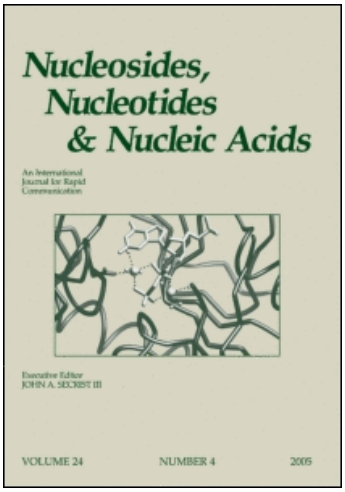

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CYANOGEN BROMIDE-INDUCED CHEMICAL LIGATION: MECHANISM AND OPTIMIZATION OF THE REACTION CONDITIONS

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Abstract: Chemical ligation induced by BrCN in the presence of N-substituted morpholine has been studied in details. It has also been shown that the phosphate activation by BrCN can be used for the synthesis of nucleotide derivatives in aqueous solutions.

INTRODUCTION

In the early 80-s chemical ligation method was originally proposed for assembling extended DNA fragments [1, 2]. Chemical ligation is based on the DNA fragment assembling on a complementary template followed by internucleotide bond formation under the action of chemical reagents. Now this method is widely adopted in nucleic acid chemistry. It allows one to prepare oligonucleotides with specific modifications of certain internucleotide bonds [3], branched DNAs [4], circular [5, 6] and chimerical [7] oligonucleotides, which are difficult to obtain by some other way. Chemical ligation is based upon two options of phosphodiester bond synthesis. The first one includes preliminary activation of a phosphomonoester group involved in the internucleotide bond formation. Reactive imidazolide [8, 9] or N-hydroxybenzotriazolide [10] oligonucleotide derivatives are usually used for this terminal phosphomonoester group activation. The second ligation option makes use of a condensing reagent [2, 11, 12]. At present time two condensing reagents - water soluble 1-ethyl-3(3'-dimethylaminopropyl)carbodiimide [2] and BrCN [11, 12], are widely used. Two variations of BrCN-induced chemical ligation have been described. The first

technique makes use of BrCN in the presence of imidazole [11], while in the second one N-morpholinoethanesulfonate (MES)-buffer containing triethylamine is used [12]. It should be emphasized that the reaction rate in the former option of ligation is comparable with the carbodiimide method while the reaction rate in the latter ligation technique is considerably higher. The chemical ligation products are usually formed in 1-3 min [12] instead of 3-20 hours [11, 13]. When BrCN was used in the presence of imidazole, the cyanoimidazole formed, as a result of their interaction, proved to be a real condensing agent [13]. With respect to BrCN-induced ligation in MES-buffer, the reaction efficiency dependence on such factors as the nature of reacting groups [3] or adjacent heterocyclic bases [14] has already been investigated, but so far the reaction mechanism remains unknown. By comparing the two techniques it becomes obvious that in MES-buffer the ligation mechanism differs from that in imidazole because of their quite different reaction rates.

The purpose of this article is to elucidate the mechanism of chemical ligation induced by BrCN in MES-buffer and to optimize the reaction conditions when applied to the synthesis of DNA fragments having an unusual structure, for instance, circular oligonucleotides.

RESULTS AND DISCUSSION

The relationship between buffer composition and chemical ligation efficiency

The phosphomonoester group activation is the first step of chemical ligation process. 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 (I) (FIG.1) by varying the buffer composition. The synthesis of the internucleotide bond leading to circular oligonucleotide formation appears to take place only in buffers containing N-substituted morpholine, namely, MES and N-methylmorpholine, and N-methylimidazole (TABLE 1). 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.

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

TABLE 1. Dependence of the chemical ligation efficiency in the duplex (I) 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

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 guanosine-5'-phosphate ethyl ester formation has been detected to be 3-5 min, the yield of this product was about 70%. When the nucleotide was treated with BrCN in the ethanol-water solution (without any amine) no phosphodiester product was formed.

It is worth noting that so far 1-ethyl-3(3'-dimethylaminopropyl)-carbodiimide was the only reagent which yielded mono- and oligonucleotide phosphodiester derivatives in aqueous media [15]. Now we can propose BrCN as another reagent for the rapid derivatization of phosphomonoester groups into phosphodiester ones.

