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Solution-Phase Study of the Deprotection and Depurination Rates of Protected Nucleotides

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ABSTRACT

Over the past decade, DNA chip technology has gained increased importance, particularly in medical research. Phosphoramidite chemistry is one method that is used to synthesize oligonucleotide probes on DNA chips. While this method allows for high coupling efficiency, the growing oligonucleotide is subject to depurination during the acidic synthesis steps. We studied the effect of different acid/solvent combinations on the rates of deprotection and depurination in solution. We used the difference between the amounts of deprotection and depurination to determine the best combination. Combinations resulting in lower amounts of available hydrogen ions were found to give the best results.

DNA chip technology is widely used for the analysis of gene expression and in the investigation of disease mechanisms. Typically, fragments of different commercially available identified DNA samples are tethered to a substrate using various techniques [1-4]. Conventional phosphoramidite chemistry for oligonucleotide synthesis can be applied in DNA chip technology to synthesize these oligonucleotide probes as this method provides high coupling efficiency. First the desired nucleoside is tethered to the substrate surface as shown in Figure 1. The 5' group is protected, typically by a dimethoxytrityl (DMT) group. The bases, which are subject to hydrolysis, must also be protected during DNA synthesis. Base protecting groups remain attached to the growing oligonucleotide throughout the synthetic process and are removed at the end of the chain assembly. The most widely used base protecting groups are benzoyl for adenine and cytosine and isobutyryl for guanine. Usually, thymine is not protected because it has few

side products in the short reaction times used. The DNA oligomerization cycle is composed of deprotection, coupling, capping and oxidation steps with intervening washes and results in the addition of one nucleotide [1].

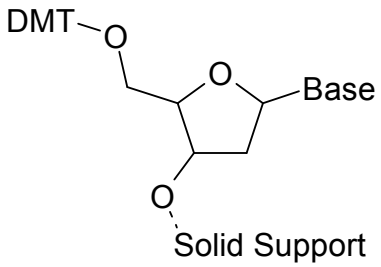

Figure 1. Typical starting point for oligonucleotide synthesis.

In the deprotection step, the 5'-protecting group (DMT in Figure 1) is removed by acid treatment. In the coupling step, tetrazole is used to activate coupling of another DMT-protected phosphoramidite to the 5'-hydroxyl groups. During the coupling step, some molecules fail to react resulting in undesired and truncated species. If these truncated sequences were allowed to react further, the result would be near full-length oligonucleotides with internal deletions. This problem is resolved by capping the free 5'-hydroxyl groups through acetylation. In the oxidation step, iodine is used to oxidize the trivalent phosphate to pentavalent phosphate. This cycle is repeated until the desired oligodeoxyribonucleic acid (oDNA) has been synthesized.

Maintaining the stability of the growing deoxynucleotide throughout the synthesis process remains an inherent problem. The N-glycosidic bond between a purine base and its deoxyribose group in DNA is very susceptible to hydrolysis [1,5]. During the deprotection step for each cycle, the growing oligonucleotide is subjected to acid conditions. The acid conditions are

necessary for deprotection, however a small number of purine rings also may be lost. This depurination is a harmful side reaction in oDNA synthesis since these bases are one of the key components in hybridization. In order to overcome this problem, efforts have been made to facilitate the removal of the DMT group by alternative reagents (acids in different solvents) and to explore new base protecting groups and the protecting groups for the 5'-OH function [6-8]. Despite recent advances in the oDNA synthesis, depurination remains a major cause of concern [1].

The concentration and dissociation constant of the acid are two key parameters affecting the rates of deprotection and depurination. High acid concentration increases both the rate of deprotection and the rate of depurination. Acids having large dissociation constants also increase the rate of these reactions. In addition, the solvent can affect the acidity of an acid. Polar solvents increase the acidity of an acid and hence the rate of deprotection and depurination. We studied the effect of various acidic reagents on the rates of deprotection and depurination of benzoyl protected deoxyadenosine in solution.

Materials and Methods

Adenine, N^6-benzoyl-5'-*O*-(4,4'-dimethoxytrityl)-2'-deoxyadenosine (Bz-DMT-dA), N^6-benzoyl-2'- deoxyadenosine (Bz-dA), benzoyl chloride, dimethoxytrityl chloride (DMTCl), trichloroacetic acid (TCA), dichloroacetic acid (DCA), chloroacetic acid, (MCA), methanesulfonic acid (MSA), methanol, methyl isobutyrate (MIB), 1-methyl-2-pyrrolidinone (NMP), tetrahydrofuran, and veratrole, were purchased from Aldrich. Acetic acid, dichloromethane (DCM) and methanol were purchased from JT Baker. Acetonitrile and

triethylamine (TEA) were purchased from EM Science. N^6-benzoyladenine (BzA) was prepared by the reaction of adenine with benzoyl chloride using NMP as the solvent and TEA as an acid scavenger followed by crystallization from methanol/water. Dimethoxytrityl hydroxide (DMTOH) was prepared as described in the literature.[9] Triethylammonium acetate (TEAOAc) buffer solution was prepared by the reaction of 7.00 g TEA with 4.00 mL acetic acid in 20 mL of methanol. Acetic acid or TEA were added to adjust the pH of the solution to 7 and methanol was added to adjust the final volume of the solution to 30 mL and a concentration of 1.2 M.

This study of the rate of deprotection and the rate of depurination used Bz-DMT-dA as the starting material. Upon treatment with acid, Bz-DMT-dA deprotects at the 5'-end forming Bz-dA, DMTOH, and other DMT containing species (DMT*) and also depurinates to form BzA and dideoxyribose (d) as shown in Figure 2.

