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Synthesis and Triple-Helix Stabilization Properties of Branched Oligonucleotides Carrying 8-Aminoadenine.

by **Anna Aviñó[a]), Marta G. Grimau[a]), Miriam Frieden[a,b]),** and **Ramon Eritja[*a]).**

[a]) Department of Structural Biology. Instituto de Biología Molecular de Barcelona. C.S.I.C., Jordi Girona 18-26, E-08034 Barcelona. Spain. (phone: +34(93)4006145; fax: +34(93)2045904; e-mail : recgma@cid.csic.es)

[b]) Present address: Cureon A/S. Fruebjergvej 3, DK-2100 Copenhagen. Denmark.

The synthesis of several branched oligonucleotides (parallel hairpins **11-14**, and Y-shape **15** oligonucleotides) is described together with their use in the formation of pyrimidine-pyrimidine-purine triple helices. Special attention was paid to the optimization of the assembly of the second strand using asymmetric branching molecules. The presence of 8-aminoadenine in the Watson-Crick purine strand and 2'-O-methyl-RNA in the Hoogsteen pyrimidine strand produced strong stabilization of the triplex.

Introduction.- Oligonucleotides bind in a sequence-specific manner to homopurine-homopyrimidine sequences of duplex and single-stranded DNA and RNA to form triplexes [1]. Nucleic acid triplexes have potential applications in diagnostics, gene analysis and therapy [1, 2]. Recently, it has been reported that oligonucleotides carrying 8-aminopurine nucleosides formed stable pyrimidine-pyrimidine-purine triplexes [3-6]. These triplex stabilization properties of 8-aminopurines can be used to capture specific polypyrimidine sequences using parallel stranded hairpins with a polypyrimidine sequence linked to the complementary purine strand carrying 8-aminopurines [7, 8]. The binding properties of parallel hairpins carrying 8-aminopurines stimulated the search for new types of hairpins with even better binding properties.

One interesting possibility is to use branched oligonucleotides [9 and references cited herein]. Most of the work dealing with branched oligonucleotides was first focused on the study of these compounds as splicing intermediates of eukaryotic mRNAs [10-12]. Moreover, branched nucleic acids have been prepared using nucleoside branching points other than the 2' and 3' positions of a ribonucleoside

such as 4'-C-(hydroxymethyl)thymidine [13, 14], *arabino*-uridine [15] or through the nucleobase [16, 17]. The complexity of the synthesis of the branching molecules and the low yields obtained triggered the search for non-nucleoside branching molecules such as derivatives of 1,2,6-hexanetriol [18], 1,3-diaminopropanol [19] and pentaerythriol [20, 21]. These branching molecules can produce dendrimeric oligonucleotides which may be used as polylabelled DNA probes to increase sensitivity in hybridization experiments [17-20]. Moreover, oligonucleotides with three arms were found to be efficient templates for the directed synthesis of trimeric structures [22]. Finally, branched oligonucleotides have been used to produce V-shape oligonucleotides with high affinity to single-stranded DNA or RNA by triplex formation [9, 14, 15, 17, 21, 23, 24] and to form alternate-strand triplexes [25, 26].

Here we report the synthesis of parallel stranded hairpins using a non-nucleoside branching molecule. We also describe their use in the formation of pyrimidine-pyrimidine-purine triple helices. The previous method of synthesis [27, 28] requires the use of reverse phosphoramidites to build the half of the molecule (scheme 1). In the present paper the hairpins are synthesized from the middle of the molecule by the extension of one branch of an asymmetric branching molecule, followed by assembly of the next branch. The combination of an acid labile group (dimetoxytrityl, $(MeO)_2Tr$) and a base labile group (9-fluorenylmethyloxycarbonyl, Fmoc) allows the assembly of the desired molecule without the need of reverse phosphoramidites. Finally, the three strands of a triplex were connected together with a three-branched Y-shape oligonucleotide using the asymmetric branching molecule. The binding properties of these molecules are discussed.

Results. - *1. Structure of the oligonucleotide derivatives* Oligonucleotides sequences of hairpins are shown in Table 1. The sequences of the hairpins are based on a triplex model sequence described previously [29, 30]. Here, the Hoogsteen polypyrimidine strand was linked to the Watson-Crick polypurine strand.

The first group of sequences (**7**-**10**) are parallel stranded and the polypurine strand is connected with the Hoogsteen polypyrimidine strand either by their 3' ends (**7, 8**) or by their 5' ends (**9, 10**) through a hexaethyleneglycol linker [$(EG)_6$]. In hairpins **8** and **9**, two adenines were replaced by two 8-aminoadenines. These hairpins were prepared by the standard method using a combination of standard and reversed phosphoramidites (scheme 1, [27, 28]). The binding properties of these hairpins have been described previously [7, 8] and they are used for comparison purposes.

The second group of sequences (**11**, **12**) are 3'-3' linked parallel hairpins like **7**. The hexaethyleneglycol loop of **7** is replaced by 4-hydroxybutyric (**11**) and 5-hydroxypentanoic (**12**) derivatives of 1,3-diaminopropanol and four thymidines (Table 1). These hairpins were prepared from the middle of the loop using an asymmetric branching molecule as outlined in part B of scheme 1. A solid support carrying the asymmetric branching molecule (scheme 2) was used for the preparation of **11**. In contrast, a phosphoramidite of the asymmetric branching molecule was used for the synthesis of **12**. This phosphoramidite was coupled to a solid support carrying a 1,3-propanediol molecule [31]. In this way the difference between **11** and **12** is a 3-hydroxypropyl phosphate in the loop. The synthesis of

11 and **12** were used to find the optimal conditions for the assembly of the sequences using asymmetric doublers. In this method, the combination of an acid labile group ($(MeO)_2Tr$) and a base labile group (Fmoc) allows the assembly of the hairpin from the middle of the loop. After the assembly of the first strand, the Fmoc group is removed and, the second strand is then synthesized.

The third group of sequences (**13** and **14**) are derivatives of **12** carrying two different types of substitutions. First, in both hairpins (**13** and **14**) two adenines were replaced by two 8-aminoadenines. In addition the whole Hoogsteen polypyrimidine strand of hairpin **13** was replaced by 2'-O-methyl-RNA backbone, and the six C in the hairpin **14** were replaced by 5-methyl-C. The introduction of RNA and 2'-O-methyl-RNA [32-36] as well as 5-methyl-C [29, 37, 38] in the Hoogsteen polypyrimidine strand have been reported to stabilize triplexes. We were interested in analyzing whether these stabilizing properties are additive to the triplex stabilizing properties of 8-aminoadenine found in parallel hairpins.