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 synthesis. 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. Triethylamine is out of play because of its high pK_a value, and N-methyl-imidazole is not suitable because of insufficient yield of the reaction product. Therefore our further investigation was aimed to the study of the chemical ligation mechanism in buffers containing N-substituted morpholines.

The influence of buffer concentration and pH on the chemical ligation efficiency

Since N-substituted morpholine is supposed to participate in the phosphate activation, investigation of the influence of its concentration on the reaction efficiency seems to be important. For this purpose we have performed the synthesis of guanosine-5'-phosphate ethyl ester in 0.25 M and 1 M MES-buffer. The yield of disubstituted phosphate is found to be about 50-55% and 70-80% respectively.

The influence of the N-substituted morpholine concentration on the efficiency of internucleotide bond synthesis has also been studied. The dependence of the yield of the product of chemical ligation in DNA-duplex (I) on the N-methylmorpholine concentration is presented in FIG. 2. The only ligation

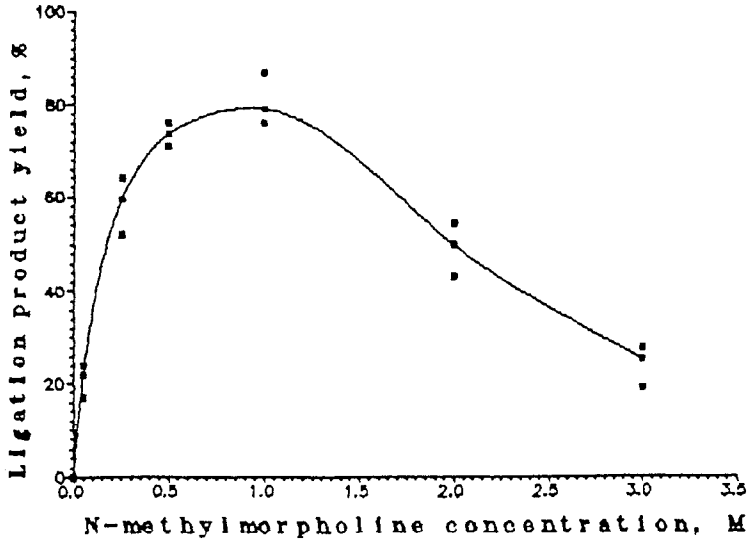


FIGURE 2. The influence of the concentration of N-methylmorpholine (initial pH 7.6) on the efficiency of chemical ligation in the duplex (I).

product in this duplex is the 38-member circular oligonucleotide. As illustrated in FIG. 2, 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 the 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 at the end of chemical ligation process. The initial pH of 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 2, the reaction mixture acidity is dependent on the N-methylmorpholine concentration in

TABLE 2. Dependence of the medium pH value after chemical ligation on the concentration of the N-methylmorpholine buffer (initial pH 7.6, BrCN concentration 0.5 M).

buffer 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

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 the pH. When increasing N-methylmorpholine concentration over 0.5 M, the unreacted amine fraction plays the role of buffer, resulting in an 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 was 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 at what stage is the reaction 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 (II) (FIG. 1) in the N-methylmorpholine buffers with different different initial pH values. As it was found earlier, pH change from 5 to 8 does not affect significantly the thermal stability of this duplex. One can see (TABLE 3) that chemical ligation proceeds 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

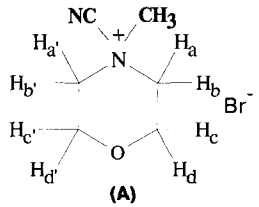
Table 3. The influence of the N-methylmorpholine buffer concentration and pH on the efficiency of chemical ligation in the duplex (II).

N-methylmorpholine concentration, M	initial pH	final pH	ligation efficiency, %
1	5.00	2.28	0
1	6.00	2.00	70-80
3	6.00	2.27	90-95
1	7.60	4.65	90-95
3	7.60	7.60	20-30

BrCN interacts only with nonprotonated N-methylmorpholine. NMR spectroscopy was used to identify the intermediate of this reaction.

Investigation of the phosphate activation mechanism using NMR spectroscopy.

As it has been found earlier [16], 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 (compound A).



(A)

Instead of two wide bands with chemical shifts of 2.70 ppm (4H, CH_2N) and 3.87 ppm (4H, CH_2O), 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 circle 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 (compound A, FIG. 3) appeared to be quite stable in aqueous medium after at least two weeks registering.

