DERIVATIZATION AND TEMPLATE-GUIDED LIGATION OF OLIGODEOXYRIBONUCLEOTIDES USING CYANOGEN BROMIDE AND N-SUBSTITUTED MORPHOLINES

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(Received 25 November 1996)

Abstract. Cyanogen bromide has been found to induce the template-guided condensation of oligonucleotides only in the presence of N-substituted morpholines. Based on ^{31}P, ^{1}H and ^{13}C NMR spectroscopy data, the mechanism of the phosphomonoester group activation by cyanogen bromide in N-substituted morpholine buffers is suggested. It has also been shown that BrCN can be used for the synthesis of oligonucleotide derivatives in aqueous solution.

1. Introduction

HCN and its derivatives can be considered as the real candidates for the role of precursors to biological molecules or prebiotic condensing agents. These compounds could be present in the atmosphere of the primitive earth and contribute to the chemical processes leading to the origins of life. The cyclization of 3'-UMP and 3'-AMP to the corresponding 2',3'- cyclic nucleotides (Ferris *et al.*, 1984) induced by a such HCN derivative as BrCN could be considered as one of these processes. It is the positive test of the suitability of this reagent for the template-directed oligonucleotide condensation. Really, our recent findings have demonstrated that BrCN can activate the phosphate group in a nick of double helix and assemble extended double-stranded DNAs (Sokolova *et al.*, 1988; Dolinnaya *et al.*, 1991) or circularize the oligonucleotides (Dolinnaya *et al.*, 1993) in aqueous solutions containing N-morpholinoethanesulfonate (MES)-buffer. The coupling capacity of BrCN has been also demonstrated in independent studies carried out by Kanaya and Yanagawa (1986). In this case BrCN was used to condence oligoadenylates on a poly(U) template. The reaction proceeded for 20–25 h at 25°C and only in the presence of imidazole and bivalent metal ions. These authors believe that the real condensing agents in this reaction are N,N'-iminodiimidazole and N-cyanoimidazole. Formation of these compounds as a result of BrCN and imidazole interaction and their efficiency for the formation of the phosphodiester bond were proved by Ferris *et al.* (1989). In our version, BrCN-mediated ligation proceeds much faster (1-3 min) and does not require the presence of imidazole.

The focus of our study is to elucidate the mechanism of template-guided condensation of oligonucleotides with BrCN in MES-buffer.

Origins of Life and Evolution of the Biosphere **27:** 555–566, 1997.

