BIOORGANIC CHEMISTRY 10, 161–168 (1981)

Selective Cleavage of O-Acyl Protecting Groups from Blocked Deoxyribotides

SURESH C. SRIVASTAVA

Department of Bioorganic Chemistry, Boston Biomedical Research Institute, Boston, Massachusetts 02114

Received March 24, 1980

Selective hydrolysis of the 3′-*O*-acetyl groups in the 5′-*P*-cyanoethyl-blocked deoxyribonucleotides, commonly used in oligomer synthesis by the diester method, can be achieved by mild hydrolysis with aqueous ammonia in pyridine for a brief period of time. The 3′-*O*-isobutyryl group is much more resistant. Conditions for 3′-unblocking of the four commonly used protected monomers as well as a model oligomer, d(CNEtpbzA-ibuG)Ac, are described.

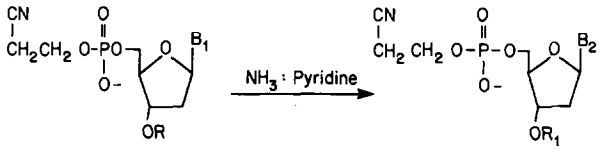
INTRODUCTION

The state of the art of oligodeoxyribonucleotide synthesis has evolved systematically by the introduction of suitable protecting groups (*1, 2*). While much current work employs phosphotriester intermediates (*3–8*), there is still considerable activity involving the use of the original diester approach (*9, 10*). When it is desired to construct oligomers bearing a phosphate at the 5′-terminus, the latter needs to be protected during condensations elsewhere. In its classical form, the cyanoethyl group has been routinely used for this purpose (*11–13*). However, this substituent is lost during the removal of the commonly used base-labile protecting group of the 3′-hydroxyl, and must be reintroduced subsequently at each chain elongation step, also causing the formation of a certain amount of pyrophosphate side product. In order to evade these difficulties, a number of alternate substituents, moderately stable to the basic conditions employed have been developed, viz., trichloroethyl (*14*), thioethyl (*15*), *N*-(*p*-methoxyphenyl) carbamoylethyl (*16*), aromatic phosphoramidate (*17, 18*), triphenylmethyl-*p*-phenylamidate (*19*), *tert*-butyldiphenyl silyl (*20*), 2-(*p*-trityl)-thioethyl or TPTE (*21*). Nevertheless, the proven ease of introduction and removal of the cyanoethyl moiety of the terminal phosphate makes it a desirable blocking group to salvage, and methods assuring its survival during synthesis were thought to be useful.

Earlier attempts to remove selectively the commonly used 3′-*O*-acetate from such models as d(CNEtpanC-bzA)Ac[1] with dilute NaOH led to substantial decyanoethylation (*13*). Kinetic studies on d(CNEtpT)Ac similarly with dilute NaOH revealed the same phenomenon, i.e., concurrent decyanoethylation and

[1] The synthetic oligonucleotides are abbreviated according to IUPAC-IUB (*22*).

0045-2068/81/020161-08$02.00/0



R = acetate or isobutyryl (see text)
and 3′-5′-phosphodiester(*N*-isobutyryl deoxyguanosine)-3′-acetate
R′ = H and 3′-5′-phosphodiester(*N*-isobutyryl deoxyguanosine)-3′-OH
B_1 or B_2 = thymine, *N*-benzoyladenine, *N*-anisoylcytosine and *N*-isobutyrylguanine

SCHEME 1

loss of acetyl group (*23*). It is conceivable on energetic grounds, however, that selective manipulation of the 3′-*O*-acetyl function is possible by soft acceptor/donor interactions, since abstraction of the α-proton next to the cyano group, which initiates decyanoethylation by β-elimination, requires hard base.[2] This hypothesis has been tested and found to be correct. When the soft base ammonia in pyridine, a reagent ideally suited to application in nucleotide chemistry by virtue of a minimum of side effects, was used, it was observed that, indeed, *O*-acyl hydrolysis proceeded much faster than the elimination reaction. This paper describes conditions for such selective unblocking. Scheme 1 illustrates application to the four major deoxyribonucleotides and a dinucleotide. The resulting partially blocked nucleotides can be conveniently isolated by reversed-phase high-performance liquid chromatography (*25, 26*).

