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FURTHER DEVELOPMENT OF OLIGORIBONUCLEOTIDE: BIS(TRIBUTYLTIN)OXIDE AS A REAGENT FOR REMOVAL OF THE INTERNUCLEOTIDIC PHENYLTHIO GROUP VIA THE PHOSPHOTRIESTER APPROACH

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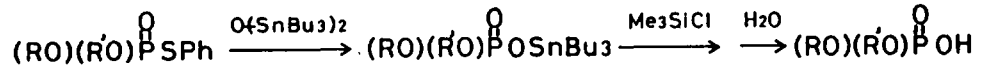
Abstract— A successful deprotection method by treatment with bis(tributyltin)oxide for the internal phosphate phenylthio group, and the synthesis of relatively long chain oligoribonucleotide GA_9 via the phosphotriester approach are described.

INTRODUCTION

In order to protect the internucleotidic phosphate group, the 2-chlorophenyl group[1] has been frequently used in oligonucleotide synthesis.[2] Reese has reported the use of tetramethylguanidium oximates for deblocking of the internal phosphate protecting group.[3-5] In his paper, 2-pyridine-syn-carboxaldoxime and 2-nitrobenzaldoxime were proved to be effective as oximes. This method has also been applied to removal of the internal phenylthio group in our oligonucleotide synthesis.[6-8] However, the oximate approach involves a risk of generation of a strong nucleophile, benzenethiolate ion, under such basic conditions. Silver ion promoted deprotection [8-12] which was a unique method to remove the phenylthio group, could be performed under neutral conditions, but required long periods of time and an additional operation of removal of silver ion. We have examined the effectiveness of several stannyl compounds as reagents for removal of the internucleotidic phenylthio group from a fully protected uridylyl(3'-5')-uridine.[13] Among them bis(tributyltin)oxide (TBT) was found to be most suitable for dephenylthiolation.



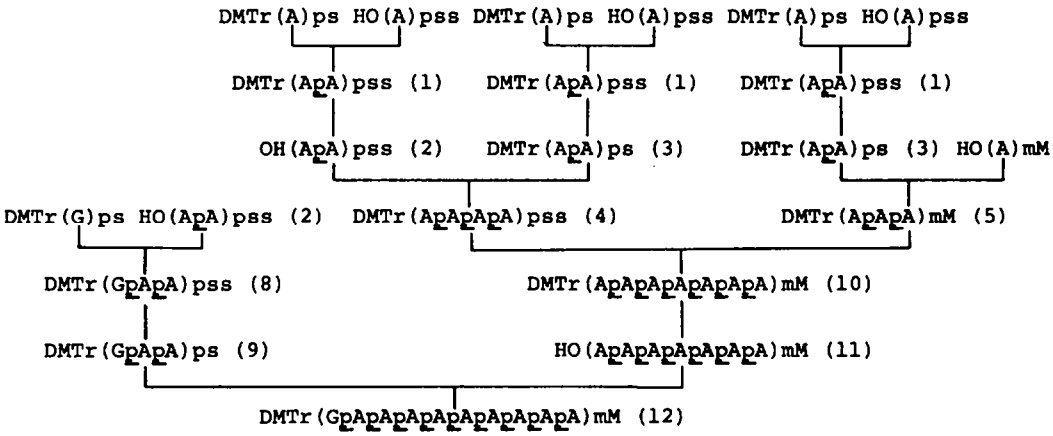
R, R' = nucleoside residues

In this paper, we wish to report the solid phase synthesis of oligoribonucleotides, GA_2, GA_3, and GA_9 on controlled pore glass (CPG) and also describe the liquid phase synthesis of GA_9 by using the TBT-mediated deprotection procedure. They were chosen as substrates to verify the so-called "C4N hypothesis",[14-17] recently proposed by Shimizu, for 1:1 complex formation of amino acids with 4 nucleotides of tRNAs. The sequence of GA_n contains GAA and A as the anticodon and discriminator, respectively, which are corresponding to L-phenylalanine tRNA.

