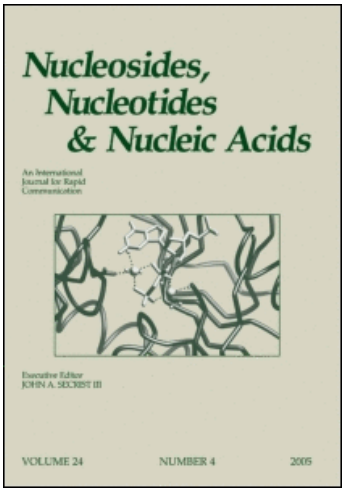

Nucleosides, Nucleotides and Nucleic Acids
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597286

Studies on Nucleotide Chemistry 15. Synthesis of Oligodeoxynucleotides Using Amidine Protected Nucleosides

Marvin H. Caruthers[a]; Lincoln J. Mcbride[a]; Laurent P. Bracco[a]; John W. Dubendorff[a]
[a] Department of Chemistry, University of Colorado, Boulder, Colorado, USA

To cite this Article Caruthers, Marvin H. , Mcbride, Lincoln J. , Bracco, Laurent P. and Dubendorff, John W.(1985) 'Studies on Nucleotide Chemistry 15. Synthesis of Oligodeoxynucleotides Using Amidine Protected Nucleosides', Nucleosides, Nucleotides and Nucleic Acids, 4: 1, 95 — 105
To link to this Article: DOI: 10.1080/07328318508077833
URL: http://dx.doi.org/10.1080/07328318508077833

NUCLEOSIDES & NUCLEOTIDES, 4(1&2), 95-105 (1985)

STUDIES ON NUCLEOTIDE CHEMISTRY 15. SYNTHESIS OF OLIGODEOXYNUCLEOTIDES USING AMIDINE PROTECTED NUCLEOSIDES

Marvin H. Caruthers*, Lincoln J. McBride, Laurent P. Bracco and John W. Dubendorff
Department of Chemistry, University of Colorado, Boulder, Colorado, USA

Summary

An *in situ* method is described for synthesizing DNA which incorporates a new series of amidine protected deoxynucleosides and bis-dialkylaminophosphines as phosphitylating agents. These procedures were used to synthesize d(GGGAATTCCC) which was digested by *Eco*RI.

For some time now, our research on nucleic acid chemistry has focused on developing a DNA synthesis methodology that is accessible to the non-chemist. A major advance toward this goal was achieved when appropriately protected deoxynucleoside phosphoramidites[1,2] were shown to be quite attractive as synthons for DNA synthesis when used with silica gel polymer supports.[3-8] Recently two major innovations have simplified this procedure further. One of these has been the development of an *in situ* approach for preparing deoxynucleoside phosphoramidites.[9,10] Another improvement has been the development of amidine protecting groups for adenine[11] and more recently for guanine, and cytosine as reported in this manuscript. We also outline an *in situ* method for synthesizing DNA using these amidine protected deoxynucleosides.

SYNTHESIS OF DEOXYNUCLEOSIDE AMIDINES

The synthesis of deoxyadenosine, deoxycytidine, and deoxyguanosine amidine derivatives proceeds generally from