MATERIALS AND METHODS

Reference compounds and/or hydrolysis substrates were prepared as follows: d(CNEtpT), d(CNEtpT)Ac were made according to literature procedures (*27, 28*). Compounds d(CNEtpanC)Ac, d(CNEtpbzA)Ac, d(CNEtpibuG)Ac, and d(CNEtpibuG)iBu were obtained via 5′-monomethoxytritylated nucleosides, acylation with acetic or isobutyric anhydride, followed by cleavage of the monomethoxytrityl group with 80% acetic acid, and silica gel-column purification to give the 5′-hydroxyl containing intermediates in 69–85% yields. The cyanoethyl phosphate addition (*29*) occurs quantitatively to yield the protected nucleotides. However, the reaction was carried out with excess nucleoside to avoid presence of inorganic phosphate in the product, while the excess nucleoside can be removed easily during ether precipitation. The overall yields in these consistently reproducible and clean operations range between 85–88%. The procedure is especially useful for the synthesis of fully protected phosphorylated deoxyguanosines, since reported isobutyrylation of dpG to dpibuG(iB) (*30*) consistently presents solubility problems. Reference compounds d(pibuG)Ac, d(pibuG)iBu, d(CNEtpibuG), d(panC)Ac, d(CNEtpanC), d(pbzA)Ac, and d(CNEtpbzA) were obtained according to published procedures (*13–23, 27, 28*). The dinucleotide d(CNEtpbzA-ibuG)Ac was obtained from d(CNEtpbzA) and d(pibuG)Ac according to Büchi and Khorana (*30*) and isolated on hplc.

[2] For nomenclature and general principles see Klopman (*24*).

TABLE 1

THE COMPOSITION OF REACTION MIXTURE ON NH_3: PYRIDINE HYDROLYSIS OF FULLY PROTECTED NUCLEOTIDES

Compound	Time (min)	Percentage of group(s) hydrolyzed			
		3'-acyl	5'-CNET	3'-acyl + 5'-CNET	None[a]
d(CNEtpT)Ac	60	60	No[b]	3	32[c]
	120	82	No	5	9
	270	87	No	6	3
	390	90	No	6.5	0.5
	840	90	No	7.5	No[b]
d(CNEtpanC)Ac	45	58.5	1.5	No	40
	60	64.5	2.0	No	32
	90	86.5	3.0	No	8.5
d(CNEtpbzA)Ac	60	75.5	2	No[b]	20
	70	82	2	6	8
	95	84	3	8	4
d(CNEtpibuG)Ac	90	72	2.5	2	18
	120	81	3.5	2.5	11
	150	84	4.5	3	8
	180	86	7.8	4	2
d(CNEtpibG)iBu	240	25	15	5	55
	360	34	22	7	32
	480	40	29	8	23
d(CNEtpbzA-ibuG)Ac	60	57	1.5	No[b]	37.5
	120	88	2.5	4	3

[a] Starting material.

[b] None observed.

[c] Recoveries of less than 100% reflect presence of minor quantities of uncharacterized materials.

5'-Deoxyguanylate 3'-isobutyrate. dGuo-5'-P, sodium salt (500 mg, 1.35 mmol) was dissolved in H_2O (1 ml) and pyridine (0.2 ml) added to the point just before precipitation occurred, followed by addition of isobutyric anhydride (4 ml). The heterogeneous reaction mixture was stirred for 15 min and vacuum dried three times with addition of dry pyridine. The residue was taken up in dry pyridine (20 ml) and isobutyric anhydride (10 ml) and the clear solution stirred for 12 hr. The mixture was cooled to −20°C and similarly chilled methanol (15 ml) was added and the solution kept at this temperature for 6 hr, followed by addition of 10% aq pyridine at 4°C, and the solution was kept at room temperature for 16 hr. It was vacuum-dried after addition of an equal volume of pyridine. The residue was shaken with dry pyridine. The solid was filtered. The filtrate was brought to 2 ml and precipitated by slow addition of ether (100 ml). The yield of centrifuged solid was 490 mg (86.4%). Paper chromatography showed a predominant spot, of R_F 0.25, with a minor spot corresponding to dGuo-5'-P (approx 5%) (*31*). The material was characterized by conversion to dGuo-5'-P with 1 *N* NaOH under