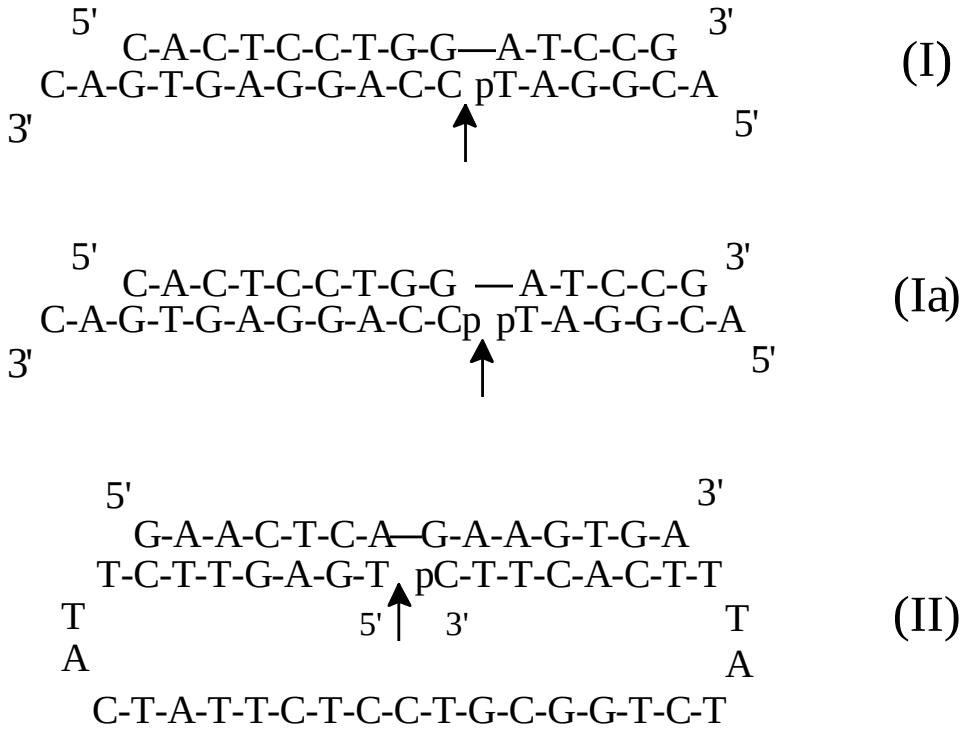

Figure 1. Sequences of DNA duplexes under study. Arrows indicate sites of new internucleotide bond formation.

2. Results and Discussion

Few experimental data on BrCN interaction with nucleosides and nucleotides are currently available. Only uridine derivatives were isolated and characterized (Ferris and Yanagawa, 1984). The vicinal 2',3'-hydroxyl groups are essential for the reaction as shown by the failure to form stable reaction products with thymidine. The phosphate derivatives of nucleotides have not been detected. However, our experiment with duplex (Ia) (Figure 1) carrying two phosphate groups at the ligation site showed that a new internucleotide (pyrophosphate) bond was formed under the action of BrCN in MES buffer containing triethylamine. It means that activated phosphate adduct occurs in fact but it is not stable in aqueous solution.

In order to understand the mechanism of the phosphate activation, the ^{31}P NMR (nuclear magnetic resonance) study of the reaction between BrCN and dpA under different conditions was carried out. As the stability of BrCN in aqueous solutions containing MES is found to be low ($T_{1/2} < 1$ min) we were forced to use an appreciable excess of this reagent. In the reaction mixture obtained by treatment of dpA in 0.25 M MES-buffer, pH 7.5, 0.02 M $MgCl_2$ with 5 eqv. of BrCN the only signal was recorded in 1–2 min at 5°C, which corresponded to the initial dpA. In absolute ethanol the signal of dpA (3.12 ppm) was transformed in 1–

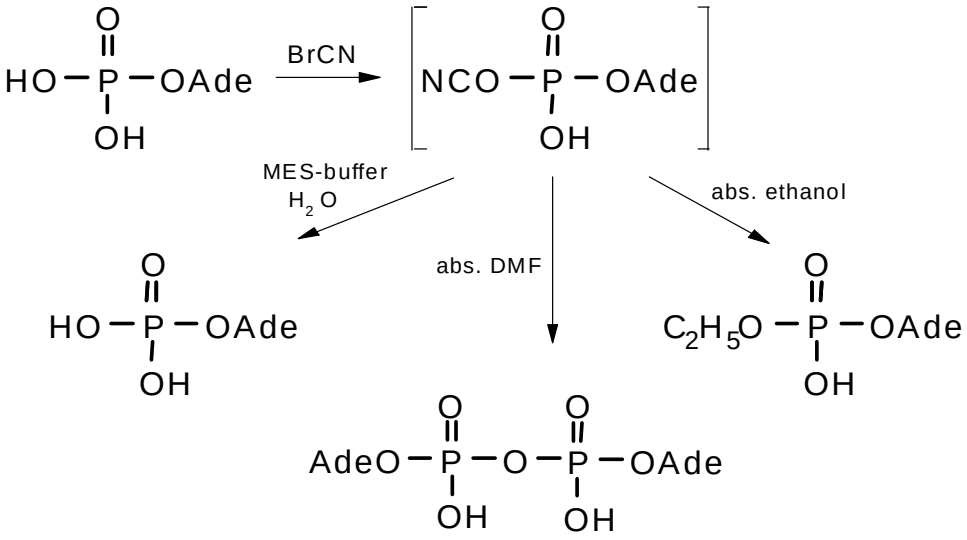

Figure 2. Scheme of dpA transformations induced by BrCN.

