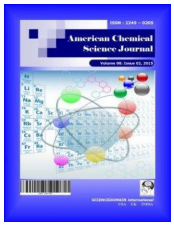

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Fluorescence Binding Assay of Atl1 Protein with the SIMA Labelled Oligonucleotide Containing the Tricyclic Nucleoside Analogue of O^6-methyl-2'-deoxyguanosine

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Authors' contributions

This work was carried out in collaboration between both authors. Both authors read and approved the final manuscript.

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ABSTRACT

The synthesis of phosphoramidite (DNA building block) containing the tricyclic nucleoside analogue of O^6-methyl-2-deoxyguanosine was carried out and its Interactions with Atl proteins was quantified through fluorescent assay (fluorescent intensity monitoring). In fluorescent assay the interaction of DNA with protein is quantified by measuring the dissociation constant, K_D. Usually the DNA is labelled with fluorophore (such as FAM, HEX or SIMA) which acts as a probe during the binding titration. Binding of protein to DNA substrate that is fluorescently labelled can cause changes in the fluorescent signal to that label since its interaction with protein changes its chemical environment from that in free solution. In this assay a series of fluorescent measurements were taken whilst increasing the amount of protein relative to the DNA. The binding curves were generated by plotting protein concentration against fluorescent intensity and the data were fitted by the non-linear least-squares regression using Kaleida Graph ($I = I_{max} + [(D+E+K_D) - ((D+E+K_D)^2 - (4DE))^{0.5}$]

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(I_{min} - I_{max})/2D). The ODN containing the tricyclic analogue bound poorly with the Atl 1 protein with the K_D = 194±29. The syntheses of the ODN with the modification at the O^6-position as well as the florescence assay were reported here.

Keywords: Fluorescence; protein; oligonucleotide; assay; tricyclic.

1. INTRODUCTION

O^6-Alkylguanine-DNA-alkyltransferases (AGTs) repair O^6-methylguanine containing DNA by the transfer of the methyl group to an active site cysteine with the regeneration of guanine. In contrast, the recently discovered alkyltransferase-like (ATL) proteins are homologues of alkyltransferases that bind to, but do not de-alkylate DNA containing O^6-alkylguanine lesions [1]. In most ATL proteins the active site cysteine is substituted by tryptophan.

The distribution of ATL proteins is diverse, it was observed in prokaryotes and lower eukaryotes but appears to be absent in multicellular organisms including humans. For example *E. coli* has two alkyltransferase proteins (*ada-C* and *ogt*) and one ATL-protein (eAtl), while on the other hand *Schizosaccharomyces pombe* (*S. pombe*) contains a single ATL (Atl1) and has no any recognisable alkyltranferase proteins [2].

Studies on both ATL proteins from *E. coli* and *S. pombe* reveal their ability to bind to short oligonucleotides containing a variety of O^6-alkylguanines such as O^6-methyl-, O^6-benzyl-, O^6-(4-bromothenyl)- or O^6-hydroxyethyl-guanine [3]. In all the cases there is no evidence for the removal of the alkyl group, base or oligonucleotides near the lesion. This suggests that ATL proteins only bind but do not repair O^6-alkylguanine-modified DNA.

However, there was no binding observed with oligonucleotides containing other types of DNA damage for example 8-oxoguanine, 5-hydroxymethylcytosine, ethenoadenine, among others [4]. This suggest that ATL proteins may be highly specific for damage recognition of O^6-alkylguanine-modifications [4].

The ATL-proteins sequence is highly homologous to that of MGMT but with tryptophan in place of cysteine. The function of this protein is still yet to be defined, but to establish whether or not it plays a role in the processing of O^6-alkylation damage in DNA a study into the effect of Atl1 deletion in *S. pombe* shows that the protein gives certain degree of protection to the organism against the toxic effect of the alkylating agents but exactly how this is achieved is yet to be defined. Also because *S. pombe* and *Thermus thermophilus* lack AGT, it was observed that the inactivation of their ATL genes (atl1 and TTHA1564 respectively) reduces their alkylation damage resistance which further suggest that ATLs protect against biological effects of DNA alkylation damage by an unidentified mechanism. However, there is no any evidence that ATLs protein repair any DNA damage but evidence shows that the protein are damage sensors instead [3]. It is part of our objective to check whether our modified nucleoside will be recognized by the Atl1 which will serve as an indicator that it could in cancer chemotherapy.