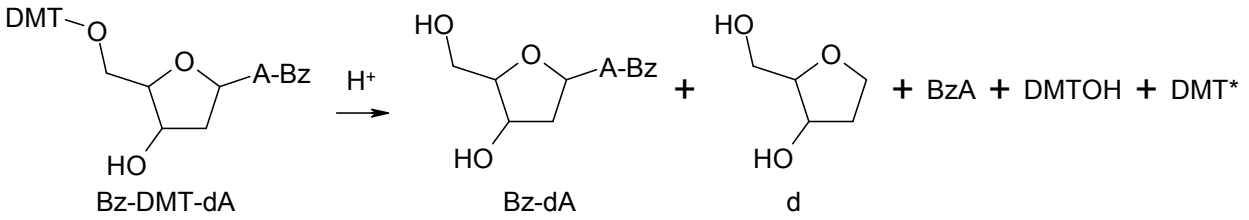

Figure 2. Reactant and products of deprotection and depurination under the experimental conditions of this study.

In order to determine the rates of deprotection and depurination, the concentration of each species was determined using an Agilent 1100 series High Pressure Liquid Chromatograph (HPLC) equipped with a C18 column and diode array detection. The temperature of the column was maintained at 25°C. The separation conditions used were 0.5 ml/min flow rate with 10-100% (over 14 min) acetonitrile gradient in 7.5 mM phosphate buffer, pH 3. Separation was

followed by a 100% acetonitrile washout for 3 minutes, gradient back to 10% acetonitrile in 3 minutes and remained at 10% for 5 minutes. The injection volume was 6 μL.

Solutions with known concentrations of Bz-DMT-dA, Bz-dA, BzA DMTOH and DMTCl were prepared separately and analyzed by HPLC to determine the response factor for each component. The HPLC detection limit for BzA was determined to be 0.000111μM BzA in the reaction mixture.

Table 1 shows the experimental matrix that was completed in this study. The starting point for each experiment was a solution containing Bz-DMT-dA, which was prepared separately in 3 ml of the solvent indicated in Table 1, with a constant amount of veratrole added as an internal standard. A solution of 1.2 M triethylammonium acetate (TEAOAc) in methanol was used to quench the reaction at the desired time.

Table 1. Experimental matrix of acids, concentrations, and solvents used for each experiment.

Acid [pK_a]	**Concentrations (M)**	**Solvent**
Chloroacetic Acid (MCA) [2.87]	0.2, 0.3, 1, 2, 3	Dichloromethane (DCM)
Dichloroacetic Acid (DCA) [1.25]	0.05, 0.1, 0.2	DCM
	0.1, 0.2	Methyl isobutyrate (MIB)
Trichloroacetic Acid (TCA) [0.77]	0.05, 0.1, 0.2	DCM
	0.1, 0.2	MIB
Methane Sulfonic Acid (MSA) [~-2]	0.02, 0.2	DCM
	0.01, 0.02, 0.1, 0.2	MIB

A 0.1 ml aliquot of the original Bz-DMT-dA solution was removed and analyzed before adding acid. This solution was analyzed as the zero reading, since it contained no acid. The remaining 2.9 ml of the reaction mixture was added to 2.9 ml of acid solution, thus resulting in the acid concentration shown in Table 1. Then 0.1 ml aliquots of the mixed solution were removed to a 1 ml vial and quenched with 0.1 ml of TEAOAc buffer at set intervals of 10 seconds, 30 seconds, 1 minute, 3 minutes, 5 minutes, 10 minute and 20 minutes and analyzed by HPLC.

Once the concentration of each species in the samples were determined, the deprotection and depurination percentages were calculated by summing the fractions of DMT containing products and the fraction of BzA, respectively.

$$\% \text{ Deprotection} = \frac{\text{Concentration of DMT products}}{\text{Total concentration of DMT}} \times 100 \qquad \text{Equation 1}$$

$$\% \text{ Depurination} = \frac{\text{Concentration of BzA}}{\text{Total concentration of adenine(A) products}} \times 100 \qquad \text{Equation 2}$$

The concentration of DMT products is the sum of the concentration of DMTOH and the concentration of DMT*. The total concentration of DMT was calculated by adding the concentrations of all the DMT containing species (Bz-DMT-dA, DMTOH, DMT*) at a particular time. Similarly, the total concentration of adenine containing products was calculated by adding the concentration of all the adenine containing species (Bz-DMT-dA, Bz-dA, BzA) at a particular time. For the purposes of this study, complete conversion was defined as 99% deprotection. Repeatability and reproducibility experiments showed the maximum experimental error was 3.2 percent for all acids studied.

Effect of Detritylating Conditions

The concentration profiles of Bz-DMT-dA, DMTOH, DMT*, Bz-dA and BzA with respect to time were generated from the HPLC data. It was observed that the concentration profiles of all the involved compounds followed the same trend under different reaction conditions. For example, as shown in Figure 3 for the case of the reaction with 0.1M TCA in MIB, as soon as the acid is added, deprotection begins, the concentration of Bz-DMT-dA decreases with time whereas the concentration of Bz-dA increases. Simultaneously, depurination begins so the concentration of Bz-dA eventually decreases and the concentration of BzA increases. The concentrations of DMTOH and DMT* increase with time and essentially reach a constant value.