TABLE 2

CHARACTERIZATION OF PROTECTED AND PARTIALLY DEPROTECTED COMPOUNDS BY HPLC ANALYSIS[a]

Compound	f	g	h	i	j	k	l	m
d(pibuG)						1.9		1.55
d(pibuG)Ac						2.3		1.75
d(pibuG)iBu						4.5		2.40
d(pG)iBu						2.5		
d(CNEtpibuG)						2.0		1.75
d(CNEtpibuG)Ac						3.0		2.20
d(CNEtpibuG)iBu						8.5		4.37
d(pbzA-ibuG)								
d(pbzA-ibuG)Ac								5.5
d(CNEtpbzA-ibuG)								6.2
d(CNEtpbzA-ibuG)Ac								10.2
d(CNEtpbzA-ibuG)iBu								
d(panC)						3.6		
d(panC)Ac						4.5		
d(CNEtpanC)							7.25	
d(CNEtpanC)Ac							12.85	3.25
d(pT)	3.2		2.0					
d(pT)Ac		8.7	4.2					
pyro-d(pT)		7.25	3.5					
pryo-d(pT)Ac		14.0		12.1				
d(CNEtpT)		8.1	4.1					
d(CNEtpT)Ac		13.0		11.0				
d(pbzA)					8.0			
d(pbzA)Ac					14.0			
d(CNEtpbzA)					16.4			
d(CNEtpbzA)					19.6			

[a] The retention time is in minutes. In the two system elution the time is measured from the start the initial system is being run for 5.0 min, followed by linear gradient for a period of 10.0 min. (f) 5% CH_3CN in 0.1 *M* TEAB, pH 7.5; (g) 5% to 10%; (h) 7%; (i) 7% to 10%; (j) 5% to 25%; (k) 15%; (l) 15% to 25%; (m) 25%.

conditions where d(pibuG) largely survives by susceptibility to bacterial alkaline phosphatase and by difference from the N-substituted, as well as N,O-disubstituted nucleotide in hplc.

Hydrolysis studies. In a typical experiment, 0.1 mmol of a completely blocked nucleotide was dissolved in a 1:1 mixture (v:v) of pyridine and concentrated ammonia (density, 0.88) was allowed to react at room temperature (23°C) for indicated periods. An aliquot was removed, concentrated to dryness *in vacuo* several times with addition of dry pyridine, dissolved in a small amount of dry pyridine, and precipitated with several milliliters of ether. The resulting solid was centrifuged, the supernatant discarded, and the residue washed with ether several times, and analyzed by hplc (Tables 1 and 2).

High-performance liquid chromatography. High-performance liquid chromatography was carried out on a μ-Bondapak C-18 reversed-phase column on a Waters Associates Model 6000 A pump and Model 450 variable wavelength uv detector, followed by a 254-nm Altex analytical uv detector and a two channel Omniscribe recorder. The eluants were thus measured at two different wavelengths (commonly 254 and 280 nm). The buffer used was triethylammonium bicarbonate (TEAB), pH 7.5. Elution times and conditions are summarized in Table 2. Quantitation was carried out by integration of eluted peaks.

RESULTS AND DISCUSSION

It is customary to examine proposed procedures in nucleotide chemistry with the monomer presenting least complications: in the deoxy series, thymidylic acid is generally used. Indeed, when 5′-cyanoethyl thymidylate 3′-acetate is subjected to soft base hydrolysis, the desired cleavage of the 3′-ester takes place in excellent yield, while concomitant β-elimination of the cyanoethyl protecting group remains well within acceptable limits (see Fig. 1).