RESULTS AND DISCUSSION

First, we synthesized a decaribonucleotide, GA_9, via the liquid phase approach. During this study, the 4,4'-dimethoxytrityl (DMTr) and tetrahydropyran-2-yl (Thp) groups were chosen as the protecting groups of the 5'- and 2'-hydroxyl groups, respectively, as reported previously.[18] N^6-Benzoyl-2',3'-O-methoxymethylideneadenosine[19] was used as the 3'-terminal unit and the fully protected decamer was synthesized from building blocks as shown in Fig 1. Mesitylenedisulfonyl chloride (MDS)[20] and 3-nitro-1,2,4-triazole (NT) were chosen as a combined coupling reagent. Detritylation and coupling reaction were performed by procedures similar to those reported previously.[7,18] The coupling conditions and yields of the fragment condensations are listed in Table 1. The conditions and yields with regard to the detritylation are summarized in Table 2.

Fig 1. Synthetic scheme for the decamer.[a]



a DMTr = 4,4'-dimethoxytrityl group, mM = methoxymethylidene group
pss = bis(phenylthio)phosphoryl group
ps = 3'-terminal triethylammonium phenylthio phosphoryl group
p = internucleotidic phenylthiophosphoryl group

Table 1. Conditions and results for the synthesis of the RNA fragments.

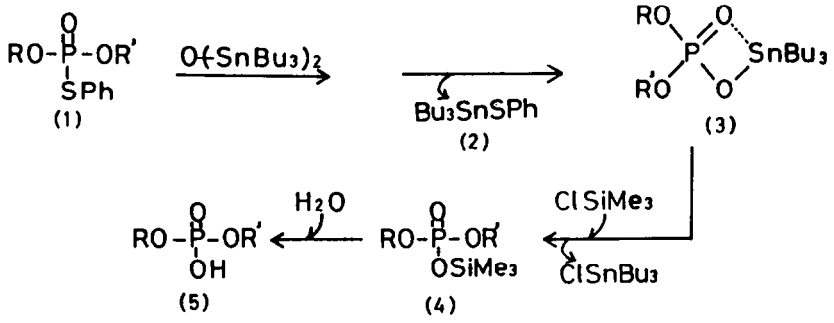
3'-phospho-diester component	(mmol)	5'-OH component	(mmol)	MDS (mmol)	NT (mmol)	time (min)	product	yield (%)
1 DMTr(A)	1.77	(A)pss	1.61	3.34	5.01	60	DMTr(AA)pss(1)	78
4 DMTr(AA)	0.37	(AA)pss	0.31	0.62	1.56	60	DMTr(AAAA)pss(4)	68
5 DMTr(AA)	0.26	(A)mM	0.24	0.48	1.20	60	DMTr(AAA)mM(5)	64
8 DMTr(G)	0.23	(AA)pss	0.15	0.30	0.75	60	DMTr(GAA)pss(8)	91
10 DMTr(AAAA)	0.21	(AAA)mM	0.15	0.33	0.50	90	DMTr(AAAAAAA)mM(10)	56
12 DMTr(GAA)	0.13	(AAAAAAA)mM	0.064	0.13	0.32	90	DMTr(GAAAAAAAAAA)mM(12)	81

Table 2. Removal of the 5'-dimethoxytrityl group with 1% TFA in CH_2Cl_2.

substrate	(mmol)	temperature	time	product	yield (%)
2 DMTr(AA)pss	0.58	0°C (5 min.)	r.t. (15 min.)	HO(AA)pss(2)	88
7 DMTr(AAA)mM	0.17	r.t.	12	HO(AAA)mM(7)	88
11 DMTr(AAAAAAA)mM	0.083	r.t.	12	HO(AAAAAAA)mM(11)	77

