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Synthesis and Properties of Oligonucleotides Carrying Cryptolepine Derivatives.

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A carboxyl derivative of the antimalarial cytotoxic drug cryptolepine was introduced into synthetic oligonucleotides by reaction of the carboxyl derivative of cryptolepine with oligonucleotides carrying an amino group either at the 3'-end or at the 5'-end. Oligonucleotides carrying the cryptolepine derivative binds their complementary sequences with greater affinity than unmodified ones. When cryptolepine is attached to a polypyrimidine oligonucleotide designed to form a parallel triplex, the triplex shows none or weak stabilization.

Introduction.- Cryptolepine (**1**, *Scheme 1*) is a naturally occurring indoloquinoline alkaloid used mainly as antimalarial drug. It has been shown to intercalate into DNA, preferably at cytosine-cytosine sites [1]. Compounds that intercalates into DNA increase the affinity of synthetic oligonucleotides for their complementary sequence when intercalating agents are attached to the 5' and 3'-ends of the synthetic oligonucleotides [2]. Acridine, anthraquinone, phenanthroline, ethidium and, ellipticine derivatives, among others, stabilize duplexes [2], while daunorubicin [3], benzopyrridoindole (BPI) and benzopyrridoquinoxaline (BPQ) [4, 5] increase the stability of triplexes. There is large interest in oligonucleotides carrying intercalating agents given their higher affinity for complementary sequences [2]. These oligonucleotides have been used in both antisense and antigene strategies for the inhibition of gene expression [6, 7]. Those carrying small DNA binding molecules have recently been reported as more efficient primers for the polymerase chain reaction (PCR) [8].

Here we described the synthesis of oligonucleotides carrying 10H-indolo[3,2-b]quinoline-11-carboxylic acid (**2**). Several sequences were designed

to study whether the presence of compound **2** at the 5'- and 3'-ends of the oligonucleotides stabilizes duplex and / or triplex DNA structures.

Results and discussion. - *1. Synthesis of oligonucleotides carrying compound* **2.** Small ligands can be incorporated into synthetic oligonucleotides at specific sites preparing oligonucleotides carrying aliphatic amino groups and performing a conjugation reaction with the carboxylic derivatives of the ligands [9, 10]. This strategy was used to incorporate the carboxyl derivative of cryptolepine (**2**) into oligonucleotides (*Schemes 2* and *3*).

Oligonucleotide sequences **3-7** (*Table 1*) carrying an amino group either at the 5'- or 3'-end were prepared using the phosphoramidite of the N^6-monomethoxytrityl (MeOTr) protected derivative of 6-aminohexanol [10] and a controlled pore glass support carrying the 2-(N-9-fluorenylmethoxycarbonyl (Fmoc)-4-aminobutyl)-1,3-propanediol linker [9] (*Scheme 3*). We used the benzoyl (Bz) group for the protection of the amino group of C and A and the isobutyryl (ibu) or the dimethylaminomethylidene (dmf) group for the protection of G.

1.1 Synthesis of oligonucleotides carrying cryptolepine at the 3'-end. The reaction with carboxyl derivative **2** with oligonucleotide sequences **3** and **7** was examined following two protocols.

a) Solid-phase coupling protocol (*Scheme 3*). The Fmoc group that protects the amino group is removed selectively with a non-nucleophillic base (DBU, 1,8-diazabicyclo[5.4.0] undece-7-ene). The carboxyl derivative **2** is reacted with the support carrying the amino-oligonucleotide using (benzotriazol-1-yloxy) trispyrrolidinophosphonium hexafluorophosphate (PyBOP) as carboxyl activator.

The excess of chemicals was washed and the solid support carrying the oligonucleotide was treated with concentrated ammonia (1 h at 50ºC for sequence **3** using the dmf protecting group for G; 2 h at room temperature for sequence **7**). The solution was analyzed by HPLC. The desired oligonucleotide carrying the cryptolepine eluted at around 14 min. Unreacted amino-oligonucleotides eluted at 9 min. The desired product was characterized by UV-spectra and mass spectrometry (*Table 1*).

b) Solution-phase coupling protocol. After the synthesis, the support carrying the amino-oligonucleotide is deprotected using concentrated ammonia. In these conditions all protecting groups including the Fmoc group are removed yielding unprotected 3'-amino oligonucleotide. This compound is treated with the carboxyl derivative **2** activated with diisopropylcarbodiimide and N-hydroxysuccinimide. The reaction is performed at room temperature overnight in aqueous sodium bicarbonate 1 M pH 9.0 /dimethylformamide (1:1). HPLC analysis revealed the presence of the desired oligonucleotide in higher yields than the solid-phase protocol (*Table 1*). Oligonucleotide sequence **5** was prepared following the solution-phase coupling protocol (*Figure 1*).