Unlike thymidylate, deoxyguanylic acid often presents problems in its chemistry. However, when d(CNEtpibuG)Ac is hydrolyzed under the present conditions, the predominant event is again hydrolysis of the ester. As shown in Fig. 2, the desired product is observed in a few hours in yields well above 80%, at times where other partial hydrolysis products do not yet build up to an important extent. Failure to observe loss of the *N*-isobutyryl group is remarkable. That this group can eventually be lost was shown in separate studies of d(pG)iBu and d(pibuG). The former is hydrolyzed to the extent of 82% (240 min) and 94% (510 min), during which times the latter is deacylated 18% and 36%, respectively. Of course, the sterically more demanding (as compared to acetate) ester is removed at a slower rate, but is still considerably more labile than the corresponding *N*-acyl unit. Nevertheless, the spread in hydrolytic cleavage rates is not sufficient for preparatory purposes in the case of dp(ibuG)iBu. In fact, from the hydrolysis rate of d(pibuG), some amide hydrolysis would have been expected for d(pibuG)Ac. Failure to observe this may be due to steric hindrance exerted by the additional blocking group.

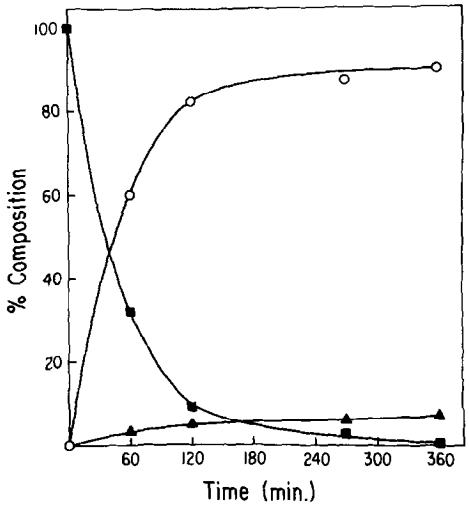


FIG. 1. Ammonia hydrolysis of d(CNEtpT)Ac. (■) Starting material, (○) d(CNEtpT), (▲) d(Thd-5'-P).

Table 1 compares the product distribution against time of exposure to ammonia hydrolysis of various substrates. The conventional blocked deoxymonomers can all be deprotected at their 3'-termini in good yield, without serious interference

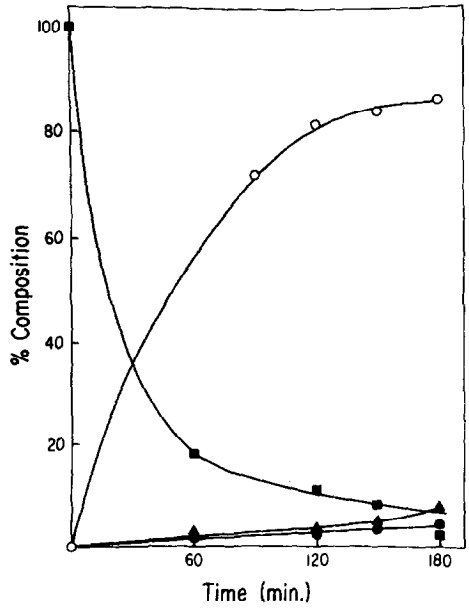


FIG. 2. Ammonia hydrolysis of d(CNEtpibuG)Ac. (■) Starting material, (○) d(CNEtpibG), (▲) d(pibuG)Ac, (●) d(pibuG).

from side products. As seen above, this behavior is due to the intrinsic lability of acetate; in this connection, use of more labile esters such as formate (*32*) may be advantageous. The individual monomers do not lose 3′-acetate at identical rates. A compromise of 2 hr seems to be a practical time period for unblocking oligomers. Indeed, measurements with the model oligomer d(CNEtpbzA-ibuG)Ac indicate a satisfactory conversion (yield 88%), with the remainder of the material distributed over surviving starting material and products of overhydrolysis, each contributing less than 5% to the mixture.