2. RESULTS AND DISCUSSION

The synthesis of the phosphoramidite of the nucleoside was carried out base on the reported literature [5,6] which was designed previously by Seela and Driller (scheme 2.22). Protection of the 2-amino group of compound **1** as its amidine was accomplished by using *N*,*N*-dimethylformamide dimethylacetal in dry DMF.(12) The ^{1}H NMR spectrum and mass spectrum revealed the presence of an amidine compound **1** and an *N*-formyl compound **2**. The most probable explanation for this may be the hydrolysis of the amidine due to the slight acidity of the silica gel from the column chromatography. Also purification was performed in 5% methanol in dichloromethane. So, it was assumed that there must have been a small amount of water present in the methanol which might react with the amidine group under the acidic conditions of the silica gel. It was therefore decided to convert the entire amidine compound into the formyl derivative before proceeding to the next step. This was achieved by stirring the amidine compound with 20% aqueous acetic acid overnight at room temperature. After converting the entire amidine compound into the formyl protected nucleoside the next step was the protection of the 5'-OH group with the dimethoxytrityl chloride (DMTCl) to give the DMT-protected nucleoside **3**. This was carried out by dissolving the formyl compound **2** in anhydrous pyridine under dry atmosphere of

argon, followed by the addition of 0.05 equivalent DMAP as a catalyst and then 1.4 equivalent triethylamine (TEA) and 1.2 equivalent of DMTCl. The reaction was completed after 2 h, based on TLC. Silica column chromatography was run using 2-5% MeOH/DCM (1% TEA). Phosphitylation of compound **4** was conveniently carried out using dry diisopropylethylamine and 2-cyanoethyl-*N, N*-diisopropylaminechlorophosphoramidite (phosphitylating reagent) in dry dichloromethane. Four equivalent of the diisopropylethylamine and 1.5 equivalent of the phosphitylating reagent were used. The least polar is the phosphoramidite **5** and it appears as a two spots very close to each other because the solvent system partially resolves the diastereomers (due to the asymmetric phosphorus atom). The reaction was worked up and silica gel column chromatography was run in 45:45:10 v/v dichloromethane: hexane: triethylamine. ^{31}P NMR was run in $CDCl_3$ pass over basic alumina to avoid the hydrolysis of the phosphoramidite. Two peaks were observed at around +148 ppm in the ^{31}P NMR which indicates the formation of the phosphoramidite. ESI+ mass spectrum also confirm the formation of compound **5**.

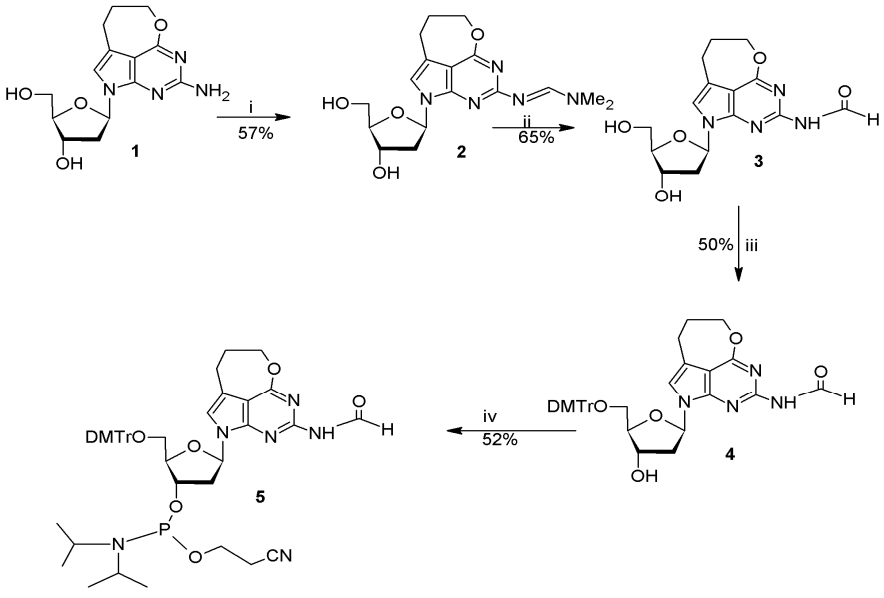
The correct route towards phosphoramidite **5** is therefore shown in scheme 1.