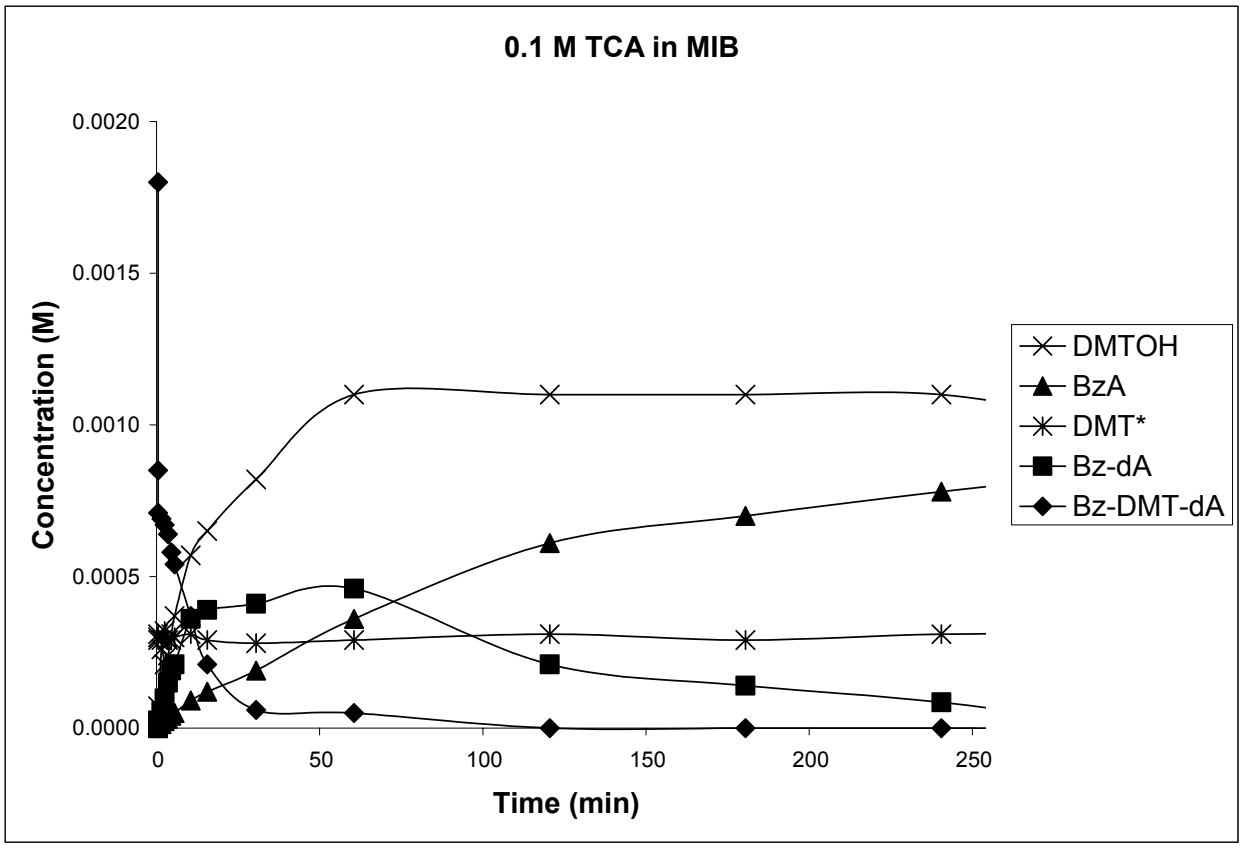

Figure 3. Typical concentration profile during reaction.

Solvent Effects. The rate of deprotection was found to be greater in DCM than in MIB for all acids studied. For example, as shown in Figure 4, with 0.1M DCA in DCM, the reaction achieves complete conversion within 30 seconds, while with the same acid in the less polar solvent, MIB, only 3.9 percent deprotection is observed in 30 seconds. A similar solvent effect on deprotection is observed for TCA. The solvent effect on depurination can be easily seen with the strong acid, MSA. However, in high concentration (0.2M MSA), as shown in Figure 5, the rate of depurination is so fast that the effect of the solvent is not observable. However, in very dilute conditions (0.02M methanesulfonic acid), the rate of depurination is still faster in DCM than in MIB.