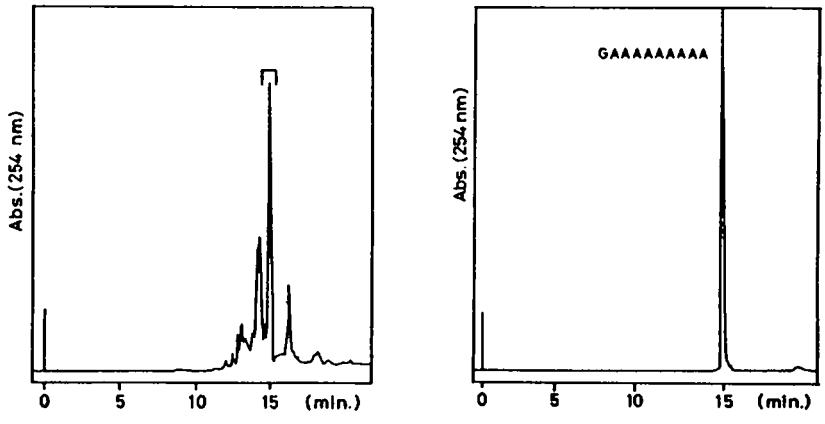
Next, removal of the internal PhS groups from the fully protected oligomer was examined in detail by using TBT. The driving force of TBT-mediated deprotection of the internucleotidic PhS group is based on the strong affinity of tin for sulfur.[21] As shown in scheme 1, the benzenethiolate ion is captured as a neutral species, i.e., tributylstannyl phenylthiolate (2). The resulting stannylphosphate (3) is easily converted to silylphosphate (4) by addition of trimethylsilyl chloride.[22] The latter is hydrolyzed to phosphodiester compound (5).

scheme 1.



Our previous paper[13] showed that the dephenylthiolation was considerably accelerated by the use of a large excess amount of TBT. Therefore, the fully protected decamer (12) was treated with 20 equivalents of TBT to one PhS group in pyridine. As a consequence, the PhS groups were found to be replaced by the Bu_3SnO groups completely within 3 h. The usual workup followed by successive treatments with conc. ammonia and with 0.01 M HCl gave a deblocked decamer GA_9, which was purified by reversed phase HPLC (Fig 2). It was also found that Sep-pak C_{18}[23] was effective for pre-purification of the partially deblocked decamers obtained prior to removal of the acid labile protecting group and the final separation using HPLC (see experimental section). The pure decamer was obtained in 3% yield. This pure decaribonucleotide was completely hydrolyzed with nuclease P_1[18] to G and pA in the correct ratio.

Fig 2. Reverse-phase HPLC profiles of (a) crude and (b) purified GAAAAAAAAA.
(a) crude GA_9 (b) purified GA_9



A column of μBondapak C_{18} and a linear gradient of acetonitrile (0—30%) in 0.1 M ammonium acetate.18)

Next, we synthesized GAn (n=2,3, and 9) on controlled pore glass (CPG)[24-26] as a solid support according to the method previously reported to test the effectiveness of the deprotection procedure by use of TBT at the solid phase system. The conditions for the manipulations are summarized in Table 3 and the coupling yields are listed in Table 4.

Table 3. Manipulation for solid phase synthesis.

step	solvent or reagent	Time
1	pyridine (washing)	1 min.
2	CH_2Cl_2 (washing)	1 min.
3	pyridine—Ac_2O—DMAP	5 min.
4	pyridine (washing)	1 min.
5	CH_2Cl_2 (washing)	1 min.
6	dry up	5 min.
7	1% TFA—CH_2Cl_2	5 sec.X3
8	CH_2Cl_2 (collecting)	1 min.X3
9	pyridine (washing)	1 min.
10	dry up	10 min.
11	coupling reaction [a]	60 min.

a Coupling reaction was performed with MSNT in CH_3CN—pyridine (4:1, v/v).