The syntheses of the ODNs were carried out on an automated DNA synthesiser employing solid phase chemistry and phosphoramidite methodology. It was performed using base-labile protecting groups for the natural bases namely; A and G using Pac protecting groups and acetyl for C. The sequence of the ODNs synthesised and their characteristic data are given in Table 1.

The interaction of Atl1 with d(5'-SIMA-GCC ATG XCT AGT A), where X is O^6-benzylguanine and guanine alone respectively as the control standards were reported.136 The former serves as a positive control (showing good binding), while the latter serves as negative control (showing poor binding).

The binding titration was done with the ODN containing the tricyclic analogue d(5'-SIMA-GCC ATG XCT AGT A) where X is the tricyclic base analogue. The curve was obtained as shown in Fig. 1.

Comparing with the standards (Table 2) it is clear that the ODN containing the tricyclic analogue bound poorly with the Atl 1 protein with the K_D = 194±29. The K_D value for **KA01** ODN (Table 2) is similar to that of guanine (negative control) which shows no much binding with Atl1.



Scheme 1. Synthesis of phosphoramidite 5

Reagents and reaction conditions: (i) N,N-dimethylformamide dimethylacetal, DMF, 50°C, 2 h, 57%; (ii) 20% AcOH, rt, overnight, 65%; (iii) DMAP, DMTCl, Et_3N,pyridine, 50%; (iv) 2-cyanoethyl-N-diisopropylamine chlorophosphoramidite, CH_2Cl_2, $^iPr_2NEt$, 52%

Table 1. The ODNs syntheses with their retention times, RP-HPLC condition, masses and sequences

Oligo	Retention time/min.	RP-HPLC Condition	Mass Spec. (MALDI)	Sequence
KA 01	20.9	0-40% B 30 min.	Exp – 4774 Obs - 4772	5'-SIMA-GCC ATG XCT AGT A
KA 02	19.1	0- 40% B 30 min	Exp – 4013 Obs - 4012	5'-GCC ATG XCT AGT A

*Where X is the modification (compound **5**) and SIMA is the hexachlorofluorescein label but with higher stability toward ammonia solution*

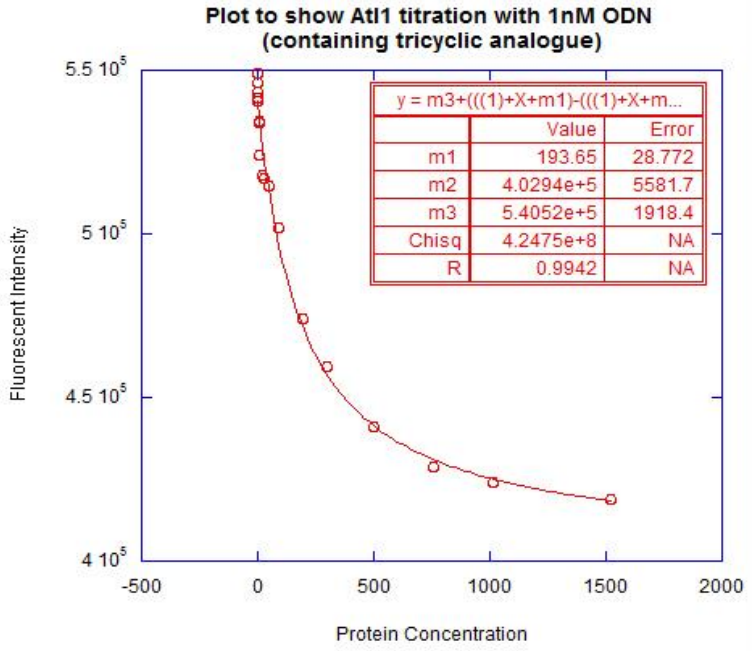


Fig. 1. Binding of Atl1 to the ODNs containing tricyclic analogue

Table 2 gives the dissociation constants of ODNs with different modifications at the O^6-position of guanine.