1.2 Synthesis of oligonucleotides carrying cryptolepine at the 5'-end. Oligonucleotide sequences carrying cryptolepine at the 5'-end (**4** and **6**) were prepared using the solution-phase coupling protocol. As described above the support carrying the amino-oligonucleotide was deprotected using concentrated ammonia. The resulting 5'-amino-oligonucleotide was treated with 10 molar excess of the carboxyl derivative **2** activated with diisopropylcarbodiimide and N-hydroxysuccinimide. The reaction was performed at room temperature overnight

in aqueous sodium bicarbonate 1M pH 9.0 /dimethylformamide (1:1) followed by the work-up described above. HPLC analysis revealed the presence of the desired oligonucleotide (*Table 1*).

2. Hybridization properties of oligonucleotides carrying cryptolepine. The hybridization properties of oligonucleotides carrying cryptolepine **2** were measured spectrophotometrically on the duplex formed by the self-complementary sequences **3-6** and on the duplex formed by sequence **7** and its complementary sequence (*Table 2*). Unmodified duplexes were included for comparison purposes. The duplex formed by sequence **3** carrying two molecules of compound **2** melted at 52 ºC, while the unmodified duplex melted at 35 ºC. This involves an increase of 8.5 ºC per intercalating molecule. The duplex formed by sequences **4-6** carrying two molecules of compound **2** melted at 46-47 ºC (increase in Tm of 5.5-6 ºC per substitution). Elevenmer sequence **7** carrying compound **2** melted at 59 ºC, while the unmodified sequence melted at 52 ºC (increase in Tm= 7 ºC).

The increase of 5.5-8.5 ºC per substitution points the strong stabilization of the duplex induced by the presence of the cryptolepine derivative **2** (for comparison similar acridine derivatives lead to increases of 3-6 ºC per substitution, [2]). In the sequence TTCCGGAA (**3** and **4**), there are differences when the crytolepine derivative is at the 3' or at the 5'-end whereas in the sequence CCAATTGG (**5** and **6**) these differences are very slight. Competition dialysis experiments on free cryptolepine indicated a preference for CC and GG sites [1]. This preference was not observed in the oligonucleotide carrying

compound **2**. On the contrary, the highest melting temperature was detected on sequence **3**, bearing two adenines near the anchoring sites.

The dependence of the duplex to random coil transition on DNA concentration was studied on oligonucleotide sequence **5**. Melting temperatures decreased with the concentration as expected for a bimolecular reaction. The plot of $1/T_m$ versus ln concentration was linear, giving a slope and a *y*-intercept from which ΔH, ΔS and ΔG were obtained (*Table 3*). The ΔG for the duplex dissociation was –20.2 Kcal/mol for the unmodified duplex, -20.8 Kcal/mol for the duplex carrying an amino group at the 3-'end and -24.6 Kcal/mol for the duplex carrying two molecules of compound **2**. The ΔG of these values differed in 4.4 Kcal/mol (2.2 Kcal/mol per substitution).

The effect of compound **2** on triplex stability was studied with sequence **7** (*Table 4*). This oligonucleotide can form a parallel triplex with the appropriate target sequences (*Table* 4). When the target oligonucleotide was a hairpin duplex with 26 bases (h26), no differences in Tm were found, when the target sequence was a larger hairpin duplex (h34), Tm increased by 3 ºC, indicating a slight stabilization due to the presence of compound **2** (*Table 4*). We can not rule out that in this triplex, the cryptolepine moiety of oligonucleotide **7** is anchored into the remaining duplex.

Conclusion. We have described the preparation of oligonucleotides carrying the cryptolepine derivative **2** either at the 3' or at the 5'-end. The addition of compound **2** to synthetic oligonucleotides increases the stability of the duplex.

