 597.18)
$^{31}$P NMR (400 MHz, $CDCl_3$) $\delta_P$: 4.53 (double peak)
$^1$H NMR (400 MHz, DMSO-$d_6$) $\delta_H$: 8.21 (1H, s, H2); 8.15 (1H, s, H8); 7.39–7.11 (10H, m, 2 × Ph); 6.12 (1H, m, C<u>H</u>CH$_3$); 5.91 (1H, s, H1′); 5.06 (2H, d, $J$ 5.5 Hz); 4.48–4.32 (4H, m, H5′ and Ph-C<u>H</u>$_2$); 3.94 (1H, d, $^3J^{HH}$ 8, H2′/H3′); 3.75 (1H, d, $^3J^{HH}$ 8, H2′/H3′); 1.27 (3H, d, $^3J^{HH}$ 7, C<u>H</u>$_3$)



## MTS Assay

15PC3 cells were seeded to a density of 12,000 cells per well in white 96-well plate (Nunc 136101) in DMEM the day prior to transfection. The next day, cells were washed once in prewarmed OptiMEM followed by addition of 72 $\mu$L OptiMEM containing 5 $\mu$g/mL Lipofectamine2000 (In Vitrogen). Cells were incubated for 7 minutes before adding 18 $\mu$L LNA phosphoramidate diluted in OptiMEM. The final LNA phosphoramidate concentration ranged from 0.1 nM to 500 nM. After 4 hours of treatment, cells were washed in OptiMEM and 100 $\mu$L serum containing DMEM was added. Following treatment with the LNA phosphoramidate compound, cells were allowed to recover for the period indicated, viable cells were measured by adding 20 $\mu$L of the tetrazolium compound [3-(4,5-dimethyl-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS] and an electron coupling reagent (phenazine ethosulfate; PES) (CellTiter 96 $AQ_{ueous}$ One Solution Cell Proliferation Assay, Promega) per 100 $\mu$L DMEM. Viable cells were measured at 490 nm in a Powerwave (Biotek Instruments). Growth rates ($\Delta$OD/h) were plotted against the concentration of the LNA phosphoramidate.

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