2 min to the signal with δ_p = 0.18 ppm corresponding to the ethyl ester of dpA. Chromatographic and electrophoretic characteristics of the dpA ethyl ester isolated from the reaction mixture were found to be the same as those of the compound obtained by the direct chemical synthesis. The treatment of dpA with BrCN in abs. DMF resulted in conversion of dpA (1.8 ppm) to compounds represented in the ^{31}P NMR spectrum by signals at –(10–12) ppm and –22 ppm. The signal at δ_p =-10.9 ppm may be assigned to adenosine pyrophosphate; two groups of signals at δ_p = –11.5 and –22 were attributed to deoxyadenosine tripolyphosphate (Zarytova, 1984). Analysis of the data obtained allowed us to suggest the following scheme of dpA transformation in the presence of BrCN (Figure 2).

In absolute DMF the unstable activated intermediate (shown in parenthesis) interacts with the strongest nucleophile present in the reaction mixture – the phosphate group of another dpA molecule resulting in nucleotide pyrophosphate or tripolyphosphate formation. This reaction is completely inhibited in absolute ethanol; in this case the main product is ethyl ester of dpA. In the aqueous medium the activated phosphate is instantaneously hydrolyzed back to dpA. However efficient internucleotide bond formation induced by BrCN in DNA-duplexes (Dolinnaya *et al.*, 1993; Kanaya and Yanagawa, 1986) demonstrates that the BrCN-activated phosphate is able to interact with close phospate or hydroxy groups.

To understand the pathways of unstable adduct formation in our reaction conditions we have studied the relationship between buffer composition and chemical ligation activity. We have decided to clear up whether BrCN can itself activate phosphate or at the first step it forms an intermediate with a buffer component. For this purpose we carried out BrCN-induced ligation in DNA duplex (II) (Fig-

Table I

Dependence of the chemical ligation efficiency in the duplex (II) on the buffer contents. Concentration of $MgCl_2$ 0.02 M, initial pH of all buffers 7.6

Buffer		Ligation efficiency, %
0.25 M MES	0.1 M triethylamine	50–60
	no triethylamine	50–60
0.25 M HEPES	0.1 M triethylamine	0
	no triethylamine	0
0.25 M N-methylmorpholine	0.1 M triethylamine	50–60
	no triethylamine	50–60
0.2 M Na-phosphate	0.1 M triethylamine	0
	no triethylamine	0
0.016 M $NaHCO_3$	0.1 M triethylamine	0
	no triethylamine	0
0.25 M N-methylimidazole	0.1 M triethylamine	8–10
	no triethylamine	8–10

Table II

Dependence of the pH value after chemical ligation on the concentration of the N-methylmorpholine (0.5 m BrCN, initial pH 7.6)

N-methylmorpholine concentration, M	0.05	0.25	0.5	1	2	3
resulting pH	3.3	2.95	2.0	4.65	5.46	7.6

ure 1) varying the buffer composition. The formation of the internucleotide bond leading to oligonucleotide circularization appears to take place only in buffers containing N-substituted morpholine, namely, MES and N-methylmorpholine, and N-methylimidazole (Table I). In phosphate and carbonate-containing buffers there is no reaction at all. The presence of triethylamine has no influence on the reaction efficiency, because it appears to be completely protonated under the reaction conditions. The efficiency of chemical ligation in buffers containing N-substituted morpholine is much higher than that in N-methylimidazole buffer. These data allow us to suppose that the joint presence of cyanogen bromide and tertiary amine is necessary for the phosphate activation.

In order to check this hypothesis we have studied the BrCN-induced reaction of guanosine 5'-phosphate with ethanol in 1M aqueous solutions of different tertiary amines: N-methylmorpholine, MES, N-methylimidazole (all with pH 7.6) and triethylamine (pH 10). The time of the of guanosine-5'-phosphate formation has been detected to be 3-5 min, with the yield of about 70%. When this reaction was carried out in the presence of BrCN and imidazole (pH 7.6) the same yield of the guanosine-5'-phosphate ethyl ester was reached in 10 hours because of

another reaction mechanism (Ferris *et al.*, 1989). When the nucleotide was treated with BrCN in the ethanol-water solution (without any amine) no phosphodiester product was formed. Thus, BrCN in appropriate conditions (example, 0.5 M BrCN in 0.02 M $MgCl_2$, containing 1 M tertiary amines, pH 7.6) can transform in 3–5 min the mononucleotides to corresponding derivatives with simple alcohols.