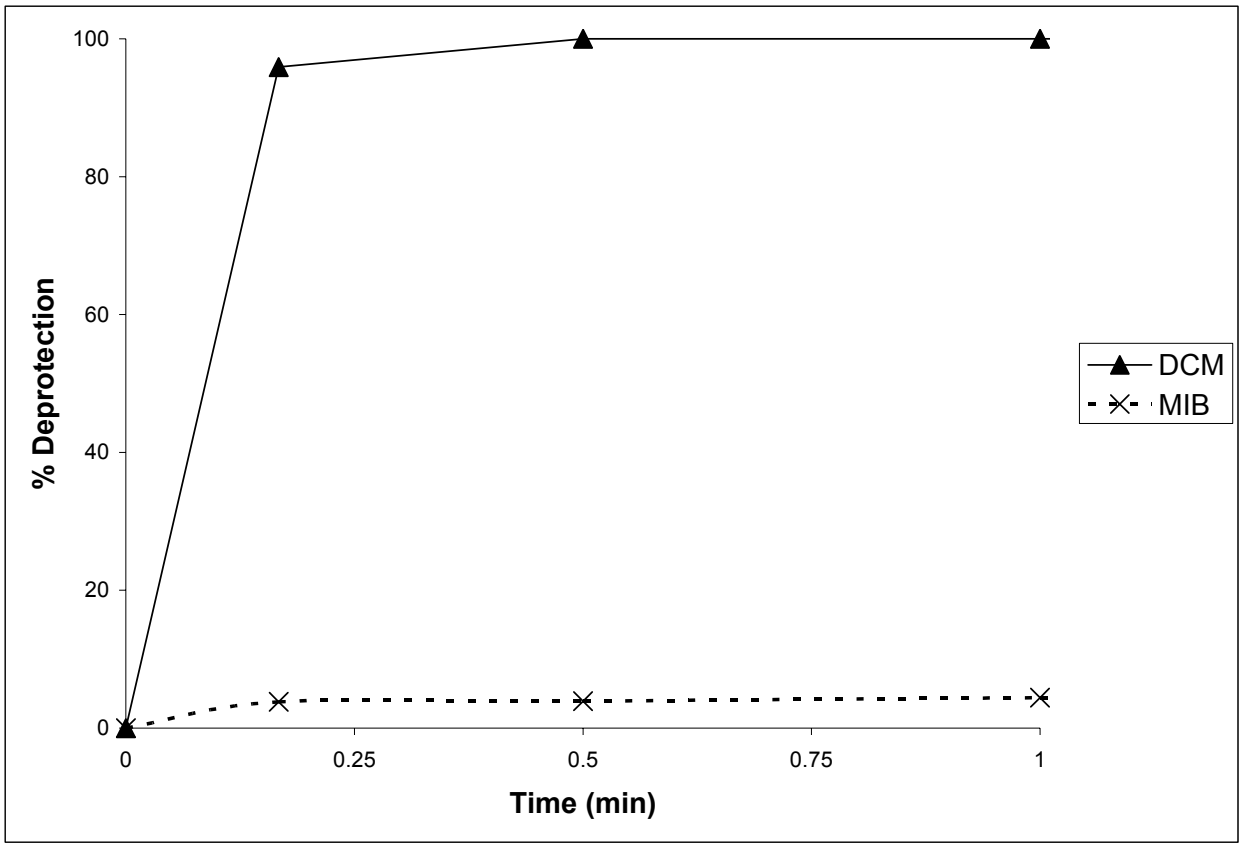

Figure 4. Effect of solvent on deprotection using 0.1 M DCA.

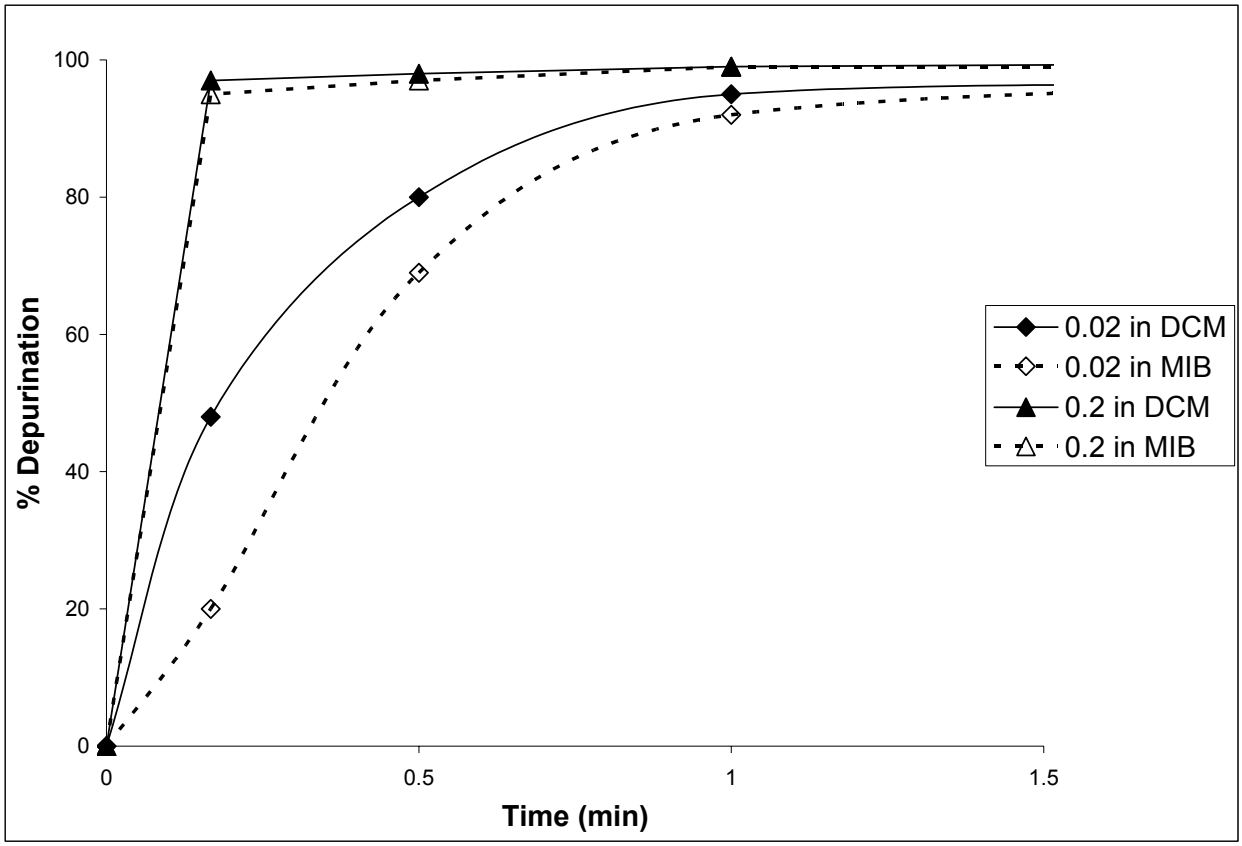

Figure 5. Effect of solvent on depurination using MSA.