Table 2. Dissociation constants of Atl1 with d(5'-SIMA-GCC ATG XCT AGT A)

Modification (X)	K_D (dissociation constant)
O^6-benzylguanine [7]	0.62±0.1
O^6-methylguanine [7]	2.20±2
Tricyclic analogue (**1**)	193.65±29
Guanine (no modification) [7]	315.97±47

3. CONCLUSION

In conclusion, synthesis of compound **5** was successfully achieved via the tricyclic nucleoside **1**. Synthesis of the ODN containing the tricyclic nucleoside was successfully achieved via phosphoramidite chemistry [8]. Florescence binding assay indicates that the ODN bind the Atl1 protien weakly with the dissociation constant, *KD* = 2.74±0.00.

4. EXPIREMENTALS

4.1 4-{[(dimethylamino) methylidene] amino}-2-(2'-deoxy-β-D-erythro-pentofuranosyl)-6-oxa-7, 8, 9-trihydro-2, 3, 5-triazabenzo [cd]azulene (2)

Compound **1** (150 mg, 490 µmol) was dissolved in anhydrous DMF (3 mL), under an argon atmosphere. After 1 h, *N,N*-dimethylformamide dimethylacetal (0.5 mL, 3.9 mmol) was added

and the solution left to stir overnight. After evaporation the residue was purified by silica gel column chromatography (0-5% MeOH in CH_2Cl_2 (1% Et_3N)) which gave **1** as a white foam (100 mg, 57%) R_f (D) = 0.47; δ_H (d_4-CD_3OD) 2.07-2.11 (2H, m, CH_2CH_2O), 2.80 (2H, t, J = 6.4 Hz, $CH_2CH_2CH_2O$), 3.00 (3H, s, N-CH_3), 3.11 (3H, s, N-CH_3), 3.45(2H, m, H2' and H2''), 3.70 (1H, m, H5'), 4.31 (1H, m, H5''), 4.39 (2H, t, J = 6.4 Hz, CH_2O), 4.93 (1H, t, J = 6.4 Hz, C4'), 5.25 (1H, d, J = 6.4 Hz, C3'), 6.05 (1H, dd, J = 3.2, 6.4 Hz, H1'), 7.15 (1H, s, H-6), 8.09 (1H, s, N=C-*H*) ppm; δ_C (d_4-CD_3OD) 25.7 (CH_2CH_2O), 28.9 ($CH_2CH_2CH_2O$), 34.0 (N-CH_3), 39.7 (N-CH_3), 40.2 (C2'), 62.3 (C5'), 71.6 (C3'), 72.7 (CH_2O), 83.6 (C1'), 87.1 (C4'), 101.3 (C-10), 114.8 (C-6a), 118.3 (C-1), 154.4 (C-2a), 157.9 (C=N), 160.6 (C-6), 165.0 (C-4) ppm; *m/z* (ESI+) 362 $[M + H]^+$; Acc Mass: 362.1827; calculated for $C_{17}H_{27}N_5O_4$ requires 362.1828 (deviation -0.3 ppm).

4.2 4-[(Formyl) amino]-2-(2'-deoxy-β-D-erythro-pentofuranosyl)-6-oxa-7, 8, 9-trihydro-2,3,5-triazabenzo[cd]azulene (3)

Compound **2** (250 mg, 692 µmol) was dissolved in 20% acetic acid (5 mL), stirred overnight at room temperature. After evaporation, the residue was purified by silica gel column chromatography (0-5% MeOH / CH_2Cl_2) which gave **3** as a white foam (150 mg, 65%). R_f (D) = 0.60; δ_H (d_4-CD_3OD) 2.15-2.25 (3H, m, CH_2CH_2O and *H*2'), 2.61 (1H, m, *H*2''), 2.90 (2H, t, J = 6.4 Hz, $CH_2CH_2CH_2O$), 3.75(2H, m, *H*5' and *H*5''), 3.94 (1H, m, *H*4'), 4.55 (3H, m, CH_2O and *H*3'), 6.70 (1H, dd, J = 3.2, 6.4 Hz, H1'), 7.28 (1H, s, *H*-6), 8.25 (1H, s, *NH*-CHO), 9.49 (1H, s, *CH*O) ppm; *m/z* (ESI+) 335 $[M + H]^+$; Acc Mass: 335.1353; calculated for $C_{15}H_{19}N_4O_5$ requires 335.1355 (deviation -0.6 ppm)

4.3 4-[(Formyl) amino]-2-(2'-deoxy-β-D-erythro-5'-O-[4, 4'-dimethoxytrityl] pentofuranosyl)-6-oxa-7, 8, 9-trihydro-2, 3, 5-triazabenzo [cd] azulene (4)