These hairpins were synthesized using the optimal conditions found in the synthesis of **12**. These optimal conditions were also used for the synthesis of a three-branched oligonucleotide having the three strands of a triplex (**15**, Table 1).

2. Synthesis of parallel hairpins. The asymmetric branching phosphoramidites was obtained from commercial sources. The synthetic scheme for the preparation of the solid support carrying asymmetric branching molecule is shown in scheme 1. 1, 3-Diamino-2-propanol was reacted with γ-butyrolactone to yield the desired triol **1** [19]. Next we tried the introduction of the $(MeO)_2Tr$ group in one position by limiting the amount of dimethoxytrityl chloride. The mono-$(MeO)_2Tr$-product was formed but the desired product was isolated from the di-$(MeO)_2Tr$- product in

very low yields. Reaction of the mono-$(MeO)_2$Tr-derivative (with and without isolation of the di-$(MeO)_2$Tr-product) with 9-fluorenylmethoxycarbonyl chloride gave the desired product but again the recovery from the silica gel purification was low. The introduction of the Fmoc- group first followed by the addition of the $(MeO)_2$Tr group also yielded little product after silica gel purification, although the Fmoc group was more stable in the silica gel chromatography than the $(MeO)_2$Tr group. Finally, we selected the more stable monomethoxytrityl (MeOTr) group for the protection of the alcohol function and the desired monofunctionalized derivative **2** was isolated in good yields. The introduction of the Fmoc group also proceeded in moderate but better yields than before. Compound **3** was further derivatized to obtain the solid support **5** (Scheme 2) following standard protocols.

Sequence **11** was used as model compound to study the synthesis of oligonucleotides having two different branches (Scheme 3). The first branch was assembled on 1 μmol-scale using standard synthesis cycle. The resulting support was acetylated and the Fmoc group was removed by treatment with 0.5 M 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) solution in acetonitrile (ACN) (10 min). Aliquots of 0.2 μmols of support were subjected to 6 different synthesis cycles as described in methods. The changes introduced were the increase of reaction times especially coupling time, double coupling protocols, introduction of an extra wash using a dimethylaminopyridine (DMAP) - tetrazole (TET) solution and non-aqueous oxidation.

In some experiments we introduced a wash in DMAP-TET between detritylation and coupling. During the removal of the Fmoc group a strong base was used (DBU) and, in these conditions, the 2-cyanoethyl (CNE) group which protects

phosphate groups was also removed. The presence of unprotected phosphates during the assembly of oligonucleotides may explain the low yields obtained during the assembly of the second branch. In a similar situation the use of DMAP-TET was recommended [39].

The progress of the assembly of the second strand was monitored by the absorption of the $(MeO)_2Tr$ group released after each coupling. The first and second coupling after branching were lower than the rest. Standard cycle (A) gave 60% overall yield. Increasing the coupling time (cycle B) and double coupling (coupling C) were the best conditions giving a 90% overall yield. The use of non-aqueous oxidation (cycle F) give no significant differences. The use of DMAP-TET gave lower yields than the standard cycle (30% overall yield).

The results obtained by the measurement of the trityl released during the acid treatments were confirmed by gel electrophoresis (Figure 1). Moreover, the truncated sequences were mainly observed near the branching point. The resulting oligonucleotide (**11**) was purified using the trityl-on and trityl-off protocols and the purified compound was characterized by mass spectrometry.

In addition we built the first strand using phosphoramidites carrying methoxy groups instead of the CNE group. Previous reports on the synthesis of branched oligonucleotides describe the use of methyl phosphoramidites [10-13]. In our model compound the use of methyl phosphoramidites was not satisfactory. The coupling efficiency of the second strand was slightly worse than using CNE-phosphoramidites and the crude product was clearly more impurified of shorter sequences near the branching point (data not shown).

Next, the assembly of sequence **12** was performed. In this sequence, the phosphoramidite of the branching molecule is added to a solid support carrying a

1,3-propandiol molecule [31]. We assayed various changes in the cycle (described in methods). As observed before, yields of the first and second coupling reactions were low, but in this case even lower than before. Using the standard cycle the overall yield was only 20%. The use of *tert*-butylhydroperoxide instead of iodine was slightly better. Increasing the coupling time increased the overall yield to 60%. Increasing the time and the concentration of the phosphoramidite or using a double coupling protocol increased the overall yield to 80-90%. The presence of the phosphate near the branching point reduced the coupling yields of the second branch, but this effect was overcome by increasing time and the concentration of the phosphoramidite.

Next, sequences **13** and **14** carrying 8-aminoadenines were prepared. First the polypurine sequence was assembled on a solid support carrying the asymmetric branching molecule coupled to 1,3-propanediol. Upon completion of the polypurine sequence, the support was removed from the synthesizer and acetylation followed by the removal of the Fmoc group was performed in a syringe. Finally, the polypyrimidine strand was assembled using a synthesis cycle with the double coupling protocol. The assembly of the second strand proceeded with an overall yield of 80%. The resulting oligonucleotides were purified using the trityl-on and trityl-off protocols and the homogeneity was checked by gel electrophoresis.

Finally, the three-branched oligonucleotide (**15**) was prepared on polystyrene supports (LV200, *Applied Biosystems*). First the Watson-Crick pyrimidine strand was assembled, followed by the addition of hexaethyleneglycol and the asymmetric branching molecule. The synthesis was continued by the addition of hexaethyleneglycol and the assembly of the Watson-Crick purine strand. At this

point, the $(MeO)_2$Tr-support was detritylated and acetylated followed by the removal of the Fmoc group as described above. A third hexaethyleneglycol molecule was then added followed by the assembly of the Hoogsteen pyrimidine strand. (Scheme 4). Standard phosphoramidites were used. The three strands were assembled using the synthesis cycle recommended for low-volume columns. A longer coupling time (480 s instead of 40 s) was used only during the assembly of the third strand. The assembly of the third strand proceeded with an overall yield of 80%. The resulting oligonucleotide (**15**) was purified using the trityl-on and trityl-off protocols and the homogeneity was checked by gel electrophoresis.