Thus, the solvent can affect the deprotection and depurination rates. The dielectric constants for DCM and MIB are 9.08 and 5.6, respectively. The larger polarity of DCM increases the acidity of the acid by dissociating and stabilizing the ions. When the acid dissociates, the resulting hydrogen ion is available for reaction and increases the rates of deprotection and depurination. As less energy is required to dissociate the acid into ions in the more polar solvent, the extent of dissociation of acid is larger in DCM. Therefore, the rates of both reactions are faster in DCM than in MIB.

Since the rates of deprotection with DCA and TCA in MIB (weaker acids in the less polar solvent) are very slow and thus undesirable for industrial synthesis, these reaction conditions were not studied further. Similarly, for the cases of 0.1M and 0.2M MSA in DCM (strong acid in more polar solvent), although the deprotection rates are very fast, the depurination rates (the undesirable reaction) are also fast, limiting the yield of the desired product. Therefore, 0.1M and 0.2M MSA in DCM were not studied further.

Acid Concentration Effects. As the acid concentration was increased, the extent of deprotection at a given time increased. For example, as shown in Figures 6, with 0.1M DCA in MIB, 6.5 percent deprotection occurs in 5 minutes and with 0.2M DCA in MIB, deprotection increases to 14 percent. Similar effects of acid concentration are observed with TCA and MSA.

Similarly, the rate of depurination increases as the acid concentration increased. As shown in Figure 7, the percent depurination after 20 minutes for 0.1M DCA in MIB and for 0.2M DCA in MIB are 2.7 percent and 8.9 percent, respectively.

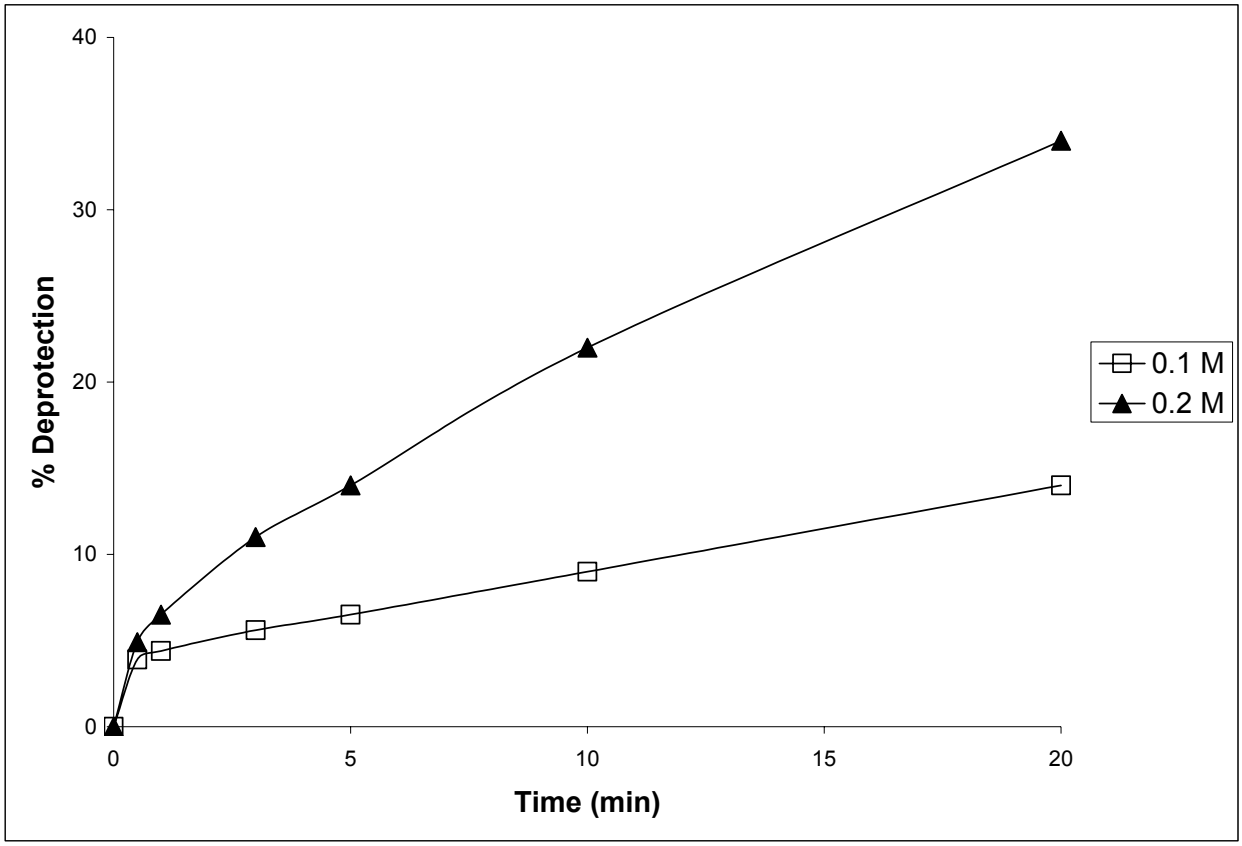

Figure 6. Effect of acid concentration on the percent deprotection using DCA in MIB