Table 4. Results for the solid phase synthesis of RNA fragments.[a,b]

							DMTr(G)ps(A)ps(A)			CPG
							82	74		(%)
						DMTr(G)ps(A)ps(A)ps(A)				CPG
						88	82	88		(%)
DMTr(G)ps(A)ps(A)ps(A)ps(A)ps(A)ps(A)ps(A)ps(A)ps(A)										CPG
99	84	75	84	86	90	94	86	90		(%)

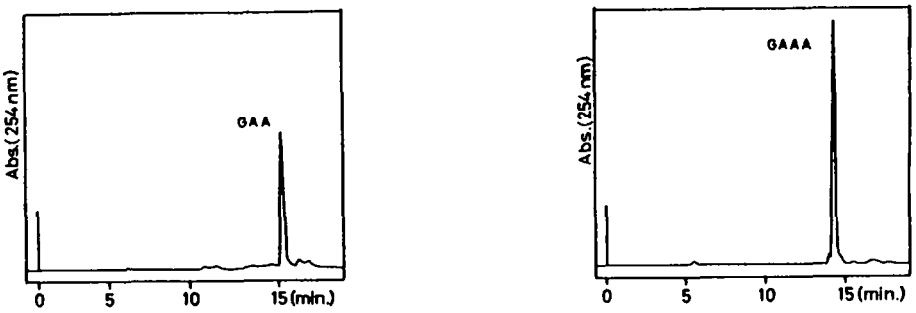
a (G) = 2'-O-(tetrahydropyran-2-yl)-N^2-propionyl-O^6-diphenylcarbamoylguanosine

(A) = 2'-O-(tetrahydropyran-2-yl)-N^6-benzoyl-adenosine

b Coupling yield was determined by the DMTr cation assay.

In the solid phase method, large excess amounts of TBT can be employed without damage of the succinate linker since the tin oxide behaves as almost neutral species in pyridine. In addition, the large excess tin reagent can be easily removed by filtration so that the whole time required for deprotection of fully protected oligomers on solid support is remakably shorten. Therefore, the resins containing oligomers were allowed to react with 50 equivalents of TBT to one PhS group for 1.5 h. Further deprotection was performed in a manner similar to that described in the liquid phase synthesis of GA_9. The pure trimer and tetramer were finally obtained in 27% and 38% yields, respectively (Fig 3). Similarly, the pure decamer was obtained in 13% by the TBT method (see experimental section). Compared with this result, the oximate method gave the decamer in 4% yield (Figs. 4 and 5). These oligoribonucleotides were completely hydrolyzed with nuclease P_1[18] to G and pA in the correct ratios. It was confirmed that the synthetic RNA oligomers were not contaminated with 2'-5' linked RNA isomers as discussed in the previous paper.[18]

Fig 3. Reverse-phase HPLC profiles of purified GA_2 and GA_3.

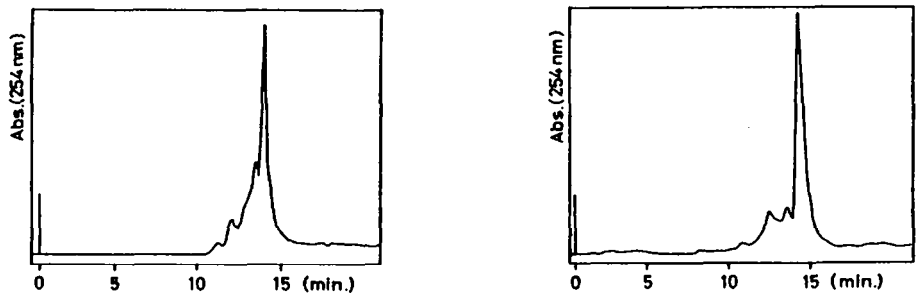


A column of μBondapak C_{18} and a linear gradient of acetonitrile (0—30%) in 0.1 M ammonium acetate.18)