3. *Thermal denaturation experiments.* The relative stability of triple helices formed by parallel hairpins and the polypyrimidine target sequences was measured spectrophotometrically. In all cases, one single transition was observed, which was assigned to the transition from triple helix to random coil [8]. Table 2 shows the melting temperatures of triplexes made by mixing parallel hairpins **11-14** with the natural polypyrimidine target sequence (S11WC), the 2'-O-methyl-RNA target sequence (S11RNA) and the 5-methyl-C substituted target sequence (S11meC). For comparison, melting temperatures of hairpins **7**-**10** are also shown. When hairpins were heated alone without the target sequence, a transition from parallel duplex to random coil is also observed. In all cases except one (**14**) this transition has a lower melting temperature and a lower hyperchromicity. Hairpin **11** has a similar melting temperature to hairpins **7** and **9**, but that of hairpin **12** is 6-7 ºC lower. Hairpins having two 8-amino-A residues have higher melting temperatures (ΔT_M 13 ºC for **10** and ΔT_M 18 ºC for **8**). The presence of two 8-amino-A and six 5-methyl-C produce a stabilization of the parallel duplex of 33

ºC. The highest stabilization of the parallel duplex was obtained in the presence of two 8-amino-A and when the backbone of the Hoogsteen strand is replaced by 2'-O-methyl-RNA (ΔT_M 40 ºC for **13**).

A similar but less pronounced trend is observed in the melting temperatures of the triplexes. Melting temperatures of triplexes formed by **7**, **9**, **11** and **12** with their natural DNA target (S11WC) are between 40-47 ºC. The presence of two 8-amino-A increase the T_M values to 51-56 ºC (**8** and **10** + S11WC), and the presence of two 8-amino-A together with the replacement of the backbone of the Hoogsteen strand by 2'-O-methyl-RNA increases the T_M values to 66 ºC (**13** + S11WC). The presence of two 8-amino-A and six 5-methyl-C gave a T_M value of only 51 ºC, the same T_M as that of the hairpin alone. A similar result was observed at pH 7.0.

When the target sequence has the 2'-O-methyl-RNA backbone (S11RNA), melting temperatures of triplexes are slightly lower than triplexes formed with the DNA target (T_M values of triplexes formed by **7**, **9**, **11** and **12** 35-42 ºC; T_M values of triplexes formed by **8** and **10** 52-55 ºC) except the melting temperature of triplex formed by the hairpin **13**, which is 71 ºC (the highest). This result is especially important because indicates that hairpins with mixed backbones bind more strongly to RNA targets.

When the target sequence has 5-methyl-C instead of C (S11MeC), melting temperatures of triplexes are between 10-12 ºC higher than triplexes formed with the natural target sequence. This is 1.7-2 ºC per substitution, consistent with the stability afforded by the presence of the methyl group at position 5 of the C in the Watson-Crick position [40].

Finally, the melting behavior of the triplex formed by the three-branch oligonucleotide (**15**) was compared with the melting behavior of the same triplex in which the strands are connected in different ways (Table 3). The triplex formed by three independent oligonucleotides (PurWC, S11WC, and S11) had two transitions at pH 6.0. The lower melting transition (20 ºC) corresponds to the dissociation of the Hoogsteen strand (S11) and the higher melting transition (50 ºC) is due to the transition from a Watson-Crick duplex to a random coil. At higher pH only the duplex-to-random-coil transition is observed. At pH 5.5 the triplex-to-duplex transition is stabilized by protonation of C residues and the transition occurs at the same time as the duplex-to-random-coil transition.

The connection of the Watson-Crick strands produces a stabilization of the Watson-Crick duplex structure without changing the stability of the triplex-to-duplex transition. The melting temperature of the duplex-to-random-coil transition of the triplex formed by H26 and S11 is 82 ºC instead of 50 ºC, and the melting temperatures of the triplex-to-duplex transitions are 40 ºC and 20 ºC at pH 5.5 and pH 6.0.

The connection of the Hoogsteen strand with the Watson-Crick purine strand produces the disappearance of the duplex-to-random-coil transition. Only one pH-dependent transition is observed at pH between 4.5-7.0 and it is assigned to the triplex-to-random-coil transition. This was expected at pH 5.5-6.0 because of the high stabilization of the Hoogsteen pairing by linking the Hoogsteen strand with the Watson-Crick purine. Two transitions were expected at pH 7.0, one being the duplex-to-random-coil at around 50 ºC. But instead, a single transition at 20 ºC (**9** + S11WC) and 32 ºC (**7** + S11WC) was observed. We believe this melting temperature is due to triplex-to-random-coil transition.

The connection of the three strands repeats the same situation found with the three independent strands, but now with strong stabilization of both transitions. Oligonucleotide **15** has two transitions. The higher melting temperature (duplex-to-random-coil transition) has the same value as the T_M observed in the triplex H26 + S11 and it is pH-independent. The lower melting temperature (triplex-to-duplex transition) shows a similar trend to the transitions observed in the triplexes **9** + S11WC and **7** + S11WC and it is pH-dependent. This behavior indicates that oligonucleotide **15** has the expected triplex structure.

Discussion. The triplex-stabilizing properties of 8-aminopurines have triggered interest in the preparation of parallel hairpins for triplex formation [8]. In this paper we described the use of Fmoc-, trityl-protected asymmetric branching molecules for the preparation of parallel hairpins and three-armed oligonucleotides. This synthetic approach allows the preparation of parallel hairpins without the need of the reversed phosphoramidites, thus facilitating the preparation of parallel hairpins carrying modified bases and backbones. Most of the previous work in this area used nucleosides as branching units [13, 15, 17, 23, 24] as the conformational rigidity imparted by the sugar could be exploited to preorganize and induce triplex formation. We preferred the use of the non-nucleoside branching unit **1** because it is easy to prepare and it is free of possible side reactions described during the synthesis of branched RNA such as phosphoryl migration and/ or chain cleavage [10-12, 24].