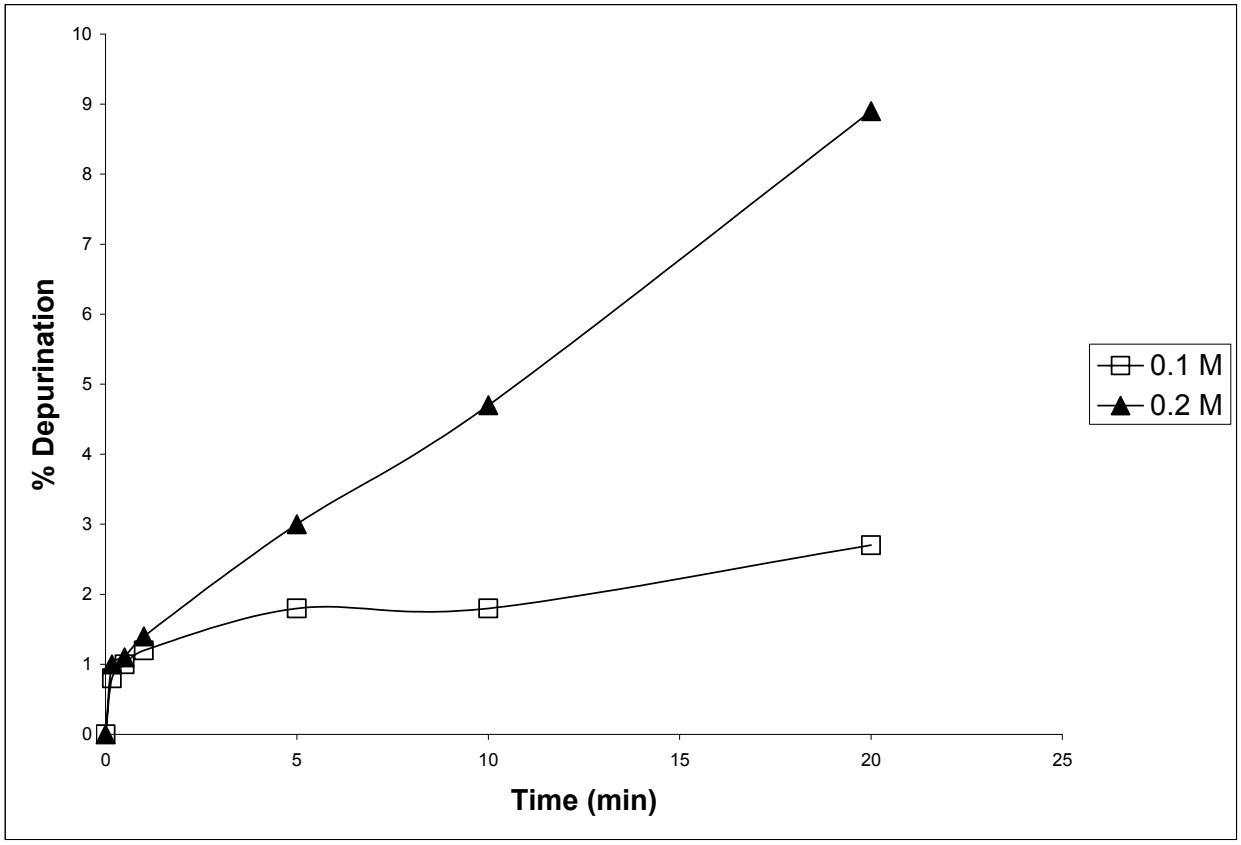

Figure 7. Effect of concentration on the percent depurination using DCA in MIB.

Acid Strength Effects. Weaker acids with higher pK_a values resulted in lower rates of deprotection in comparison to the stronger acids with lower pK_a values. For example, as shown in Figure 8, the reaction with 0.1M TCA in MIB achieves 44 percent conversion within 1 minute, however the reaction with 0.1M DCA in MIB achieves only 4.4 percent deprotection in the same time interval. Similarly, the rate of depurination also decreases as the pK_a of an acid is increased. As shown in Figure 9, the reaction with 0.1M TCA in MIB achieves 25 percent depurination in 20 minutes, however the reaction with 0.1M DCA in MIB achieves only 2.7 percent depurination. Acids with higher acidity (lower pK_a) dissociate easier, resulting in a higher concentration of H^+ than those with lower acidity (higher pK_a). Hence, as acidity

increases, the rates of deprotection and depurination increase. Among the acids used, both the rates of deprotection and depurination increase in the order of MCA<DCA< TCA < MSA.