 0732-8311/85/0401-0095$3.50/0

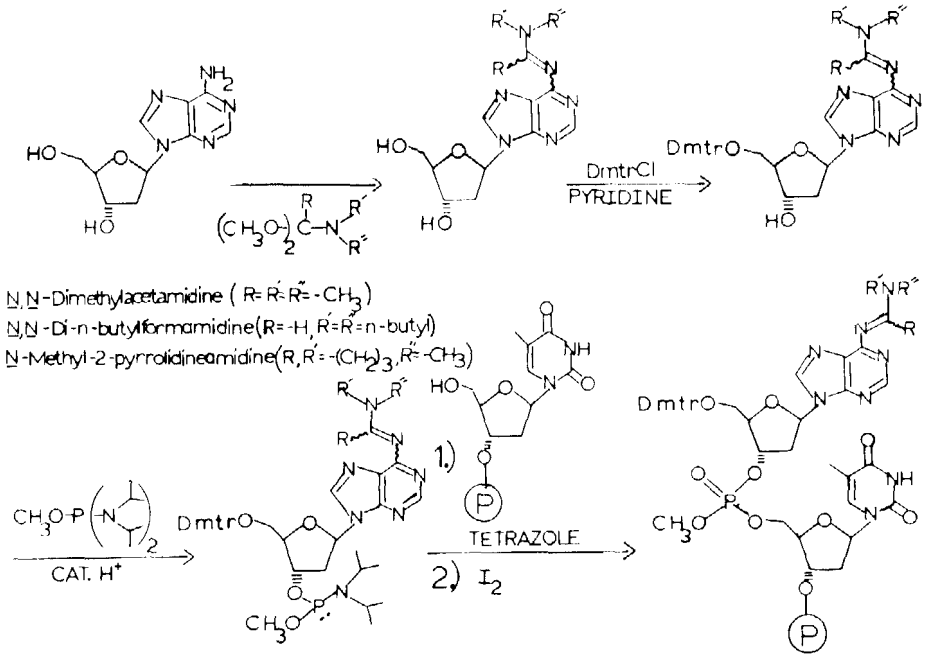

FIG. 1. Preparation of N^6 amidines of deoxyadenosine and their use *in situ* for DNA synthesis on polymer supports. The top part illustrates schematically the synthesis of amidines while the bottom part outlines the *in situ* synthesis of DNA. (P), silica gel polymer support.

commercially available N,N-dimethylacetamide dimethylacetal or N,N-dimethylformamide dimethylacetal. Thus N^6(*N,N*-dimethylacetamidine)deoxyadenosine, dA(aca), was prepared from deoxyadenosine and N,N-dimethylacetamide dimethylacetal via a one step procedure (see FIG. 1). Deoxyadenosine was first co-evaporated from pyridine to remove water and then 3 equivalents of *N,N*-dimethylacetamide dimethylacetal in dry methanol was added. After a three day reaction at room temperature, the amidine derivative can be converted directly without isolation to 5'-dimethoxytrityl-N^6(*N,N*-dimethylacetamidine)deoxyadenosine. This reaction proceeds by neutralizing excess amide acetal with water, co-evaporating with pyridine to render the product anhydrous, and then adding 1.2 equivalents of dimethoxytrityl chloride. This general procedure as developed initially for the synthesis

of N^6-(*N*-methyl-2-pyrrolidine amidine)deoxyadenosine[11] is quite attractive as a one flask method for protecting deoxynucleosides and has been used for the amidines listed in TABLE 2. The synthesis of N^2(*N,N*-dimethylacetamidine)deoxyguanosine, dG(aca), was completed by first converting *N,N*-dimethylacetamide dimethylacetal to *N,N*-dimethylacetamide diethylacetal. This was accomplished by repeatedly adding absolute ethanol to the dimethylacetal and distilling at atmospheric pressure to remove alcohols. If *N,N*-dimethylacetamide dimethylacetal was used, then methylation of guanosine at N^1 was observed (see FIG. 2). For the preparation of *N*-methyl-2-pyrrolidine amidine (pya) and *N,N*-di-*n*-butylformamidine (dnb) derivatives of deoxyadenosine, deoxycytidine, and deoxyguanosine, similar published procedures were used.[11,12]

Various amidine deoxyadenosines were generally more stable toward depurination than the N-benzoylamide (bz)