All asymmetric branching molecules prepared carry two hydroxyl groups protected by orthogonal groups: one acid labile and one base labile. The acid labile group is always a trityl (MeOTr or $(MeO)_2Tr$) derivative while the base

labile group is frequently levulinyl group [10-21] and in some cases the tert-butyldimethylsilyl group [24]. We believe that the extensive use of the levulinyl group is due to the optimal use in the synthesis of branched RNA to avoid phosphoryl migration. In our case this side reaction could not happen and we selected the Fmoc group as base labile group. Although the conditions for the removal of the Fmoc group are very mild, this approach implies the removal of the Fmoc group in the middle of the synthesis to allow the second strand to grow. In this step, in addition to the Fmoc group, the CNE group of the phosphates of the assembled DNA half is also removed. Little data was available on the possible consequences of the premature removal of the CNE groups of phosphates although the general feeling was positive due to the fact that during the synthesis of branched RNA the branching nucleoside should have unprotected phosphodiester function to prevent phosphoryl migration [24]. We found that coupling reactions are slower after branching. These difficulties in coupling last a few bases after branching, but after three or four bases, coupling becomes easier (as observed by the lack of the corresponding truncated sequences). This is consistent with results found during the synthesis of branched RNA [10-24] and it indicates that steric hindrance and electronwithdrawing effects of the groups at the branching site slow the coupling reactions more than the presence of unprotected phosphate groups. For these reasons difficulties in coupling at the branching point are easily overcome by increasing the coupling time, or by increasing the phosphoramidite concentration. On the other hand, changing the CNE group to a methyl group, changing oxidation conditions and addition of an extra washing step with DMAP-TET does not significantly improve coupling at the branching point.

We have also measured the relative stability of purine-pyrimidine-pyrimidine triplexes formed by the parallel hairpins prepared in this work. RNA/DNA hybrid hairpins carrying 8-aminopurines and 2'-O-methyl-RNA at the Hoogsteen strand were found to have the best affinity for both DNA and 2'-O-methyl-RNA polypyrimidine targets. On the other hand, substitution of C by 5-methyl-C at the Hoogsteen position induced a high stabilization of the parallel structure but not of the triplex. During the preparation of this manuscript, the synthesis of 3'-3'-linked oligonucleotides branched by a pentaerythritol linker was described [21]. These authors also showed a high stabilization of triplexes formed by single-stranded DNA or RNA sequences with branched oligonucleotides composed of 2'-deoxyribonucleotides and 2'-O-methylribonucleotides. Thus, our results are in agreement with these authors and indicate that parallel hairpins carrying 2'-O-methyl-RNA and 8-aminoadenine have enhanced binding properties towards single-stranded polypyrimidine sequences by triplex formation.

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Experimental Part

General. 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). Long chain amino controlled pore glass (LCAA-CPG) was purchased from CPG (*CPG,* Inc., *New Jersey,* USA). Solvents were from *S.D.S.* (*S.D.S.,* France). N,N'-Bis(4-hydroxybutyryl)-1,3-diamido-2-propanol (**1**) was prepared as described elsewhere [19]. *Instrumentation.* Oligonucleotide sequences were synthesized on a *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 spectra and melting experiments were recorded on a *Shimadzu* UV-2101PC spectrophotometer equipped with temperature controller SPR-8. ^{1}H-, and ^{13}C-NMR spectra were recorded on a *Varian* Gemini 200MHz.

N-[4-(4-methoxytrityloxy)butyryl]-N'-(4-hydroxybutyryl)-1,3-diamido-2-propanol (**2**). N,N'-Bis(4-hydroxybutyryl)-1,3-diamido-2-propanol (**1**, 5 g, 19 mmol) was dried after repeated evaporation of a solution in anhydrous pyridine and dissolved in 20 ml of pyridine. The solution was cooled over an ice-bath and treated dropwise with a solution of 2.89 g (9.3 mmol) of 4-methoxytrityl chloride in 20 ml of dichloromethane (DCM) (over 30 min). After the addition the reaction mixture was gradually warmed to room temperature and stirred for 90 min. The reaction was stopped with the addition of 0.5 ml of metanol (MeOH) and the solution was concentrated to dryness. The residue was dissolved in 200 ml of DCM, and the organic solution was washed in 100 ml of 5% Na_2CO_3 aqueous solution, and 100 ml of saturated NaCl, dried (anhydrous Na_2SO_4) and concentrated to dryness. The residue was purified by column chromatography on silica gel. The column was packed with a 0.5% pyridine solution in DCM and

eluted with a 0 to 5% gradient of MeOH in DCM to give 2.51 g (50%) of the desired compound. TLC (5% MeOH in DCM) Rf = 0.37. Anal. Calcd. For $C_{31}H_{38}N_2O_6$.2 H_2O: C, 65.25; H, 7.42; N, 4.91. Found C, 65.84; H, 7.84; N, 4.92. Mass spectra (FAB+): 557.2 (M+Na), 273.1 (MeOTr+) expected for $C_{31}H_{38}N_2O_6$ 534.6. ^{1}H-NMR (DMSO-d_6) δ (ppm): 7.4-6.4 (m, 16 H, MeOTr, 2 NH), 4.6 (wide s, 1H, CHO<u>H</u>), 3.71 (s, 3H, OCH_3), 3.6, 3.4, 3.2 (m, 8H, O<u>H</u>, C<u>H</u>OH, C<u>H_2</u>OH, C<u>H_2</u>NH), 3.0 (t, 2H, CH_2O MeOTr), 2.3 (m, 4H, C<u>H_2</u>CO), 1.8 (m, 4H, CH_2C<u>H_2</u>CH_2). ^{13}C-NMR (Cl_3CD) δ (ppm): 174.1, 173.7 (2 CONH), 157.4 (MeOTr), 143.6 (MeOTr), 134.8 (MeOTr), 129.2 (MeOTr), 127.3 (MeOTr), 126.8 (MeOTr), 125.8 (MeOTr), 112.0 (MeOTr), 85.2 (Cq, MeOTr), 68.6 (CHOH), 60.8, 61.4 (CH_2OH and CH_2O MeOTr), 54.2 (OCH_3 MeOTr), 42.0 (CH_2NH), 32.4 (<u>C</u>H_2CONH), 27.2 and 25.0 (CH_2<u>C</u>H_2CH_2).