This reaction was used to obtain phosphodiester derivatives of oligonucleotides. The only method to synthesize these compounds in aqueous medium is based on the water-soluble carbodiimide – induced condensation of phosphorylated oligonucleotides and alcohols (Ivanovskaya *et al.*, 1987). The reaction time is about 6–10 hours. Using BrCN-induced phosphate activation we shortened the time of oligonucleotide phosphodiesters synthesis to 3–5 min. The reaction efficiency is about 60% for iso-propyl alcohol and 90–95% for primary alcohols used: methyl and ethyl alcohols and ethylene glycol (Figure 3).

These results support the idea that phosphate activation does really require the joint presence of cyanogen bromide and tertiary amine. The fact that triethylamine triggers the guanosine-5'-phosphate ethyl ester synthesis but not chemical ligation reaction may be interpreted as indicating that only free (unprotonated) amino group can participate in phosphate activation. It should be noted that the rate of mononucleotide ester formation is as high as the rate of internucleotide bond formation. It allows us to hypothesize that the phosphate activation mechanism is the same both inside and outside the DNA duplex. Among all the amines under consideration N-substituted morpholines appear to be the most appropriate for the chemical ligation in DNA duplexes.

The influence of the N-substituted morpholine concentration on the efficiency of internucleotide bond formation has been also studied. The dependence of the cyclization yield in DNA duplex (II) on the N-methylmorpholine concentration is presented in Figure 4. The only ligation product in this duplex is the 38-member cyclic oligonucleotide. As illustrated in Figure 4, when the N-methylmorpholine concentration was increased from 0.05 M to 1 M, the circular product yield also increased; further increase of the amine concentration decreased the chemical ligation efficiency. In 3 M buffer the circular oligonucleotide yield appeared to be negligible. Increasing MES concentration from 0.05 M to 1 M we observed a similar dependence as for N-methylmorpholine. Further increasing of the MES concentration was impossible because of its limited solubility in water.

To explain the results obtained we have analyzed the pH change of the N-methylmorpholine buffers in the end of chemical ligation process. The initial pH value for all buffers was 7.6. BrCN-induced chemical ligation is always followed by the acidification of the reaction mixture. This effect has been supposed to be caused by HBr evolution as a consequence of BrCN interaction with H_2O and phosphate groups. If N-methylmorpholine only fulfilled the role of buffer in chemical ligation, the smooth fall of the reaction medium acidity should be observed when increasing N-methylmorpholine concentration. As it is shown in Table II, the reaction mixture acidity is dependent on the N-methylmorpholine concentration in