Fig 4. Reverse-phase HPLC profiles of crude GA_9 which was chain elongated on CPG and obtained after the full deprotection.

a) TBT method

b) oximate method

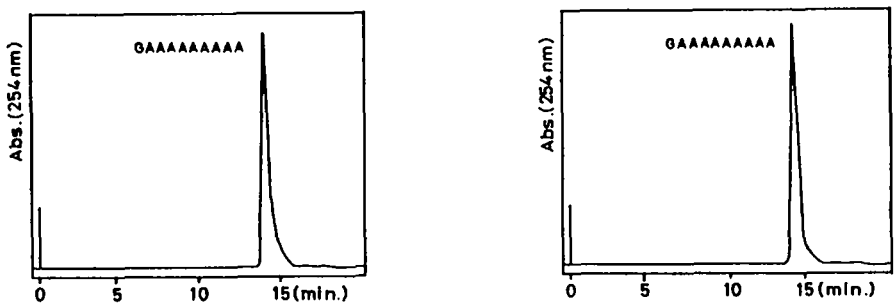


A column of μBondapak C_{18} and a linear gradient of acetonitrile (0—30%) in 0.1 M ammonium acetate.18)

Fig 5. Reverse-phase HPLC profiles of purified GA_9.

a) TBT method

b) oximate method



A column of μBondapak C_{18} and a linear gradient of acetonitrile (0—30%) in 0.1 M ammonium acetate.18)

CONCLUSION

The oximate method has been mainly employed for deprotection of the 2-chlorophenyl group in the phosphotriester approach. Since this method was usually employed under strong basic conditions using tetramethylguanidine in aqueous solution, selective removal of the internucleotidic 2-chlorophenyl

group without loss of the linker bond was not accomplished. In order to leave the succinate linker anhydrous conditions have been employed. However these conditions required relatively long periods of time (16 h).[27] The combined use of the 2-chlorophenyl group and the oximate method is time consuming at the stage of deprotection in the phosphotriester approach.

In this paper, it was shown that the time required for dephenylthiolation under almost neutral conditions was shortened and bothersome post-treatment was omitted by use of bis(tributyltin)oxide, especially in the solid phase synthesis. Recently, Efimov has reported that new catalysts such as pyridine N-oxides[28] were so effective that coupling time could be cut down in the phosphotriester method. If this approach is employed together with our TBT-mediate deprotection procedure, it will provide a rapid and practical oligonucleotide synthesis.

EXPERIMENTAL

^{1}H-NMR spectra were recorded at 100MHz on a JNM-PS-100 spectrometer. UV spectra were obtained on a Hitachi 220A spectrometer. Column chromatograhy was performed with silica gel C-200 purchased from Wako Co. Ltd., and a mini-pump for a goldfish bowl was conveniently used to attain a medium pressure for rapid chromatographic separation. HPLC was performed on a μBondapak C_{18} column using 0.1 M ammonium acetate (pH 7.0) at the flow rate of 1.5 ml/min. Pyridine was distilled twice from p-toluenesulfonyl chloride and from CaH_2 and then stored over molecular sieves 4A.

General procedure for synthesis of fully protected decaribonucleotide GA_9 in the liquid phase method