N-[4-(9-fluorenylmethoxycarbonyloxy)butyryl]-N'-[4-(4-methoxytrityloxy) butyryl]-1,3-diamido-2-propanol (**3**). Compound **2** (0.63 g, 1.18 mmol) was dissolved in 10 ml of pyridine and reacted with 9-fluorenylmethoxycarbonyl chloroformate (0.31 g, 1.18 mmol). After 3.5 h stirring at room temperature, the reaction was stopped by addition of 0.5 ml of MeOH and the solution was concentrated to dryness. The residue was dissolved in 100 ml of DCM, and the organic solution was washed in 100 ml of saturated NaCl, dried (anhydrous Na_2SO_4) and concentrated to dryness. The residue was purified by column chromatography on silica gel. The column was packed with a 0.5% pyridine solution in DCM and eluted with a 0 to 5% gradient of MeOH in DCM to give 0.2 g (23%) of the desired compound. TLC (5% MeOH in DCM) Rf = 0.78. Anal. Calcd. For $C_{46}H_{49}N_2O_8$: C, 72.90; H, 6.52; N, 3.70. Found C, 72.32; H, 6.46; N,

3.37. Mass spectra (FAB+): 779.5 (M+Na), 273.2 (MeOTr+) expected for $C_{46}H_{49}N_2O_8$ 757.9. ^{1}H-NMR (DMSO-d_6) δ (ppm): 7.9-6.8 (m, 24 H, MeOTr, Fmoc, 2 NH), 4.9 (d, 1H, CHOH), 4.4 (m, 2H, CH_2 Fmoc), 4.18 (m, 1H, CH Fmoc), 3.75 (s, 3H, OCH_3), 3.5 (m, 1H, CHOH), 3.4-2.9 (m, 8H, CH_2O-MeOTr, CH_2O-Fmoc, CH_2NH), 2.4-2.2 (m, 4H, CH_2CO), 2.1-1.8 (m, 4H, $CH_2CH_2CH_2$). ^{13}C-NMR (DMSO-d_6) δ (ppm): 173.4, 172.5 (CONH), 157.4 (MeOTr), 143.4 (MeOTr), 142.6, 141.4, 140.2 (Fmoc), 129.5 (MeOTr), 127.0 (MeOTr, Fmoc), 126.6 (MeOTr), 126.2, 125.9 (MeOTr, Fmoc), 124.2 (Fmoc), 118.9 (Fmoc), 85.2 (Cq MeOTr), 69.7, 68.9, 66.4, 61.5 (CHOH, CH_2O MeOTr, CH_2OFmoc, CH_2 Fmoc), 54.5 (OCH_3, MeOTr), 45.9 (CH Fmoc), 41.8 (CH_2NH), 32.8, 31.1 (CH_2CO), 25.4, 23.8 ($CH_2CH_2CH_2$).

*Preparation of the solid support carrying the asymmetric doubler (**5**).*

Synthesis of the hemisuccinate **4**. Compound **3** (0.2 g, 0.26 mmol) was reacted with 31 mg (0.31 mmol) of succinic anhydride in the presence of 38 mg (0.31 mmol) of DMAP in 25 ml of DCM. The mixture was stirred for 20 h at room temperature and the resulting solution was diluted with 75 ml of DCM. The organic solution was washed in 75 ml of a 0.1 M NaH_2PO_4 (pH 5) followed by a saturated NaCl solution, dried (anhydrous Na_2SO_4) and concentrated to dryness. The residue was dissolved with the minimum amount of DCM and precipitated with hexane. Yield 0.2 g (0.23 mmol, 87%). The resulting white solid was partially contaminated with DMAP but it was used in the following step without further purification. ^{1}H-NMR (DMSO-d_6) δ (ppm): similar spectra to compound **3** with the exception of a signal at 2.8 (m, 2H, HOOC-CH_2).

Coupling of the hemisuccinate to the solid support. The support carrying the asymmetric doubler was prepared by the coupling of the hemisuccinate **4** to sarcosyl-LCAA-CPG [41] using Ph_3P, 2,2'-dithiobis(5-nitropyridine) and DMAP as condensing agents [42]. Coupling time was increased from 5 min to 45 min, which gave a functionalization of 34 μmol per g.

Synthesis of branched oligonucleotides. Phosphoramidites of the natural bases ($dA^{benzoyl}$, $dC^{benzoyl}$, $dG^{isobutyryl}$, and T) were from commercial sources. The phosphoramidite of 8-aminoadenine protected with the dimethylformamidine group was prepared as described elsewhere [3, 4]. 5-Methyl-$dC^{benzoyl}$, 2'-O-methyl-U and 2'-O-methyl-$C^{benzoyl}$, $(MeO)_2Tr$ -hexaethyleneglycol, and asymmetric doubler phosphoramidites were from commercial sources. 3'-O-$(MeO)_2Tr$ -1,3-propanediol-succinyl-CPG support was prepared as described elsewhere [31]. Oligonucleotides were synthesized on a 1 and 0.2 μmol scale using a DNA synthesizer. The supports carrying the first half of the parallel hairpin were synthesized at 1 μmol scale. After the assembly of the first half of hairpins the $(MeO)_2Tr$ group at the 5'-end was removed and the resulting supports were treated as follows: A) Acetylation of the 5'-end: treatment of the trityl-off supports with 2 ml of a 1:1 mixture of the capping solutions from the DNA synthesizer (A: acetic anhydride / lutidine / tetrahydrofuran (THF) 1:1:8) and B: 10% N-methylimidazole in THF) for 10 min. B) Removal of the Fmoc group: treatment of the supports with 2 ml of 0.5 M DBU solution in ACN for 10 min. and washes in 1% triethylamine in ACN and ACN to eliminate excess of DBU. These supports were divided in several aliquots to test the assembly of the second half of the hairpins using several cycles of synthesis. The trials were performed at

a 0.2 μmol scale. The following synthesis cycles were tried: I) Standard cycle (coupling time 30 s, capping time 15 s, oxidation time 30 s). II) Increased coupling time cycle: coupling time 5 min, capping time 75 s, oxidation time 75 s. III) Double coupling cycle: Two coupling reactions per base of 5 min, capping time 75 s, oxidation time 75 s. IV) Neutralization cycle: the standard cycle in which an additional step of neutralization with a solution of DMAP-TET was included between detritylation and coupling steps. V) Neutralization and increased coupling time cycle: The increased coupling time cycle in which an additional step of neutralization with a solution of DMAP-TET was included between detritylation and coupling steps. VI) Neutralization and double coupling cycle: the double coupling cycle in which an additional step of neutralization with a solution of DMAP-TET was included between detritylation and coupling steps. VII) Anhydrous oxidation cycle. The standard cycle in which iodine solution was substituted by a *t*-butylhydroperoxide solution (20 ml of the commercially available solution in decane (5-6 M) was mixed with 40 ml of dichloromethane), oxidation time was increased to 1 min. Finally the phosphoramidite concentration was 0.1 M but in some cases a 0.2 M solution was also used.