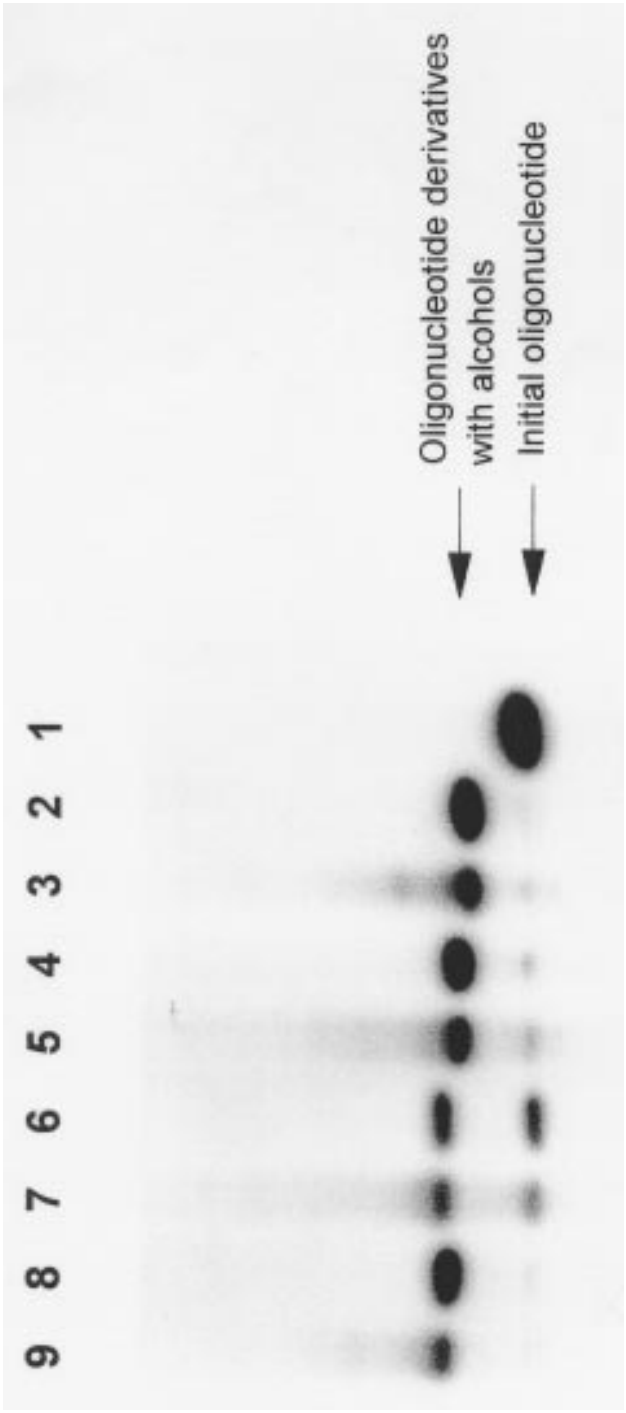

Figure 3. Autoradiograph of a 20% denaturing polyacrylamide gel representing carbodiimide- (lanes 3, 5, 7, 9) and BrCN-induced (lanes 2, 4, 6, 8) synthesis of octanucleotide (lane 1) derivatives with methanol (lanes 2, 3), ethanol (lanes 4, 5) iso-propanol (lanes 6, 7) and ethylene glycol (lanes 8, 9).

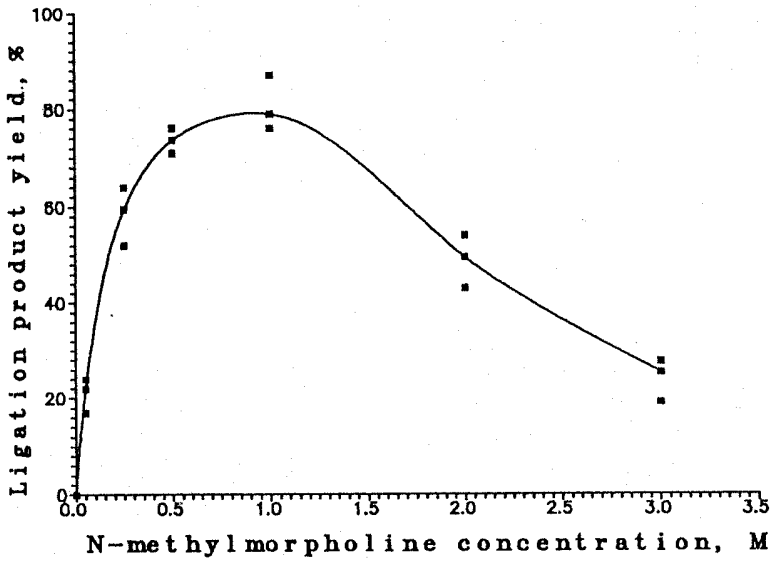

Figure 4. The influence of the concentration of N-methylmorpholine (initial pH 7.6) on the efficiency of chemical ligation in the duplex (II).

a more complicated way. In all experiments the oligonucleotide concentration was constant. Therefore, pH change in the reaction medium could be explained only by supposing that the interaction between BrCN and N-methylmorpholine leads to HBr evolution. Actually, the lowest pH value was observed when using 0.5 M buffer. In this case the relation between N-methylmorpholine and BrCN is equimolar. Consequently, on one hand, the amount of the evolved acid is maximal, and on the other hand, in the reaction mixture there is no more free N-methylmorpholine able to maintain pH. When increasing N-methylmorpholine concentration over 0.5 M, the unreacted part of the amine serves as buffer, resulting in the acidity decrease.