Compound **3** (150 mg, 449 µmol) was dissolved in anhydrous pyridine (5 mL) under an argon atmosphere with stirring. 4, 4-Dimethoxytrityl chloride (183 mg, 539 µmol) and 4-methyldiaminopyridine (2 mg, 15 µmol) were added. After 4 h the mixture was evaporated, redissolved in ethylacetate (20 mL) and extracted with saturated sodium acetate (10 mL), water (10 mL), brine (10 mL) and dried ($NaSO_4$). Evaporation and purification of the resulting residue by silica gel chromatography (0-5% MeOH / DCM (1% Et_3N)) gave **4** as a light yellow foam (140 mg, 50%). R_f (C) = 0.6, UV active, stained orange with anisaldehyde; δ_H ($CDCl_3$) 2.17 (2H, m, CH_2CH_2O), 2.36-2.44 (2H, m, *H*2' and *H*2''), 2.71 (2H, t, J = 6.6 Hz, $CH_2CH_2CH_2O$), 3.33 (2H, m, *H*5' and *H*5''), 3.76 (6H, s, 2x -OCH_3, DMT), 4.12 (1H, t, J = 6.6 Hz, *H*4'), 4.44 (2H, t, J = 6.6 Hz, CH_2O), 4.51 (1H, d, J = 6.6 Hz, *H*3'), 6.15 (1H, dd, J = 3.2, 6.6 Hz, *H*1'), 6.32 (4H, m, Ar-*H*, DMT), 6.92 (1H, s, H-6), 7.20-7.91 (9H, m, Ar-*H*, DMT) 8.05 (1H, s, *NH*-CHO), 9.50 (1H, s, *CH*O) ppm; δ_C ($CDCl_3$) 26.5 (CH_2CH_2O), 29.3 (C2'), 40.9 ($CH_2CH_2CH_2O$), 46.5 (C5'), 55.6 (2x -OCH_3), 64.5 (CH_2O), 72.8 (C3'), 73.6 (allyl-*C*, DMT), 83.5 (C1'), 86.1 (C4'), 86.9 (C-10), 113.5 (C-1), 115.5 (C-6a), (118.7, 127.3, 128.3, 128.7, 130.5, 136.1, 136.2, 145.0, 152.0, DMT), 154.0 (C-2a), 158.9 (C-6), 163.6 (*CH*O), 165.5 (C-4) ppm; *m/z* (ESI+) 637 $[M + H]^+$; Acc Mass: 637.2654; calculated for $C_{36}H_{37}N_4O_7$ requires 637.2662 (deviation -1.3 ppm).

4.4 4-[(Formyl) amino]-2-(2'-deoxy-β-D-erythro-5'-O-[4, 4'-dimethoxytrityl] pentofuranosyl)-6-oxa-7, 8, 9-trihydro-2, 3, 5-triazabenzo [cd]azulene-3'-(2-β-cyanoethyl-N, N-diisopropyl) phosphoramidite (5)

Compound **4** (110 mg, 173 µmol) was dissolved in distilled dichloromethane (2 mL, 0.1 M) under an argon atmosphere. This was followed by addition of dry diisopropylethylamine (120 µL, 694 µmol) and finally 2-cyanoethyl-*N*-diisopropylamine chloro phosphomidite (50 µL, 208 µmol) was added drowise over 15 min period. After 1 h benzyl alcohol solid support (2.5 mmol/g average) was added and the resulting suspension was stirred for 30 min. further. The reaction was then filtered to remove the solid support, the filtrate was washed with 10% Na_2CO_3 (10 ml), brine (10 mL) and dried (Na_2SO_4). The residue was evaporated and purified by silica gel column chromatography under nitrogen with 45% dichloromethane / hexane (10% Et_3N) to give phosphoramidite **16** as a white foam (75 mg, 52%); R_f (F) = 0.55; ^{31}P NMR δ ($CDCl_3$) +148.5 and +148.6 phosphoramidite diastereoisomers, ppm; *m/z*

(ESI+) 837 $[M+H]^+$; Acc Mass: 837.3737; calculated for $C_{45}H_{54}N_6O_8P$ requires 837.3741 (deviation -0.3 ppm).