An appropriate fully protected oligoribonucleotide was dissolved in a 4 M solution of pyridinium phosphinate (30 equiv.) in pyridine. To the solution was added triethylamine (15 equiv.). The resulting mixture was warmed to 40°C and kept for 1 h. Then pyridine—water (1:1, v/v) was added and the aqueous solution was washed three times with hexane—ether (2:1,v/v) to remove thiophenol and the unreacted starting material. The remaining aqueous layer was extracted three times with $CHCl_3$, and the $CHCl_3$ extracts were combined and washed with a 0.25 M triethylammonium hydrogencarbonate (TEAB) solution. The organic phase was dried over $MgSO_4$, filtered, and evaporated under reduced pressure. The residue was mixed with a hydroxyl component and 3-nitro-1,2,4-triazole and rendered anhydrous by repeated coevapration with dry pyridine. Finally, the mixture was dissolved in dry pyridine and MDS was added. The mixture was stirred at room temperature until the reaction was completed. The reaction time is listed in Table 1. Then pyridine—water (1:1, v/v) was added and the product was extracted three times with $CHCl_3$. The combined $CHCl_3$ extracts were washed three times with a 0.25 M TEAB solution and then with water. The organic layer was dried over $MgSO_4$ and the solvent was removed by evaporation under reduced pressure. The residue was chromatographed with CH_2Cl_2—MeOH to give the coupling product. The final concentrations of MeOH in CH_2Cl_2 were 2, 3 and 4% in the case of dimer, trimer and tetramer, respectively. The heptamer and decamer were separated by preparative thin layer chromatography with CH_2Cl_2—MeOH (85:15, v/v).

Deprotection of fully protected GA_9

To a solution of the fully protected decamer (13 mg, 2 μmol) in pyridine (360 μl) was added bis(tributyltin)oxide (183 μl, 360 μmol). The solution was kept at room temperature for 3 h. Then the mixture was treated with trimethylsilyl chloride (51 μl, 400 μmol) for 10 min. Pyridine (1 ml) and concentrated ammonia (10 ml) was successively added to the solution. The resulting mixture was sealed and kept at 60°C for 5 h and then at room temperature for 12 h. The solution was evaporated under reduced pressure. During this evaporation pyridine was added three times to avoid partial loss of the Thp and DMTr groups. The residue was dissolved in sterilized water (50 ml). The aqueous layer was washed with ether (50 ml X 3) and concentrated under reduced pressure. Then the residue was passed slowly through a mini column of Sep-pak C_{18}, and the column was first washed with 5% MeOH in sterilized water (100 ml) and eluted with 50% aqueous MeOH. The eluants were combined and evaporated under reduced pressure. The residue containing oligoribonucleotides with the DMTr, Thp and mM groups were further dissolved in a 0.01 M HCl solution (10 ml). The solution was adjusted to pH 2.0 by addition of 0.1 M HCl and then kept at room temperature for 30 h. A small amount of diluted ammonia was added to neutralize the solution. The aqueous solution was evaporated under reduced pressure. The residue was passed slowly through a mini column of Sep-pak C_{18}, and the column was first washed with sterilized

water (100 ml) and eluted with 50% aqueous MeOH. The eluants were combined and evaporated under reduced pressure. Final purification was performed by reversed phase HPLC (μBondapak C_{18} column). The pure decamer GA_9 (7 OD) was obtained in 3% yield by assuming the hypochromicity of 25%.

General procedure for the loading of nucleosides on CPG-Biogel
The CPG gel (Bio 500 Amino Propyl-CPG Mean Pore Dia (A) 524, Particle size 120/200, 1.0 g) having an aminopropyl functionality of 133 μmol/g was shaken with 2'-O-(tetrahydropyran-2-yl)-5'-O-dimethoxytrityl-N^6-benzoyladenosine-3'-O-succinate (343 mg, 400 μmol), DCC(413 mg, 2.0 mmol), triethylamine (56 μl, 400 μmol) in the presence of dimethylaminopyridine (5 mg) in DMF (2 ml) for 30 h. The support was filtered and washed successively with DMF (10 ml), pyridine (10 ml), methanol (10 ml) and then ether (10 ml). The unreacted amino groups were capped by treatment with acetic anhydride—pyridine (1:9, v/v) in the presence of dimethylaminopyridine (2 mg) for 30 min followed by extensive washing of the support with pyridine, methanol and ether. The CPG gel was dried over P_4O_{10} by a vacum pump. The amount of loading of the first nucleoside on the support was measured according to the published procedure.[18]