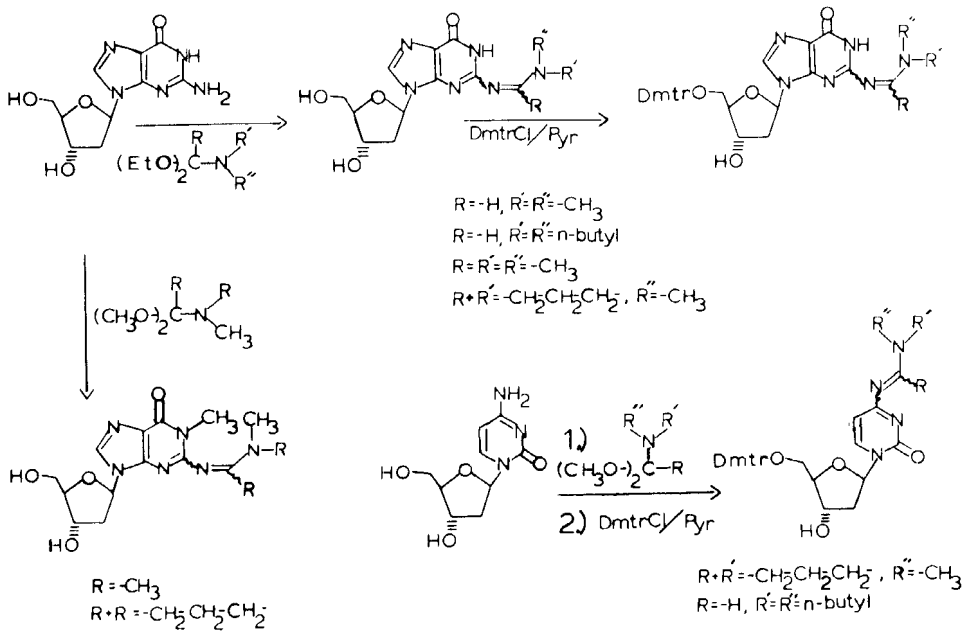

FIG. 2. Preparation of N^2 and N^4 amidines of deoxyguanosine and deoxycytidine, respectively.

TABLE 1. Depurination rates at 28°C of various protected deoxynucleosides covalently attached to a polymer support (Fractosil-500)[a]

	$t_{\frac{1}{2}}$ (h) in 2% (v/v) DCA/CH_2Cl_2[b]	%Depurination per synthetic cycle (2 min)
dA(bz)	1.7	1.0
dA(aca)	34	0.067
dA(dnb)	30	0.077
dG(ib)	23	0.10
dG(aca)	11	0.21
dG(dnb)	21	0.11

[a]Depurination rates were assayed on protected deoxynucleoside covalently joined to silica gel (ref 4). Dichloroacetic acid solution was added to each sample contained in a teflon sealed cuvette and the absorbance of purine liberated from the support monitored at the λmax of the derivative.
[b]DCA (dichloroacetic acid) was freshly distilled at reduced pressure to remove HCl.

derivative. For example, dA(dnb) and dA(aca) were observed to be 18 to 20 times more stable, respectively, toward dichloroacetic acid (TABLE 1). The depurination rate of N^2(*N,N*-di-*n*-butylformamidine)deoxyguanosine, dG(dnb), was approximately the same as for N^2-isobutyrldeoxyguanosine, dG(ib). In contrast, dG(aca) depurinated approximately two to threefold faster than dG(ib). These results suggested that the preferred amidine derivatives for deoxyadenosine and deoxyguanosine were *N,N*-dimethylacetamidine and *N,N*-di-n-butylformamidine, respectively.

In order to be useful in DNA synthesis, amidine protecting groups should be labile using extremely mild conditions. We have observed that this was indeed the case (TABLE 2). Using concentrated ammonium hydroxide at 50°C, dA(aca), dC(pya), dG(aca) and dG(dnb) were removed under conditions comparable to those usually employed for removal of amide protecting groups (10-24 hrs at 50°C).

TABLE 2. Deprotection of various amine-protected deoxynucleosides with conc NH_3 (aq) at 50°C[a]

	$t_{\frac{1}{2}}$[b]
dA(aca)	2.5 hrs
dA(dnb)	5 hrs
dA(pya)	24 hrs
dC(pya)	10 min
5-Methyl-dC(pya)	30 min
dC(dnb)	10 min
dG(dmf)	10 min[c]
dG(aca)	30 min
dG(ib)	30 min
dG(pya)	5 hrs
dG(dnb)	15 min

[a]Deprotection assays were completed in screw cap vials fitted with Pierce teflon discs. Cold, concentrated ammonium hydroxide was added to the protected deoxynucleosides of deoxycytidine and deoxyadenosine and the rates monitored at 50°C spectrophotometrically. For deoxyguanosine derivatives, aliquots were quenched with acetic acid and then measured spectrophotometrically.
[b]Determined spectrophotometrically and by TLC.
[c]N^2(*N,N*-dimethylformamidine)deoxyguanosine, dG(dmf). All other abbreviations listed in this table are defined in the text.