5. OLIGODEOXYRIBO- NUCLEOTIDE SYNTHESIS

The oligodeoxyribonucleotides **KA 01** and **KA 02** were synthesised on an Applied Biosystems DNA automated synthesiser (Model 394) employing the regular phosphoramidites [9]. Synthesis was carried out on solid support CPG in a 1 µM scale, 0.1 M solutions of unmodified phosphoramidites and 0.15 M of the modified phosphoramidites were used for the coupling steps. Oligomers containing modified nucleotide were synthesised using mild/fast deprotection phosphoramidites for unmodified parts of the sequence. Depending on the type of modified DNA made the final dimethoxytrityl group (DMT) was removed (DMT-OFF) or left on by programming the synthesiser (DMT-ON). Removal of the oligonucleotide from the solid support was carried out with concentrated ammonia at room temperature by the Applied Biosystems 394 automated synthesiser through the machine own deprotection programme. Further incubation at 65ºC for 24 hour removed the formyl protecting group. The 5'-SIMA label and -DMT protected oligomers were purified by reverse phase high performance liquid chromatography (RP-HPLC). Detritylation was performed by treatment with 20% acetic acid for 1h. After removal of the acid the oligomer was further dissolved in water 1 mL and extracted with diethyl ether. The deprotected oligomer was then re-purified by RP-HPLC employing gradient system of TEAB (triethylammonium bicarbonate) in acetonitrile.

6. FLUORESCENCE TITRATION

Fluorescence intensity titrations were carried out using a Horiba Jobin Yvon FluoroMx-3 Fluorimeter. The machine had a thermostatically controlled chamber. The experiment was carried out at 25ºC in quartz curvet with four clear sides and a path length of 10 mm manufactured by Starna Scientific. The excitation wave length used was 530 nm and the emission was recorded at 560 nm. The excitation and emission slits were set to 5 nm and 1 accumulation scan with an integration time of 1 second was used for each reading. The ODN was labelled with 5'-SIMA which allow the ODN concentration to be used in the range of 1-5 nM. The protein aliquots were added with a glass syringe.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Pegg AE. Repair of O(6)-alkylguanine by alkyltransferases. Mutat Res. 2000;462: 83-100.
2. Margison GP, Butt A, Pearson SJ, Wharton S, Watson AJ, Marriott A, Caetano CM, Hollins JJ, Rukazenkova N, Begum G, et al. Alkyltransferase-like proteins. DNA Repair. 2007;6:1222-1228.
3. Pearson SJ, Wharton S, Watson AJ, Begum G, Butt A, Glynn N, Williams DM, Shibata T, Santibanez-Koref MF, Margison GP. A novel DNA damage recognition protein in Schizosaccharomyces pombe. Nucleic Acids Res. 2006;34:2347-2354.
4. Pearson SJ, Ferguson J, Santibanez-Koref M, Margison GP. Inhibition of O6-methylguanine-DNA methyltransferase by an alkyltransferase-like protein from *Escherichia coli*. Nucleic Acids Res. 2005;33:3837-3844.
5. Abdu K, Williams DM. Synthesis of tricyclic Nucleoside analogue of O6-methyl-2'-deoxyguanosine. Int. J. Chem. Eng. Appl. 2013;4(2):69-73.
6. Abdu K, et al. Synthesis of oligodeoxy-ribonuclueotide containing the conformationally locked anti analogue of O6-methyl-2'-deoxyguanosine and their recognition for MGMT and Atl. Chem. Comm. 2012;48(91):11214-11216.
7. Williams DM, Yakovlev DY, Brown DM. The synthesis of a tricyclic pyrrolopyrimidine related to N6-hydroxyadenine. J. Chem. Soc. Perkin Trans. 1997;1:1171-1178.
8. McBride LJ, Caruthers MH. An investigation of several deoxynucleoside phosphoramidites useful for synthesizing

deoxyoligonucleotides. Tetrahedron Letters. 1983;24:245-248.

9. Sinha ND, Biernat J, McManus J, Koster H. Polymer support oligonucleotide synthesis XVIII: use of beta-cyanoethyl-N, N-dialkylamino-/ N-morpholino phosphoramidite of deoxynucleosides for the synthesis of DNA fragments simplifying deprotection and isolation of the final product. Nucleic Acids Res. 1984;12: 4539-4557.

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