Thus, the increase of the ligation product yield following the increase of the N-substituted morpholine concentration could be explained by the assumption that the concentration of the phosphate activating reagent in the reaction mixture also increases. It looks as if this reagent is a product of the interaction between BrCN and N-substituted morpholine. It is important that chemical ligation efficiency decreased if the N-methylmorpholine concentration had been increased to 2–3 M with a consequent increase of the reaction medium pH. Nevertheless, it was necessary to clear up what stage of the reaction is adversely affected by the pH value increase: BrCN interaction with N-methylmorpholine, phosphomonoester group activation, or activated phosphate reaction with nucleophile.

To solve this problem we have investigated chemical ligation process in the DNA duplex (Ia) (Figure 1) in the N-methylmorpholine buffers with different initial pH values. As it was found ealier, pH change from 5 to 8 does not affect

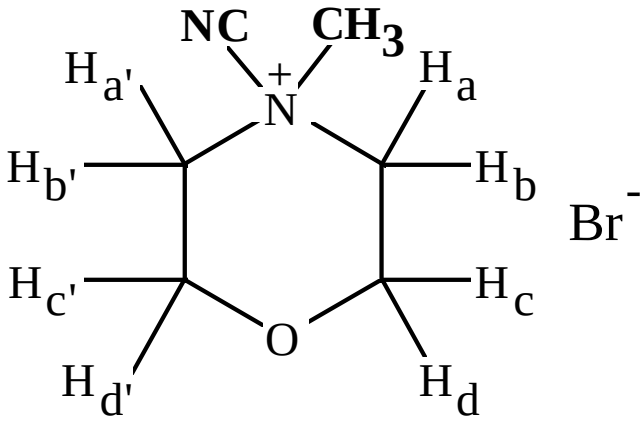

Figure 5. The product of BrCN interaction with N-methylmorpholine.

significantly the thermal stability of this duplex. Chemical ligation was shown to proceed efficiently if the initial pH value is not lower than 6 as well as if the resulting pH is not higher than 5. These data suggest that BrCN interacts only with nonprotonated N-methylmorpholine. NMR spectroscopy was employed to identify the intermediate of this reaction.

As it has been found earlier (Brawn, 1918), when N-methylmorpholine is treated by BrCN in absolute organic medium the morpholine cycle opens and picks up a CN-group to form a compound having the following structure: $BrCH_2CH_2OCH_2CH_2N(CH_3)CN$. We assumed this process in an aqueous solution to be hardly probable. The use of ^1H and ^{13}C-NMR spectroscopy allowed us to ascertain that BrCN addition to N-methylmorpholine buffer (pH 7.6) leads to an immediate formation of a new compound having a PMR spectrum which differs from the spectrum of nonprotonated N-methylmorpholine. This new compound has been found to be a product of the CN-group coupling to a tertiary N-atom of N-methylmorpholine (Figure 5).

Instead of two wide bands with chemical shifts of 2.70 ppm (4H, C$\underline{H_2}$N) and 3.87 ppm (4H, C$\underline{H_2}$O), characteristic for nonprotonated N-methylmorpholine, four two-proton bands appeared. They had the following chemical shifts: 4.00 ppm (2H), 3.68 ppm (2H) (H_c, $H_{c'}$, H_d, $H_{d'}$), 3.37 ppm (2H) and 3.08 ppm (2H) (H_a, $H_{a'}$, H_b, $H_{b'}$). CH_3N-group band displaced from 2.30 ppm to 2.80 ppm. This PMR spectrum resembles the spectrum of protonated N-methylmorpholine (therefore the morpholine cycle does not open when N-methylmorpholine interacts with BrCN), but has the more high resolution. This is due to the absence of the CH_2-protons splitting at H-atom bound to quarternary N-atom in protonated N-methylmorpholine. In the spectrum of protonated morpholine we do not observe the resolution of 4 bands just because of this splitting. Thus, the presence of four non-equivalent protons indicates the N-atom substitution, at the same time the

high spectrum resolution shows that this substitution is a result of the CN-group coupling to a tertiary N-atom of N-methylmorpholine but not its protonation.