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Experimental Part

General. The phosphoramidites and ancillary reagents used during oligonucleotide synthesis were from *Applied Biosystems* (*PE Biosystems Hispania S.A.,* Spain), *Cruachem* (*Cruachem Ltd.,* Scotland) and *Glen Research* (*Glen Research Inc*., USA). The rest of the chemicals were purchased from *Aldrich, Sigma* or *Fluka* (*Sigma-Aldrich Química S.A.,* Spain). Solvents were from *S.D.S*. (*S.D.S.*, France). NAP-10 columns (Sephadex G-25) were purchased from Pharmacia Biotech. *Instrumentation.* Oligonucleotide sequences were synthesized on an *Applied Biosystems* DNA synthesizer model 392 (*Applied Biosystems,* USA). Mass spectra (matrix-assisted laser desorption ionization time-of-flight, MALDI-TOF) were provided by the Mass spectrometry service at the University of Barcelona. UV-Visible spectra were recorded on a *Shimadzu* UV-2101PC spectrophotometer.

*Synthesis of oligonucleotides carrying amino groups***.** Oligonucleotide sequences carrying an amino group either at the 5'-end or at the 3'-end were assembled on a 1 μmol scale. The phosphoramidite derivative of 6-aminohexanol carrying the monomethoxytrityl group (*Glen Research* and *Cruachem* [10]) was used for the introduction of the amino group at the 5'-end. The 3'amino C7 modifier

controlled pore glass (CPG) [9] (*Glen Research*) was used for the preparation of oligonucleotides carrying amino groups at the 3'-end. The benzoyl (Bz) group was used for the protection of the amino group of C and A and the dimethylaminomethylidene (dmf) or the isobutyryl (ibu) group for the protection of G. Coupling yields were >95%. The last $(MeO)_2Tr$ protecting group was removed.

Synthesis of oligonucleotides carrying cryptolepine derivative **2** *using the solid-phase coupling protocol*. Oligonucleotide sequences **3** and **7** carrying an amino group at the 3' end were prepared as described above using the dmf group for the protection of G. The resulting solid supports were treated with a 0.1M soln. of 1,8-diazabiciclo[5.4.0] undece-7-ene (DBU) in dry acetonitrile (2 min, room temperature). Thus, the Fmoc group that protects the amino group was removed selectively with a non-nucleophilic base. The resulting supports were washed with acetonitrile and reacted with 10H-indolo[3,2-b]quinoline-11-carboxylic acid (**2**) as follows. A mixture containing 10 molar excess of 10H-indolo[3,2-b]quinoline-11-carboxylic acid, 20 molar excess of diisopropylethylamine (DIEA) and 10 molar excess of (benzotriazol-1-yloxy) trispyrrolidinophosphonium hexafluorophosphate (PyBOP) was prepared in dry dimethylformamide (DMF) (0.2 ml). The mixture was left for 2 min at room temperature and added to the support. After 1 h at room temperature, the mixture was filtered and washed with DMF and acetonitrile (ACN). The support was dried and the concentrated ammonia (1 ml) was added. The ammonia solution was left for 1 h at 50 ºC (or for 2 h at room temperature in sequence **7**). The mixture was filtered and the ammonia solution was concentrated to dryness. The residue was dissolved in

water and desalted by a NAP-10 (Sephadex G-25) column eluted with water. Finally, the oligonucleotide fractions were analyzed by HPLC. HPLC solutions were solvent A: 5% ACN in 100mM triethylammonium acetate pH 6.5 and solvent B: 70% ACN in 100mM triethylammonium acetate pH 6.5. Column: PRP-1 (*Hamilton*), 250 x 10 mm. Flow rate: 3 ml/min. A 20 min lineal gradient from 5-35% ACN. The desired oligonucleotide carrying the 5-methyl-5H-quidoline at 14 min The unreacted amino-oligonucleotide eluted at around 9 min. The desired product was characterized by UV and mass spectra (*Table 1*).

Synthesis of oligonucleotides carrying cryptolepine derivative **2** *using the solution-phase coupling protocol*. Oligonucleotide sequences **3**-**7** carrying an amino group either at the 3'- or at the 5'-end were prepared as described above. The solid support obtained after the assembly of the sequence was treated with concentrated ammonia for 1 h at 50ºC (when using the dmf group for the protection of G) or for 16 h at 50ºC (when using the ibu group for the protection of G). The mixture was filtered and the ammonia solution was concentrated to dryness. The residue was passed over a Dowex 50x4 (Na+ form) column to exchange ammonium ions for sodium ions. The resulting amino-oligonucleotide was dissolved in 0.1 ml of water, and mixed with 0.1 ml of 1M sodium carbonate aqueous buffer pH 9. In a separate container 10H-indolo[3,2-b]quinoline-11-carboxylic acid (10 molar excess) was dissolved in 0.1 ml of DMF and mixed with N-hydroxysuccinimide (10 molar excess) and N,N-diisopropylcarbodiimide (10 molar excess). The mixture was left for 10 min at room temperature and added to the aqueous solution of the amino-oligonucleotide. The reaction mixture was kept at room temperature overnight. The mixture was concentrated to dryness

and the residue was dissolved in water. The solution was passed through a NAP-10 column. The fractions containing oligonucleotide were analyzed by HPLC as described above. The desired product was characterized by UV-spectra and mass spectrometry (MALDI-TOF) (*Table 1*)