The three-branched oligonucleotide (**15**) was prepared on 0.2 μmol using polystyrene supports (LV200 columns). The first two branches were assembled using the standard cycle recommended for these columns. After capping and removal of the Fmoc group as described above, the third branch was assembled using the same cycle but with increased coupling time (8 min).

After the assembly of the sequences, oligonucleotide-supports were treated with 32% aqueous ammonia at 55 ºC. When O-methyl phosphoramidites were used, supports were first treated with 1 ml of thiophenol / triethylamine /dioxane (1:1:2)

for 2 h at room temperature, washed in ACN and dried. The resulting supports were then treated with concentrated ammonia. Ammonia solutions were concentrated to dryness and the products were purified by reversed-phase HPLC. Oligonucleotides were synthesized with the last $(MeO)_2Tr$ group at the 5' end (trityl-on protocol) to facilitate reverse-phase purification. HPLC conditions: Solvent A: 5% ACN in 100 mM triethylammonium acetate (TEAA) pH 6.5 and solvent B: 70% ACN in 100 mM TEAA pH 6.5. Columns: PRP-1 (Hamilton), 250 x 10 mm. Flow rate: 3 ml/min. A 30 min linear gradient from 10-80% B (trityl on) or a 30 min linear gradient from 0-50% B (trityl off). MS (MALDI-TOF) of sequence **11**: 8358.9 ($M+4Na^+$) expected for $C_{265}H_{323}N_{93}O_{165}P_{26}$ 8275.4). Oligonucleotides were also checked by denaturing gel electrophoresis. In this case, a small aliquot of the $(MeO)_2Tr$-oligonucleotide was detritylated with 80% acetic acid and dried. The resulting product was loaded in the gel using glycerol loading buffer for the analysis of the purity.

Yields after purification. **11** (200 nmols), 8 OD units (cycle II). **12** (200 nmols), 7 OD units (cycle III). **13** (200 nmols), 3 OD units. **14** (200 nmols), 2 OD units, **15** (200 nmols), 4 OD units. Sequences **7**-**10** were prepared in polystyrene supports (200 nmols) and the amount obtained after the same HPLC purification were between 5-10 OD units [8].

*Melting experiments***.** Melting experiments with triple helices were performed as follows. Solutions of equimolar amounts of hairpins and the target Watson-Crick pyrimidine strand (11-mer) were mixed in 0.1 M sodium phosphate / citric acid buffer of pH ranging from 4.5 to 7.0 and 1M NaCl. The 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, 15000 and, 15000 M^{-1} cm^{-1} for C, T, G, A and, 8-amino-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. Melts were run on duplex concentration of 3-4 μM at 260 nm.

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Table 1: Sequences of parallel hairpins and a three-branched oligonucleotide prepared.

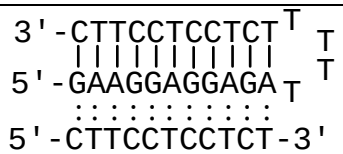
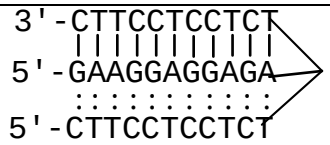
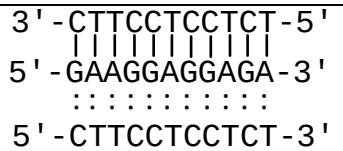
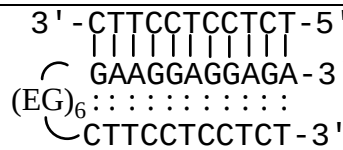
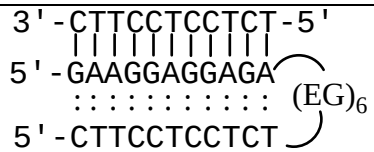
Number	Sequence[a)]
7	5’-GAAGGAGGAGA-heg-TCTCCTCCTTC-5’
8	5’-GAAGGNGGNGA-heg-TCTCCTCCTTC -3’
9	3’-AGAGGAGGAAG-heg-CTTCCTCCTCT-3’
10	3’-AGNGGNGGAAG-heg-CTTCCTCCTCT-3’
11	5’-GAAGGAGGAGA-TT-bpa-TT-TCTCCTCCTTC-5’
12	5’-GAAGGAGGAGA-TT-bppd-TT-TCTCCTCCTTC-5’
13	5’- GAAGGNGGNGA -TT-bppd-*UU-UCUCCUCCUUC*-5’
14	5’- GAAGGNGGNGA -TT-bppd-TT-TMTMMTMMTTM-5’
15	5’-GAAGGAGGAGA-heg-bpp-(heg-CTTCCTCCTCT-5’)-heg-TCTCCTCCTTC-5’

[a)]heg: hexaethyleneglycol, bpa: [-PO_3-$O(CH_2)_3$-CONH-CH_2]$_2$-CHOH, bppd: [-PO_3-$O(CH_2)_4$-CONH-CH_2]$_2$-$CHOPO_2OCH_2CH_2CH_2OH$, bpp: [-PO_3-$O(CH_2)_4$-CONH-CH_2]$_2$-$CHOPO_3$-, N: 8-aminoadenine, M: 5-methylcytosine, *U,C*: 2-O’-methyl-RNA

Table 2. Melting temperatures (ºC) of parallel hairpins and triplexes in 0.1 M sodium phosphate and citric acid pH 6.0 and pH 7.0 (last entry), 1 M NaCl. S11: 5'-CTTCCTCCTCT-3'; S11RNA: 5'-*CUUCCUCCUCU*-3'; S11MeC: 5'-MTTMMTMMTMT-3'. *C,U*: 2'-O-methyl-RNA and M: 5-methylcytidine; the rest of the sequences are described in Table 1.