A particularly important consideration as well was the reactivity of protected nucleoside bases when used as synthons for DNA synthesis. For example, important side reactions were observed when thymine and *N*-isobutyrldeoxyguanosine were used in the phosphate triester chemistry.[13] The reactivity of amidine derivatives of adenine, guanine, and cytosine toward phosphitylation were therefore investigated using ^{31}P NMR. Results with guanine are presented in FIG. 3. 5'-Dimethoxytrityl-N^2(*N,N*-dimethylformamidine)deoxyguanosine-3'-*N,N*-diisopropylaminomethoxyphosphine was prepared from 5'-dimethoxytrityl-N^2(*N,N*-dimethylformamidine)deoxyguanosine and bis-(*N,N*-diisopropylamino)methoxyphosphine using a published procedure.[9] The ^{31}P NMR in acetonitrile of the purified material showed a pair of diastereomers at 148.9 and 148.6 ppm. When this solution was treated with excess tetrazole, in addition to

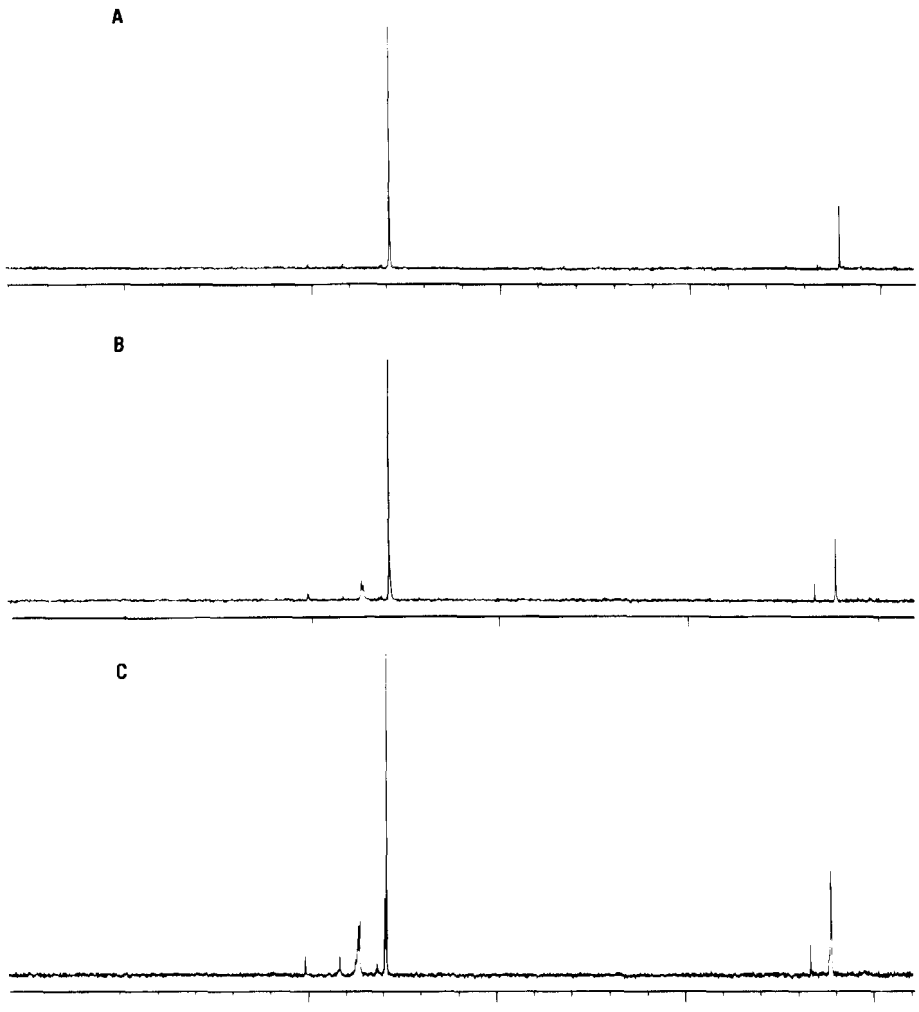

FIG. 3. ^{31}P NMR spectra of the following in 0.4 M tetrazole in acetonitrile:
A) 3'-(*N,N*-diisopropylaminomethoxyphosphinyl)-5'-Dimethoxytrityl-N^1-methyl-N^2-(*N,N*-dimethylacetamidine)deoxyguanosine.
B) 3'-(*N,N*-diisopropylaminomethoxyphosphinyl)-5'-Dimethoxytrityl-N^2-(*N,N*-dimethlformamidine)deoxyguanosine.
C) 3'-(*N,N*-diisopropylaminomethoxyphosphinyl)-5'-Dimethoxytrityl-N^2-isobutyrldeoxyguanosine.

the formation of the intermediate reactive species (presumably either the protonated amidite or the tetrazolide) at 126.9 ppm, there appeared a pair of broad singlets at 134.3