*Melting experiments***.** Melting experiments were performed as follows. Solutions of equimolar amounts of the appropriate oligonucleotides were mixed either in a soln. containing 1M NaCl, 10mM sodium phosphate buffer of pH 7.0 (in experiments on duplex formation) or in a soln. containing 1M NaCl and 0.1M sodium phosphate / citric acid buffer of pH 6.0 (in experiments related on triplex formation). DNA concentration was determined by UV absorbance measurements (260 nm) at 90 ºC, using for the DNA coil state the following extinction coefficients: 7500, 8500, 12500 and 15000 M^{-1} cm^{-1} for C, T, G and A, respectively. The solutions were heated to 90 ºC, allowed to cool slowly to room temperature and stored at 4ºC until UV was measured. UV absorption spectra and melting experiments (absorbance vs temperature) were recorded in 1 cm path-length cells using a spectrophotometer, with a temperature controller and a programmed temperature increase rate of 0.5 ºC/min. Melting curves were recorded at 260 nm and melting temperatures were measured at the maximum of the first derivatives of the melting curves. Results: see *Tables 2-4*.

The samples used on the thermodynamic studies were prepared in a similar way but melting experiments were recorded at 260 nm using 0.1, 0.5 and 1 cm path-length cells. Melting curves were obtained at concentrations ranging from 1 to 35 μM of duplex. Melting temperatures (Tm) were measured at the maximum of the first derivative of the melting curve. The plot of 1/Tm versus lnC was

linear. Linear regression of the data gave the slope and the y-intercept, from which ΔH and ΔS were obtained. The free energy was obtained from the standard equation: $\Delta G = \Delta H - T\Delta S$.

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Table 1. Synthesis and characterization of oligonucleotides carrying the cryptolepine derivative **2**.

Sequence (5'-3')	Protocol	yield	UV(max)	EM(Found)	Expected
3: TTCCGGAA-**2**	Solid phase	7%	258, 348	2861 ($M-H^+$)	2862
3: TTCCGGAA-**2**	Solution	18%	258, 348	2861 ($M-H^+$)	2862
4: **2**-TTCCGGAA	Solution	12%	260, 349	2831 ($M-H^+$)	2832
5: CCAATTGG-**2**	Solution	15%	260, 349	2831 ($M-H^+$)	2832
6: **2**-CCAATTGG	Solution	22%	270, 349	2861 ($M-H^+$)	2862
7: CTTCCTCCTCT-**2**	Solid phase	5%	273, 349	3646 ($M-H^+$)	3647
7: CTTCCTCCTCT-**2**	Solution	20%	273, 349	3646 ($M-H^+$)	3647

Table 2. Melting temperatures of duplexes carrying compound **2**

Sequence (5'-3')	Tm (ºC)*	ΔTm (ºC)
TTCCGGAA	35	--
3, TTCCGGAA-**2**	52	17
4, **2**-TTCCGGAA	47	12
CCAATTGG	35	--
5, CCAATTGG-**2**	46	11
6, **2**-CCAATTGG	47	12
CTTCCTCCTCT[#]	52	--
7, CTTCCTCCTCT-**2**[#]	59	7

*1M NaCl, 0.1 M sodium phosphate pH 7.0 at 4 μM concentration.