Besides, ^{13}C-NMR spectrum of the initial N-methylmorpholine contained three bands: 45.32 ppm ($\underline{C}H_3N$), 54.69 ppm (2C, $\underline{C}H_2N$) and 66.53 ppm (2C, $\underline{C}H_2O$). These band positions were almost invariant with respect to the addition of BrCN (the resulting values: 44.32, 54.29 and 64.97 ppm), but there a new band appeared at δ 82.43 ppm, which could be assigned to CN-group. The chemical shift value of CN-group in BrCN is found to be 80.17 ppm in D_2O and 76.21 ppm in $CDCl_3$.

It is important to notice that the obtained quaternary ammonium base (Figure 5) appeared to be quite stable in aqueous medium after monitoring for two weeks. Methylphosphate addition to this compound resulted in instantaneous disappearance of the bands characteristic for this CN-containing substituted morpholine and appearance of the bands of protonated N-methylmorpholine. Moreover, we have registered the disappearance of the CN-group band in ^{13}C spectrum. It is connected with the rapid hydrolysis of the cyanic acid formed in the reaction yielding carbon dioxide and ammonia (Figure 6).

Hence according to NMR-spectroscopy data, we conclude that the phosphate group activation occurs when methylphosphate interacts with the preformed compound (A), but not with BrCN. N-Methylmorpholine acts as a CN-group transferor. This assumption is in accord with the data concerning to BrCN-induced chemical ligation occurring in the presence of other amines: N-methylimidazole and MES.

3. Materials and Methods

Oligonucleotides were synthesized on a DNA synthesizer Applied Biosystems 380B using the standard phosphoramidite procedures and purified by phase-reversed HPLC. Cyanogen bromide (5 M solution in dry acetonitrile) was from Aldrich. Colorimetric control of BrCN concentration under chemical ligation conditions was made as in (Kohn and Wilchek, 1978).

^{31}P NMR spectra were recorded on a Bruker HXS-270 spectrometer operating at 121.5 MHz equipped with a variable temperature unit. The chemical shifts are given in ppm relative to 85% phosphoric acid as an external standard.

^{1}H and ^{13}C NMR spectra were obtained with a VXR-400 spectrometer. Bands were attributed using the APT, COSY and HETCOR techniques. Spectra were obtained using the following solutions: 0.5 M N-methylmorpholine in D_2O, adjusted DCl to pH 7.5, 0.5 M BrCN in CD_3CN and 0.1 M methylphosphate in D_2O.

Reactions of dpA with BrCN was carried out according the following procedures.

In aqueous solution: to solution of dilitium salt of dpA (77 mg, 0.225 mmol) in 850 μl of 0.25 M MES-buffer, pH 7.5, 0.02 M $MgCl_2$ at 0–5°C the solution of BrCN (120 mg, 1.125 mmol) in 150 μl abs. DMF was added and ^{31}P NMR spectra were recorded.