and 133.8 ppm (FIG. 3B). These signals appear to be a result of phosphitylation at the O^6-position since the N'-methyl derivative did not produce these signals (FIG. 3A). Of particular interest was a comparison of these results with the ^{31}P NMR spectrum obtained when the comparable *N*-isobutyrldeoxyguanosine derivative was activated with tetrazole (FIG. 3C). Clearly the amidine derivative was less susceptible to ring-adduct formation. These observations were generally true for the other amidine protected deoxynucleoside phosphoramidites. Although we have no evidence that these ring adducts lead to modified bases, a chemistry which essentially eliminates this potential problem is quite attractive.

SYNTHESIS OF OLIGODEOXYNUCLEOTIDES WITH AMIDINE PROTECTED NUCLEOSIDES

Amidine protected deoxynucleosides were also shown to be synthons for preparing deoxyoligonucleotides (FIG. 1). The first step is synthesis of bis-diisopropylaminomethoxyphosphine. Excess diisopropylamine was added to a solution of methyldichlorophosphite. Removal of the amine salt and fractional distillation of the crude liquid afforded a 77% yield. This bis-aminophosphine is very stable when stored at -10°C. Even with repeated sampling of the phosphine, the ^{31}P NMR spectrum was unchanged after one month. When oxygen was bubbled through a solution in dichloromethane for 24 h, the ^{31}P NMR indicated approximately 8% degradation and 11% hydrolysis.

Phosphoramidites of $(MeO)_2$TrdT, $(MeO)_2$TrdA(aca), $(MeO)_2$-TrdC(pya) and $(MeO)_2$TrdG(aca) were each prepared as 0.1 M solutions in dry acetonitrile containing bis-diisopropylaminomethoxyphosphine (1.0 eq) and diisopropyl ammonium tetrazolide (0.5 eq). These phosphoramidites prepared *in situ* were then used to synthesize deoxyoligonucleotides via the following general procedure. To a suspension of the appropriately derivatized silica support[3] in 450 µl of a 0.4 M solution of tetrazole in acetonitrile was added 450 µl