Hairpin	No target	Target S11WC	Target S11RNA	Target S11MeC
9	25	45	38	55
7	25	47	42	58
12	18	40	35	52
11	24	43	36	53
8	43	56	55	--
10	38	51	52	62
13	58	66	71	--
14, pH 6.0	51	51	--	63
14, pH 7.0	29	32	--	--

Table 3. Melting temperatures (ºC) of triplexes having different constrains in 0.1 M sodium phosphate and citric acid, 1 M NaCl. H26: 5'-GAAGGAGGAGA-T_4-TCTCCTCCTTC-3'; S11: 5'-CTTCCTCCTCT-3'; S11WC: 5'-TCTCCTCCTTC-3'; PurWC: 5'-GAAGGAGGAGA-3'; the rest of the sequences are described in Table 1.

Triplex		pH 5.5	pH 6.0	pH 6.5	pH 7.0
H26+S11		40 / 82	20 / 82	-- / 82	-- / 82
15		54 / 80	38 / 80	27 / 82	15 / 80
PurWC+ S11WC+S11		44*	20 / 50	-- / 50	-- / 50
9+S11WC		54*	45*	33*	20*
7+S11WC		56*	47*	36*	32*

*Triplex-to-random-coil transition

LEGENDS

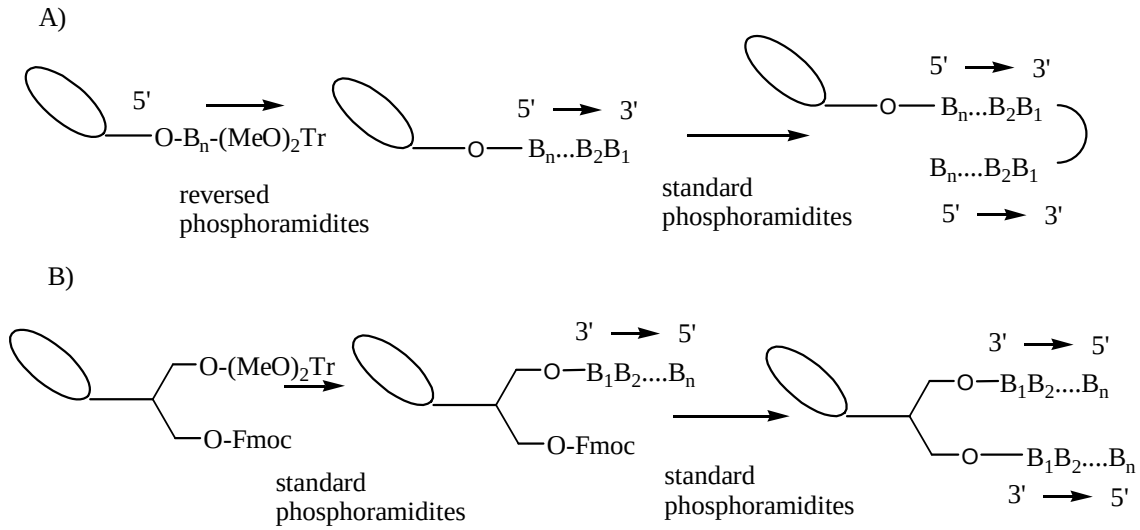
Scheme 1. Scheme of two possible ways to assemble 3'-3' linked DNA hairpins with the two arms in parallel orientation. A) Standard method. The synthesis starts at one end of the hairpin. The first half is assembled using reversed phosphoramidites followed by the assembly of the second half using standard phosphoramidites. B) Method using branching molecules. The hairpin is assembled from the loop using a branching molecule which carries two hydroxyl groups protected by orthogonal groups: the acid labile dimethoxytrityl ($(MeO)_2Tr$) and the base labile fluorenylmethoxycarbonyl (Fmoc). This last method does not require the use of reversed phosphoramidites.

Scheme 2. Synthesis of the support carrying the asymmetric branching molecule.

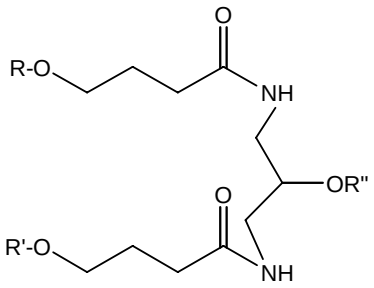
Scheme 3. Outline of the synthesis of parallel hairpin **11**.

Scheme 4. Outline of the synthesis of oligonucleotide **15**.

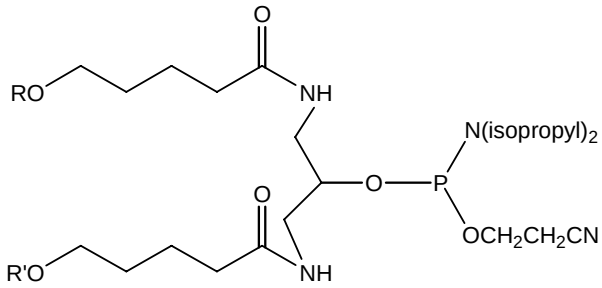
Figure 1. Analysis by gel electrophoresis (20% acrylamide, 8M urea) of the products obtained after the assembly of the parallel hairpin **11** obtained using: A) standard cycle; B) Increased coupling time; C) double coupling; D) same as A but adding a wash in DMAP-TET; E) same as B but adding a wash in DMAP-TET; F) same as C but adding a wash in DMAP-TET.