Methylphosphate addition to this compound (A) resulted in instantaneous disappearance of the bands characteristic for compound (A) and appearance of the bands of protonated N-methylmorpholine. Moreover, we have noticed the disappearance of the CN-group band in ^{13}C-spectrum. It is related to the rapid hydrolysis of the cyanic acid formed in the reaction yielding carbon dioxide and ammonia (FIG. 3).

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 the BrCN-induced chemical ligation occurring in the presence of other amines: N-methylimidazole and MES.

All the results obtained imply that the phosphomonoester group activation followed by phosphodiester bond synthesis proceeds according to the scheme presented in FIG. 3. Cyanogen bromide with a tertiary amine forms a quaternary ammonium base, which was shown to be very stable in water, but able to react rapidly with monosubstituted phosphate. The reactive intermediate formed in this reaction quickly interacts with any nucleophile: it could be a mixed anhydride with the cyanic acid.

Relying on our data, we can recommend the following optimal conditions of chemical ligation using BrCN: 1M MES or N-methylmorpholine buffer, pH

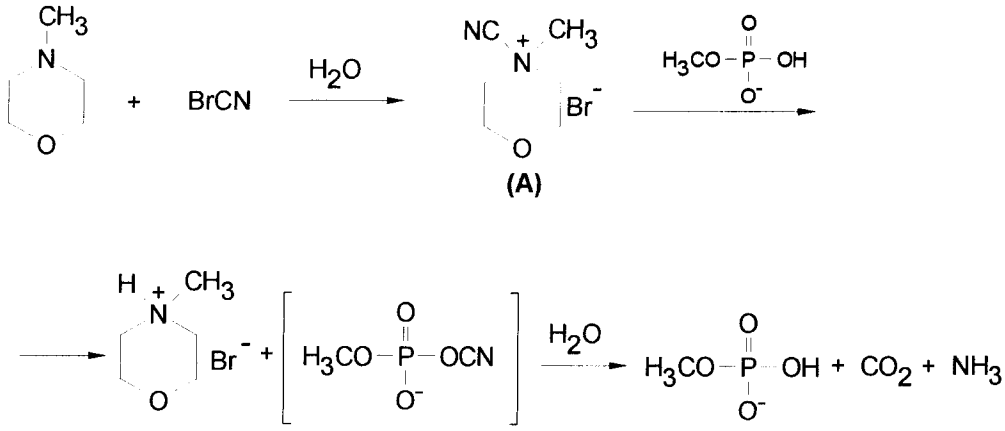


FIGURE 3. Activation of the phosphomonoester group by BrCN in the N-methylmorpholine buffer.

7.6, containing 0.02 M $MgCl_2$, 0.5 M BrCN, temperature 0°C, the reaction time 1-5 min. Pushing the ligation under these conditions, the circular oligonucleotide yield in duplex (I) was found to be 80%. This yield is increased by 20% when compared to the reaction conditions proposed earlier [6].

MATERIALS AND METHODS

5'-O-dimethoxytrityl-3-(N,N-diisopropylamido)-β-cyanoethyl phosphites of 2'-deoxyribonucleosides were from Applied Biosystems. Cyanogen bromide (5 M solution in dry acetonitrile) was from Aldrich.

Oligonucleotides were synthesized in an automated DNA synthesizer Applied Biosystems 380B. Small Scale dN CPG (Applied Biosystems) was used as a polymer support. Synthesized oligonuleotides were deprotected by routine phosphoramidite procedures and purified by phase-reversed HPLC.

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.

Chemical ligation was performed in buffers, listed in Tables 1 and 2. In the duplex (I) 38-member linear oligonucleotide and 14-member template were mixed

in the ratio 1:2. In the duplex (II) all oligonucleotides were mixed in equimolar ratio. The mixtures were lyophilized and dissolved in 100 μl of the corresponding buffer to obtain an oligonucleotide concentration of 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 100 μl of 2 M water solution of $LiClO_4$ and 1 ml of 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 1ml/min, 45°C.

To synthesize guanosine-5'-phosphate ethyl ester, 1 mg of the Na-salt of 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 3-60 min at 0°C. To follow the reaction thin-layer chromatography on Cellulose F254 (Merck, Germany) in ethanol:0.1 M ammonium acetate (7:3) was used.

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