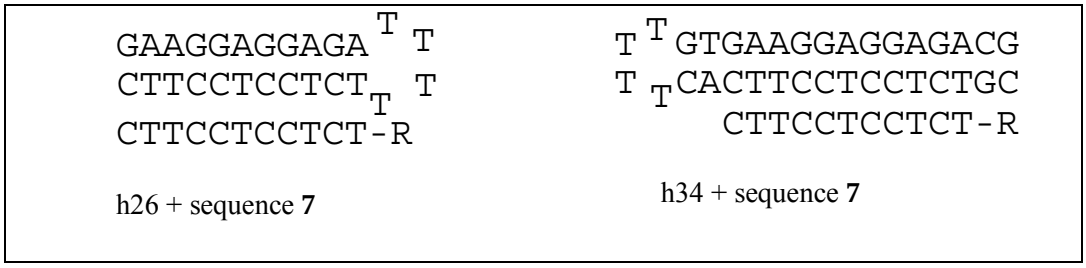
[#]complementary sequence 5'-AGAGGAGGAAG-3'

Table 3. Thermodynamic parameters for duplex to random coil transitions in 1M NaCl, 0.1 M sodium phosphate pH 7.0 from the slope of the plot $1/T_m$ versus $\ln C$[a].

duplex	Tm (ºC)[b)]	ΔH (kcal/mol)	ΔS (cal/mol K)	ΔG (kcal/ mol)
5'-CCAATTGG-3'	34.7	-107	-293	-20.2
5'-CCAATTGG-NH_2-3'	35.8	-111	-303	-20.8
5, 5'-CCAATTGG-**2**-3'	45.8	-121	-323	-24.6

[a)] ΔH, ΔS and ΔG are given as round number, ΔG is calculated at 25ºC, with the assumption that ΔH and ΔS do not depend on temperature; analysis was carried out using melting temperatures obtained from denaturation curves; [b)] at 4 μM duplex concentration.

Table 4. Melting temperatures of triplexes carrying compound **2**.



Sequence	R=	Tm(triplex)*	Tm(duplex)*	ΔTm(triplex)
h26+ unmodified	H	26 ºC	77 ºC	--
h26+ sequence **7**	**2**	26 ºC	77 ºC	0
h34+ unmodified	H	26 ºC	>80 ºC	--
h34+ sequence **7**	**2**	29 ºC	>80 ºC	3 ºC

*1M NaCl, 0.1 M sodium phosphate-citric acid pH 6.0 at 4 μM concentration.

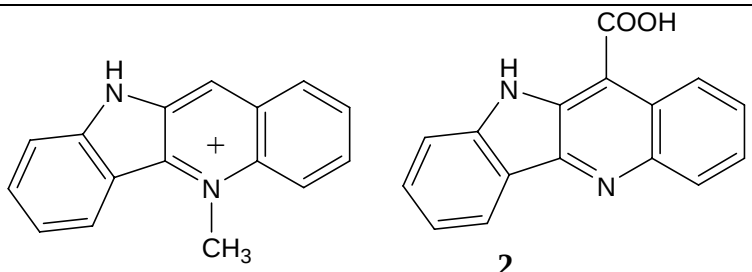
LEGENDS

Scheme 1: Cryptolepine (**1**) and the carboxyl derivative **2** used for the derivatization of oligonucleotides.

Scheme 2: Synthesis of oligonucleotides carrying cryptolepine at the 5'-end by recation of a 5'-amino-oligonucleotide with the carboxyl derivative of cryptolepine **2**.

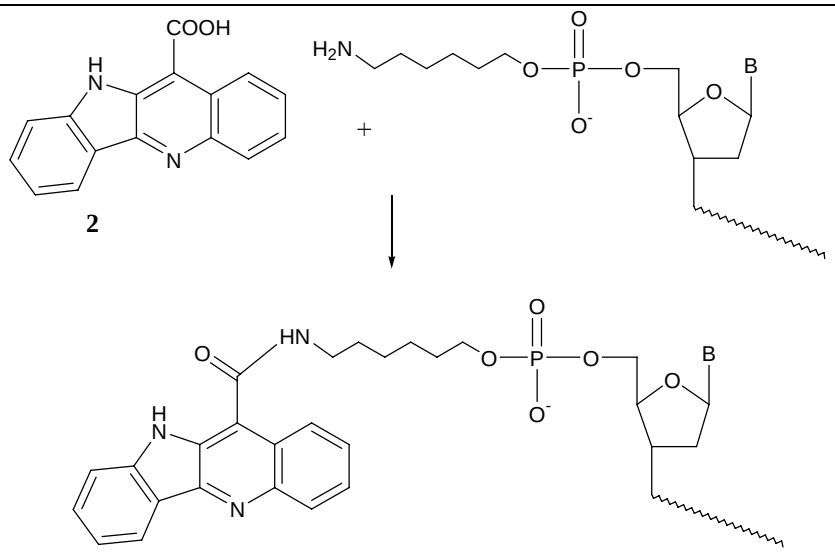
Scheme 3: Synthesis of oligonucleotide carrying cryptolepine derivative **2** at the 3'-end.