A)
5'
O-Bn-(MeO)2Tr
reversed
phosphoramidites
5' → 3'
O Bn...B2B1
standard
phosphoramidites
5' → 3'
O Bn...B2B1
Bn....B2B1
5' → 3'
B)
O-(MeO)2Tr
O-Fmoc
standard
phosphoramidites
3' → 5'
O B1B2....Bn
O-Fmoc
standard
phosphoramidites
3' → 5'
O B1B2....Bn
O B1B2....Bn
3' → 5'

Scheme 1

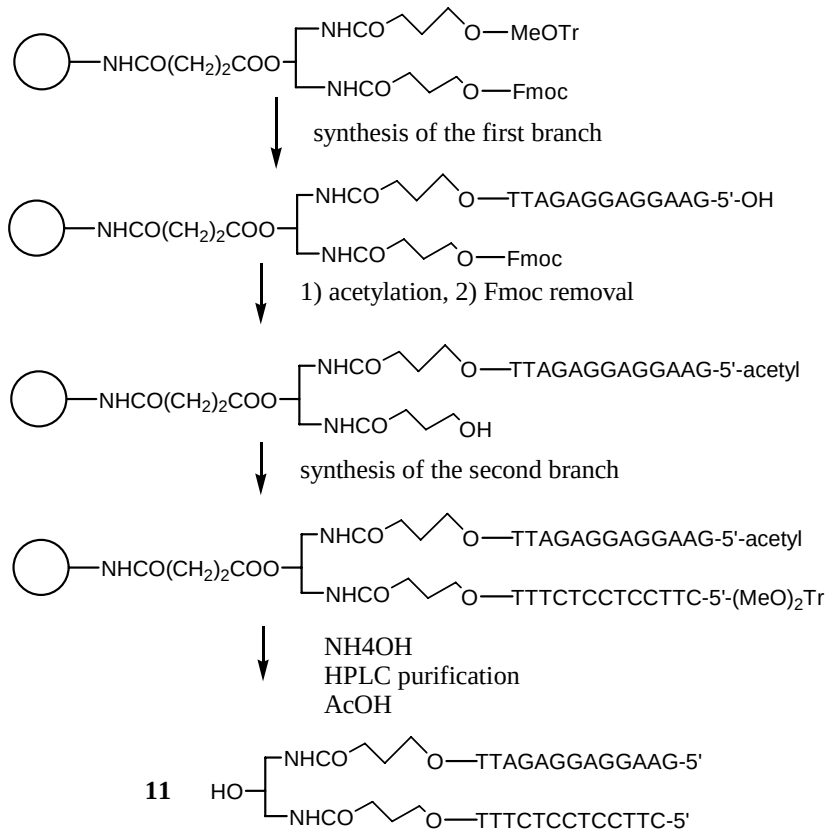


1, R=R'=R"=H
2, R=MeOTr, R'=R"=H
3, R=MeOTr, R'=Fmoc, R"=H
4, R=MeOTr, R'=Fmoc, R"=$COCH_2CH_2COOH$
5, R=MeOTr, R'=Fmoc, R"=$COCH_2CH_2CONH$-LCAA-CPG

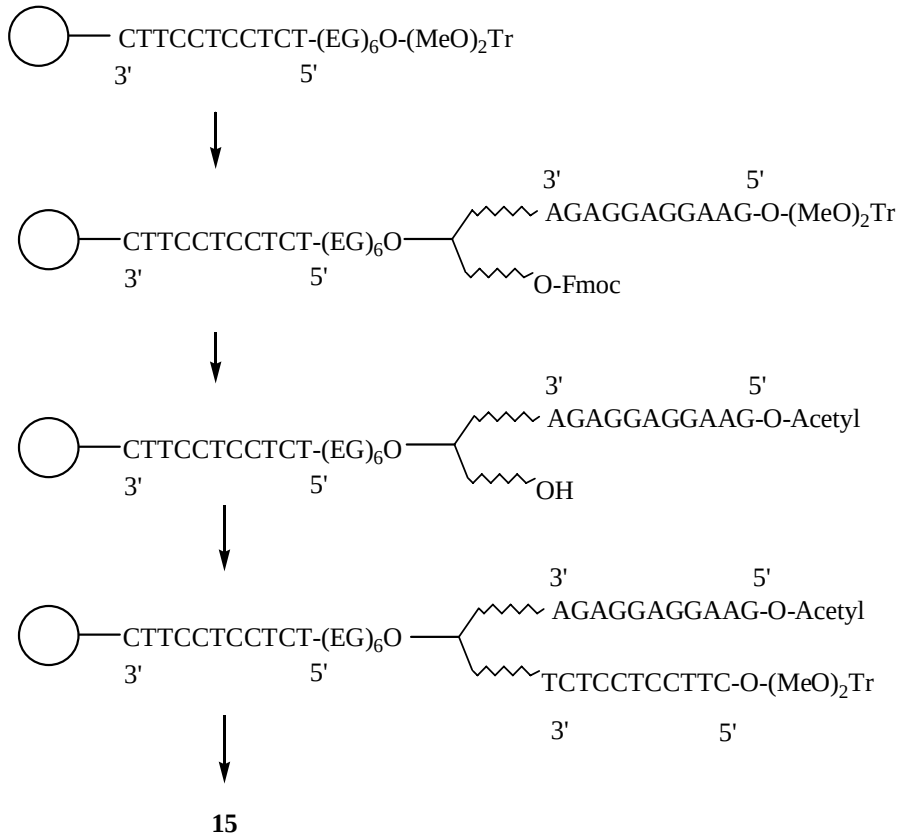


6, R=$(MeO)_2$Tr, R'=Fmoc

Scheme 2


NHCO
O—MeOTr
NHCO(CH2)2COO
NHCO
O—Fmoc
synthesis of the first branch
NHCO
O—TTAGAGGAGGAAG-5'-OH
NHCO(CH2)2COO
NHCO
O—Fmoc
1) acetylation, 2) Fmoc removal
NHCO
O—TTAGAGGAGGAAG-5'-acetyl
NHCO(CH2)2COO
NHCO
OH
synthesis of the second branch
NHCO
O—TTAGAGGAGGAAG-5'-acetyl
NHCO(CH2)2COO
NHCO
O—TTTCTCCTCCTTC-5'-(MeO)2Tr
NH4OH
HPLC purification
AcOH
11
NHCO
O—TTAGAGGAGGAAG-5'
HO
NHCO
O—TTTCTCCTCCTTC-5'

Scheme 3


CTTCCTCCTCT-(EG)6O-(MeO)2Tr
3'
5'
3'
5'
AGAGGAGGAAG-O-(MeO)2Tr
CTTCCTCCTCT-(EG)6O
3'
5'
O-Fmoc
3'
5'
AGAGGAGGAAG-O-Acetyl
CTTCCTCCTCT-(EG)6O
3'
5'
OH
3'
5'
AGAGGAGGAAG-O-Acetyl
CTTCCTCCTCT-(EG)6O
3'
5'
TCTCCTCCTTC-O-(MeO)2Tr
3'
5'
15

Scheme 4

Figure 1