General procedure for synthesis of the decamer in the solid phase method
The CPG gel which was loaded with the 3'-terminal adenosine unit (capacity of 50 μmol/g, 200 mg) was packed in a column of the manual synthesizer. All manipulation for one complete nucleotide chain elongation cycle are summarized in Table 3. The CPG gel was treated with TFA (1%) in CH_2Cl_2 three times for 5 sec, and it was immediately washed with CH_2Cl_2 for 1 min. These solutions were combined for determination of coupling yield. The CPG gel was dried under reduced pressure. Then a 3'-phosphodiester unit (10 equiv. 100 μmol), pre-dried by repeated coevaporation with pyridine, was treated with MSNT (200 μmol) in CH_3CN—pyridine (4:1, v/v, 500 μl). The resulting mixture was added into the column. After 1 h, the gel was washed with pyridine for 1 min and then was allowed to react with pyridine—acetic anhydride (9:1, v/v, 1 ml) in the presence of a catalytic ammount of DMAP (2 mg) for 5 min. The CPG gel was washed successively with pyridine and CH_2Cl_2 for 1 min. Then the gel was dried over P_4O_{10} in vacuo. The repetition of the above nucleotide chain elongation cycle gave the protected decamer GA_9 on CPG.

Deprotection of the fully protected decamer on the solid support
a) bis(tributyltin)oxide method
To the protected decamer on CPG (70 mg, 0.88 μmol) was added pyridine (400 μl) and bis(tributyltin)oxide (204 μl, 400 μmol). The mixture was kept at room temperature for 1.5 h. Then trimethylsilyl chloride (63 μl, 500 μmol) was added to the solution. After the solution was kept for 10 min, water was added (100 μl). The CPG gel was filtered off and washed with pyridine (20 ml). This support was poured into a mixture of pyridine (1 ml) and concentrated ammonia (10 ml). The solution was sealed and kept at 60°C for 5 h and then at room temperature for 12 h. The mixture was filtered, the eluant was evaporated under reduced pressure. During the evaporation, pyridine was added three times to avoid partial loss of the Thp and DMTr groups. The residue was dissolved in sterilized water (50 ml). The aqueous solution was washed with ether—ethyl acetate (3:2, v/v) (50 ml X 3) and concentrated under reduced pressure. The residue was passed slowly through a mini column of Sep-pak C_{18} and the column was first washed with 5% MeOH in sterilized water (100 ml) and eluted with 50% aqueous MeOH. The eluant was evaporated under reduced pressure. The residue containing the decamer protected partially with the DMTr and Thp groups were further dissolved in a 0.01 M solution of HCl in sterilized water (10 ml) and the solution was adjusted to pH 2.0 and then kept at room temperature for 30 h. A small amount of diluted ammonia was added to neutralize the solution. The aqueous solution was concentrated under the reduced pressure. The residue was passed slowly through a mini column of Sep-pak C_{18} and the mini column was first washed with sterilized water (100 ml) and eluted with 50% aqueous MeOH. The eluant was evaporated under reduced pressure. Final purification was performed by HPLC (μBondapak C_{18} column). The pure decamer GA_9 (13 OD) was obtained in 13% yield by assuming the hypochromicity of 25%.

b) oximate method
A 0.5 M solution of N^1,N^1,N^3,N^3-tetramethylguanidium 4-nitrobenzaldoximate in pyridine—water (9:1, v/v, 1.0 ml) was added to the decamer linked to the CPG (70 mg, 0.88 μmol), and the mixture was kept at room temperature for 6 h. Then the mixture was poured into pyridine (1 ml) and concentrated ammonia (10 ml). The solution was sealed and kept at 60°C for 6 h, and at room temperature for 10 h. The resulting mixture was worked up as described in the above experiment. The pure decamer GA_9 (4 OD) was obtained in 4% yield. This yield was estimated by assuming the hypochromicity of 25%.

Acknowledgments

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