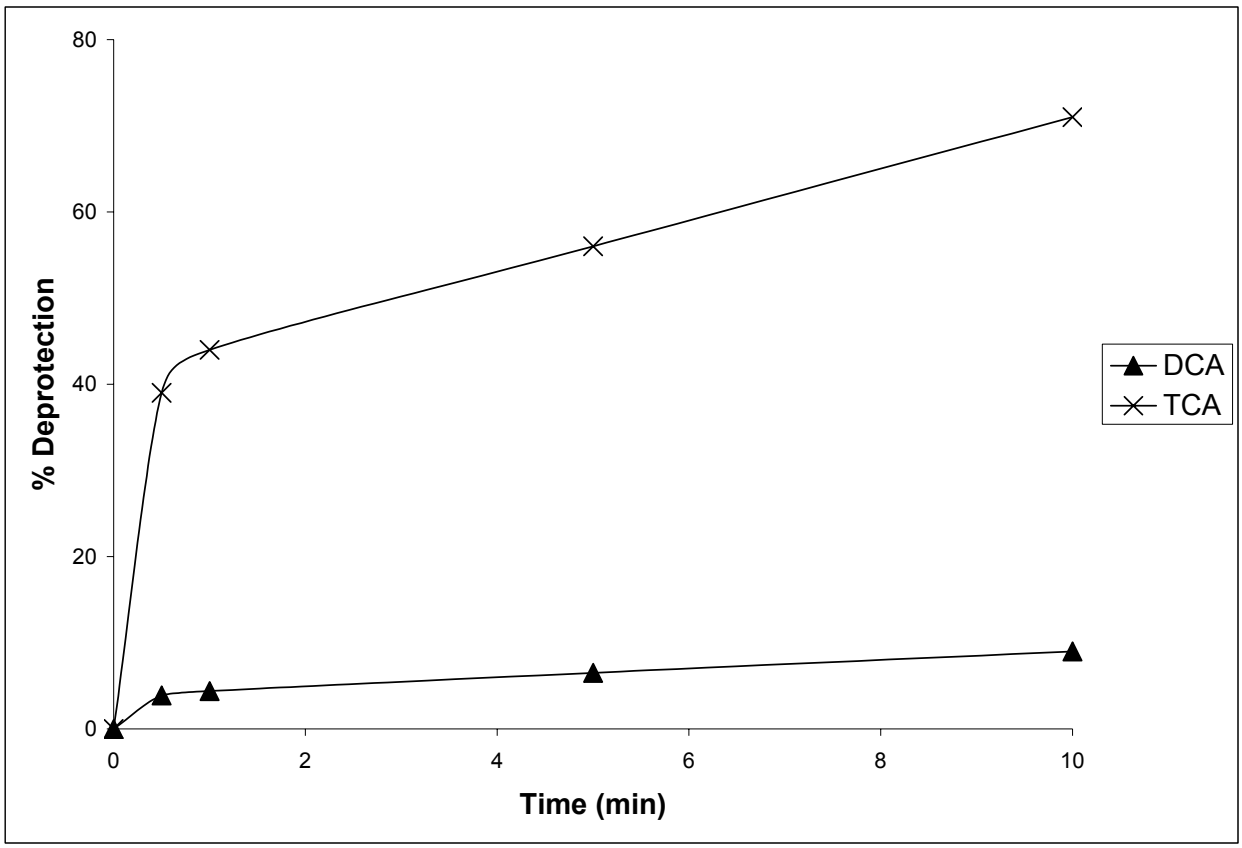

Figure 8. Effect of pK_a on the percent deprotection in MIB

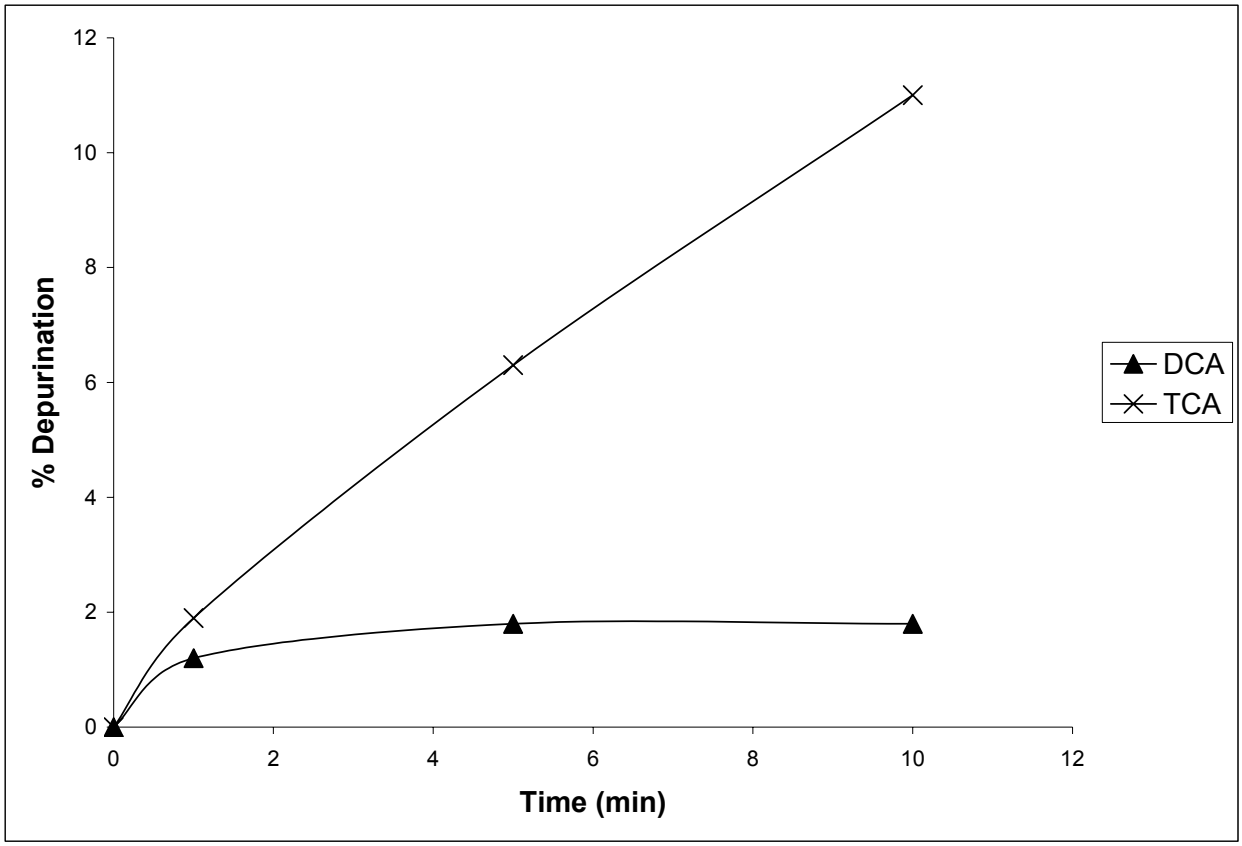

Figure 9. Effect of pK_a on percent depurination.