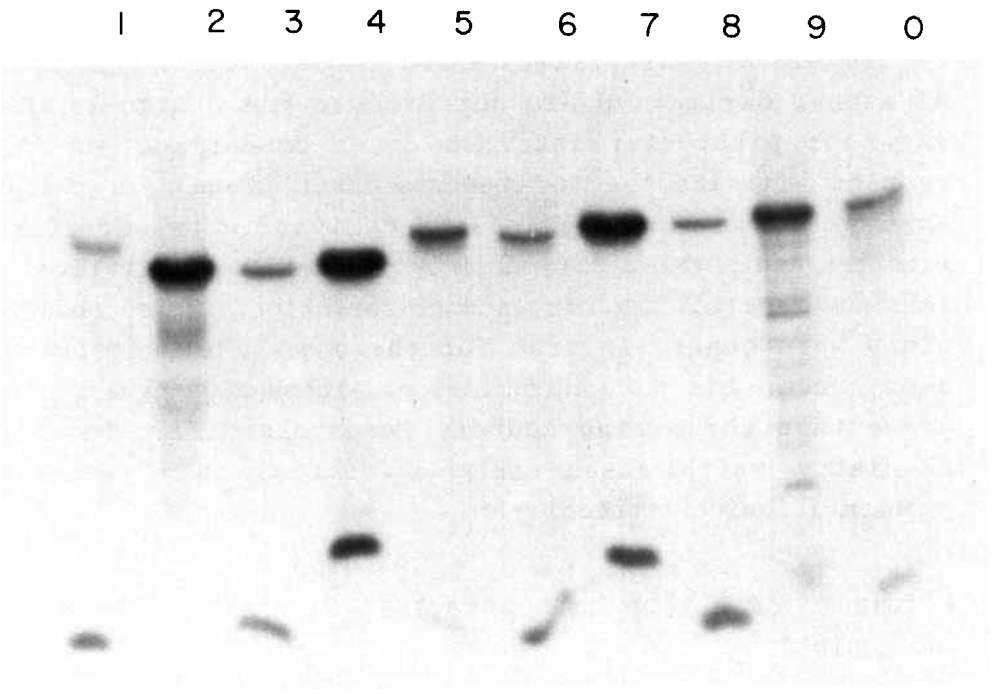

FIG. 4. Polyacrylamide gel showing the results of DNA synthesis *in situ* using deoxynucleoside amidines. The deprotected deoxyoligonucleotides (6) were phosphorylated using T4-kinase and γ-^{32}P ATP. Lanes 1, 5, 6, and 10, marker deoxyoligonucleotides having base compositions $A_4T_3G_3C_4$, $A_6T_3G_3C_2$, $A_4T_4G_1C_5$, and $A_3T_6G_3C_2$, respectively; Lane 2, crude reaction mixture from the synthesis of d(TCAAGGCCGTAA); Lanes 3 and 4, reaction mixture from the synthesis of d(TCAAGGCCGTAA) purified on reverse phase hplc; Lane 9, crude reaction mixture from the synthesis of d(CTTGATGAATCGCC); Lanes 7 and 8, reaction mixture from the synthesis of d(CTTGATGAATCGCC) purified on reverse phase hplc.

(45 μmol, 20 eq) of a 0.1 M solution of the appropriate deoxynucleoside phosphoramidite generated *in situ*. The mixture was allowed to stand five minutes. The solution was then removed by filtration and the silica was washed with dry acetonitrile. After acetylation, oxidation, and detritylation,[6] the cycle was repeated until the synthesis was complete. Using $(MeO)_2$TrdA(aca), this chemistry is illustrated in FIG. 1. Results as presented in FIG. 4 demonstrate

the utility of this method. Lanes 2 and 9 show the results following synthesis of deoxyoligonucleotides containing 12 and 14 mononucleotides respectively. As can be seen after phosphorylation of these crude, unpurified reaction mixtures, the product on a molar basis in each case was the major band observed on the gel and corresponds to approximately 60% of the overall yield. Isolated yields after purifiation via reverse phase hplc were 10-20%. These compounds have subsequently been used for synthesizing mutants of *cro* repressor.[14]

These amidine-protected deoxynucleosides were also used to synthesize d(GGGAATTCCC) which contains an *Eco*RI site.[9] After deprotection with concentrated ammonium hydroxide at 60°C for 16 h, the deoxyoligonucleotide was 5'-labeled using γ-^{32}P ATP and T4-kinase, isolated by gel electrophoresis (20% polyacrylamide/7 M urea), and then degraded with *Eco*RI. As can be seen from the results presented in FIG. 5, at least 95% of the compound is cleaved suggesting that this *in situ* approach using amidine protecting groups is at least comparable to our previous procedures[5,6].