Figure 1: HPLC profiles of oligonucleotide sequence **5** carrying cryptolepine at the 3'-end. A) Oligonucleotide carrying an amino group at the 3'-end before conjugation. B) Mixture obtained after the reaction with compound **2**. The desired oligonucleotide carrying cryptolepine eluted at 14 min.

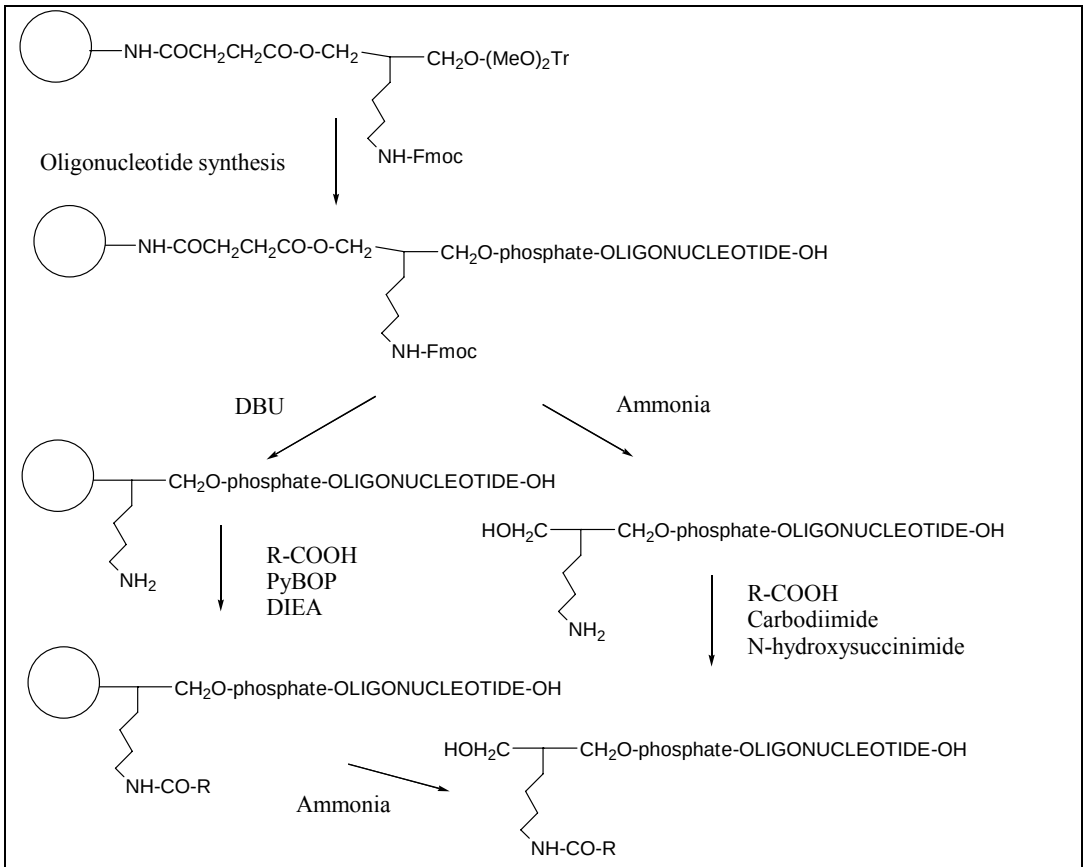


1 **2**

Scheme 1



Scheme 2


NH-COCH2CH2CO-O-CH2
CH2O-(MeO)2Tr
NH-Fmoc
Oligonucleotide synthesis
NH-COCH2CH2CO-O-CH2
CH2O-phosphate-OLIGONUCLEOTIDE-OH
NH-Fmoc
DBU
Ammonia
CH2O-phosphate-OLIGONUCLEOTIDE-OH
NH2
R-COOH
PyBOP
DIEA
HOH2C
CH2O-phosphate-OLIGONUCLEOTIDE-OH
NH2
R-COOH
Carbodiimide
N-hydroxysuccinimide
CH2O-phosphate-OLIGONUCLEOTIDE-OH
NH-CO-R
Ammonia
HOH2C
CH2O-phosphate-OLIGONUCLEOTIDE-OH
NH-CO-R

Scheme 3

Graphical illustration for the table of contents