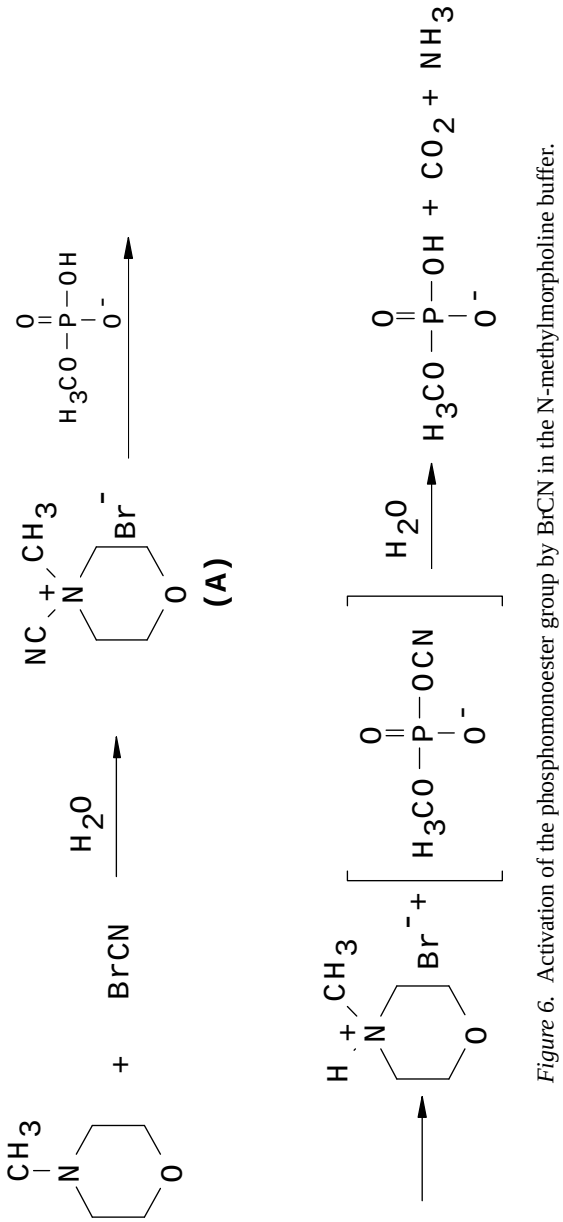

Figure 6. Activation of the phosphomonoester group by BrCN in the N-methylmorpholine buffer.

In absolute DMF: to a solution of triethyl ammonium salt of dpA (65 mg, 0.15 mmol) in 750 μl of abs. DMF at 0-5°C the solution of BrCN (80 mg, 0.75 mmol) in 100 μl of abs. DMF was added and ^{31}P NMR spectra were recorded.
In absolute ethanol: the reaction was carried out as it is described for abs. DMF. Only the absolute ethanol was used as a solvent.

To synthesize guanosine-5'-phosphate ethyl ester, 1 mg of the Na-salt of the mononucleotide was dissolved in 50 μl of the corresponding buffer. Then 50 μl of ethanol and 10 μl of BrCN solution were added. The reaction mixtures were incubated during 3–10 min at 0°C for all the amines except imidazole (incubation time for imidazole is equal to 10 h). To follow the reaction thin-layer chromatography on Cellulose F_{254} (Merck, Germany) in ethanol: 1 M ammonium acetate (7:3) was used.

To prepare the oligonucleotide derivatives with simple alcohols, an octanucleotide d(GGAAGCTTp) was dissolved in 50 μl of 1 M N-methylmorpholine buffer, pH 7.6, containing 0.02 M $MgCl_2$; oligonucleotide concentration (per monomer) was 1–5 mM. Then 50 μl of the corresponding alcohol and 10 μl of 5 M solution of BrCN in acetonitrile were added into the reaction mixture. The reaction proceeded for 3–5 min under room temperature. Then reaction products were precipitated by adding 2% $LiClO_4$ and analyzed by electrophoresis in 20% denaturing polyacrylamide gel.

Chemical ligation was performed in buffers, listed in Tables I and II. In the duplex (II) 38-member linear oligonucleotide and 14-member template were mixed in the ratio 1:2. In the duplex (I) all oligonucleotides were mixed in equimolar ratio. The mixtures were lyophilized and dissolved in 100 μl of the corresponding buffer to obtain the oligonucleotide concentration 10^{-4} M per monomer. The reaction solutions were heated to 90°C and then allowed to cool slowly to 0°C. Then 10 μl of BrCN solution was added and the reaction mixtures were incubated during 1–5 min at 0°C. DNA-fragments were precipitated by adding of 1 ml of 2% $LiClO_4$ solution in acetone.

Chemical ligation products were analyzed by electrophoresis in 20% PAGE, 7 M urea. Gels were stained by ethidium bromide or exposed to X-ray film.

Quantitative analysis of the chemical ligation efficiency was carried out by ion-pair HPLC (Waters) in acetonitrile gradient (5–40%) in 48 mM potassium phosphate buffer, pH 7, containing 2 mM tetrabutylammonium dihydrophosphate, elution rate 1 ml/min, 45°C.

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