Yield

Since the depurination and deprotection rates are fast for all acid conditions, it is important to determine the yield of the desired product. The yield was calculated as the difference between the amount of deprotection and the amount of depurination. As shown in Figure 10, the yield increases with time, reaches a maximum and then decreases with time depending upon the concentration and acidity of the acid. Stronger acids or higher concentration of the same acid, cause the yield to decrease immediately after reaching a maximum, but with weaker acids or with low concentration, yield decreases very slowly. This is because as the acid concentration or the acidity (due to the solvent) increases, both the rate of deprotection and the rate of

depurination become very fast and that results in a lower yield in comparison to that for weak acids or for acids with low concentration.

The reaction with MCA in DCM (weak acid in a more polar solvent) was also studied. In the case of 0.3M MCA, although the yield increased and reached near completion, the reaction took much longer to achieve complete conversion. Therefore, MCA in DCM was not studied further.

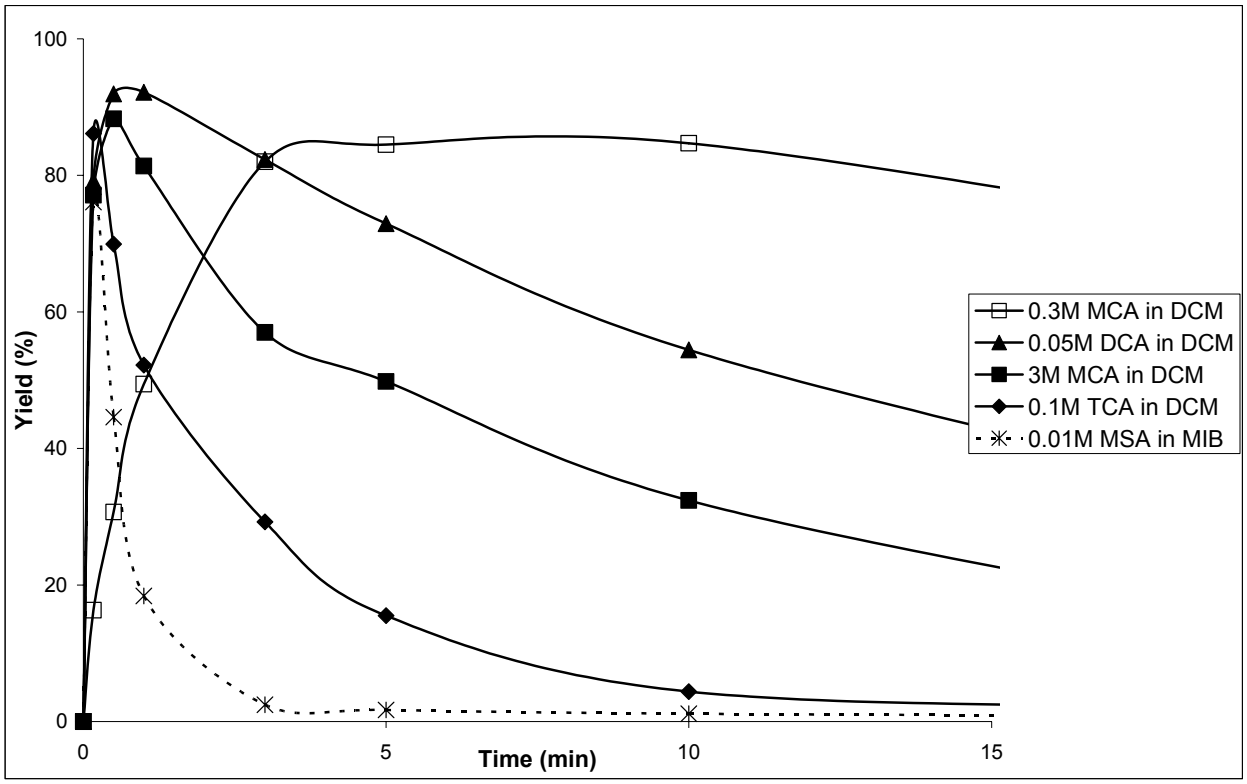

Figure 10. Yield comparisons.

Conclusions

The results of this study show that the rate of deprotection and the rate of depurination increased as the acid strength and/or dissociation constant increased. In addition, both reaction rates

increased as the solvent polarity increased. Among the acids investigated, the rate of deprotection was lowest with MCA and highest with MSA.

Although efficient detritylation is an advantage, it is not sufficient. The undesired depurination reaction also increased, reducing the overall yield. Therefore, the overall yield was determined for all reaction conditions to determine the best conditions, maximum deprotection with minimum depurination. It was found that with concentrated or stronger acids, depurination started immediately; hence, the yield started decreasing immediately after reaching a maximum. However, with weaker acids such as MCA, the yield increased slowly, remained at the maximum for some time and then started decreasing slowly. The results showed that DCM was the most suitable solvent for MCA, DCA and TCA. However, MIB was more suitable than DCM for MSA because the depurination rate was so fast in DCM; resulting in a very low yield.

These results help us understand and attain maximum deprotection with minimum depurination in oligonucleotide synthesis. However, this study was based on the reaction mechanism with a mononucleoside. In order to understand the implication of detritylating conditions for oligonucleotide synthesis, further studies are required for fully protected CPG-immobilized mononucleotides and their dimers, trimers and oligomers.

Acknowledgment

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