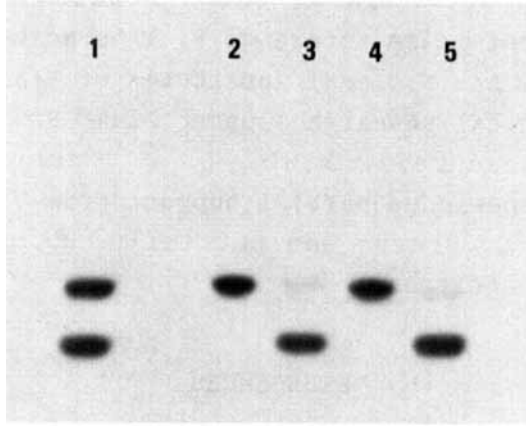

FIG. 5. The gel electrophoresis pattern of *Eco*RI digestion of d(GGGAATTCCC) prepared by the *in situ* method (Lanes 4 and 5) using amide protected bases (9) and by the *in situ* method using amidine protected deoxynucleosides (Lanes 2 and 3).

These results show that a stable dialkylaminophosphine can be used as a phosphitylating reagent to form deoxynucleoside phosphoramidites cleanly and in good yields. The reactions were catalytic with the corresponding amine hydrotetrazolide. Moreover appropriately protected amidine derivatives of deoxyadenosine, deoxyguanosine, and deoxycytidine were shown for the first time to be useful intermediates compatible with the phosphoramidite methodology for DNA synthesis. By substituting amidines for benzoylamide on deoxyadenosine, significantly less depurination in acid was also observed. This improvement should be especially useful for chemical syntheses of long oligomers (>50) where depurination can drastically reduce overall yield. These results therefore illustrate an extremely simple methodology for DNA synthesis. (1) All four deoxynucleosides can be protected by one flask procedures. (2) These protected deoxynucleosides next are converted *in situ* to phosphoramidites and used directly for deoxyoligonucleotide synthesis on silica gel supports.

ACKNOWLEDGMENTS

This is paper 15 in a series Studies on Nucleotide Chemistry. Paper 14 is reference 9. This work was supported by a grant from the National Institutes of Health (GM25680), and the Biomedical Research Support Grant Program, Division of Research Resources, NIH. L. J. McBride and J. W. Dubendorff acknowledge partial support from Upjohn graduate fellowships. L. Bracco was partially supported through a grant from Elf Aquitaine.

REFERENCES

1. Beaucage, S. L. and Caruthers, M. H. (1981) *Tetrahedron Lett.* 22, 1859-1862.
2. McBride, L. J. and Caruthers, M. H. (1983) *ibid.* 24, 245-248.
3. Matteucci, M. D. and Caruthers, M. H. (1980) *ibid.* 21, 3243-3246.

4. Matteucci, M. D. and Caruthers, M. H. (1981) *J. Am. Chem. Soc.* 103, 3185-3191.
5. Caruthers, M. H. Beaucage, S. L., Becker, C., Efcavitch, J. W., Fisher, E. F., Galluppi, G., Goldman, R. A., deHaseth, P. L., Martin, F., Matteucci, M. D. and Stabinsky, Y. (1982) in *Genetic Engineering*, Setlow, J. and Hollaender, A Eds., Vol. 4, pp. 1-17, Plenum Press, New York.
6. Caruthers, M. H. (1982) in *Chemical and Enzymatic Synthesis of Gene Fragments, A Laboratory Manual*, Gassen, H. G. and Lang, A. Eds., pp. 71-79. Verlag-Chemie, Weinheim, Federal Republic of Germany.
7. Caruthers, M. H. (1982) in Recombinant DNA, Proceedings of the Third Cleveland Symposium on Macromolecules, ed. Walton, A. G., (Elsevier Scientific Publishing Company, Amsterdam) pp. 261-272.
8. deHaseth, P. L., Goldman, R. A., Cech, C. L. and Caruthers, M. H. (1983) *Nucleic Acids Res.* 11, 773-787.
9. Barone, A. D., Tang, J.-Y and Caruthers, M. H. (1984) *Nucleic Acds Res.* 12, 4051-4061.
10. Beaucage, S. L. (1984) *Tetrahedron Lett.* 25, 375-378.
11. McBride, L. J. and Caruthers, M. H. (1983) *Tetrahedron Lett.* 24, 2953-2956.
12. Froehler, B. C. and Matteucci, M. D. (1983) *Nucleic Acids Res.* 11, 831-836.
13. Reese, C. B. and Ubasawa, A. (1980) *Tetrahedron Lett.* 21, 719-722.
14. Bracco, L., unpublished research.