The selective removal of 3′-terminal acetate blocking groups, a conventional step in deoxyribonucleoside phosphodiester technology, thus appears to be practical. The procedure is very mild and relatively rapid, as demonstrated by the cleavage reaction on each of the four suitably protected monomers. The reaction is very clean, and the small amounts of side products can easily be removed by high-performance liquid chromatography. Thus, the well-established 5′-terminal cyanoethyl blocking group can be carried through several cycles of a phosphodiester synthetic sequence without the necessity of periodic reintroduction, avoiding the need for alternate protecting substituents.

While this work was in progress, there was a report (*33*) on the selective cleavage of the acyl group from d(CNEtpT)Ac using methanolic ammonia.

ACKNOWLEDGMENTS

The author wishes to thank Dr. Alexander L. Nussbaum for his encouragement and help in preparation of the manuscript. This work was carried under a grant from the National Institutes of Health (GM 24 369) to A.L.N.

REFERENCES

1. H. Kössel and H. Seliger, *Progr. Chem. Org. Nat. Prod.* **32,** 298 (1975).
2. V. Amarnath and A. D. Broom, *Chem. Rev.* **77,** 183 (1977).
3. R. L. Letsinger and K. K. Ogilvie, *J. Amer. Chem. Soc.* **89,** 4801 (1967).
4. R. Arentzen and C. B. Reese, *J. Chem. Soc. Perkin I,* 445 (1977).
5. J. C. Catlin and F. Cramer, *J. Org. Chem.* **38,** 245 (1972).
6. J. Stawinski, T. Hozumi, S. A. Narang, C. P. Bahl, and R. Wu, *Nucl. Acids Res.* **4,** 353 (1977). T. Hirose, R. Crea, and K. Itakura, *Tetrahedron Lett.* **28,** 2449 (1978).
7. R. Crea, T. Hirose, and K. Itakura, *Tetrahedron Lett.* **5,** 395 (1979).
8. K. L. Agarwal and F. Riftina, *Nucl. Acids Res.* **5,** 2809 (1978).
9. D. V. Goeddel, D. G. Yansura, and M. H. Caruthers, *Proc. Nat. Acad. Sci. USA* **75,** 3578 (1978).
10. M. Mevarech, E. N. Noyes, and K. L. Agarwal, *J. Biol. Chem.* **254,** 7472 (1979).
11. G. Weimann and H. G. Khorana, *J. Amer. Chem. Soc.* **84,** 419 (1962).
12. S. A. Narang, T. M. Jacob, and H. G. Khorana, *J. Amer. Chem. Soc.* **89,** 2158 (1967).
13. H. Schaller and H. G. Khorana, *J. Amer. Chem. Soc.* **85,** 3841 (1963).
14. F. Eckstein, *Angew. Chem. Int. Ed. Engl.* **5,** 671 (1966).
15. A. F. Cook, M. J. Holman, and A. L. Nussbaum, *J. Amer. Chem. Soc.* **91,** 6479 (1969).
16. S. A. Narang, O. S. Bhanot, J. Goodchild, J. Michniewicz, R. H. Wightman, and S. K. Dheer, *Chem. Commun.*, 516 (1970).

17. J. G. MOFFAT AND H. G. KHORANA, *J. Amer. Chem. Soc.* **83**, 649 (1961).
18. E. OHTSUKA, K. MURAO, M. UBASAWA, AND M. IKEHARA, *J. Amer. Chem. Soc.* **92**, 3441 (1970).
19. K. L. AGARWAL, A. YAMAZAKI, AND H. G. KHORANA, *J. Amer. Chem. Soc.* **93**, 2754 (1971).
20. R. A. JONES, H. J. FRITZ, AND H. G. KHORANA, *Biochemistry* **17**, 1268 (1978).
21. K. L. AGARWAL, Y. A. BERLIN, H. J. FRITZ, M. J. GAIT, D. G. KLEID, R. G. LEES, K. E. NORRIS, B. RAMAMOORTHY, AND H. G. KHORANA, *J. Amer. Chem. Soc.* **98**, 1065 (1976).
22. IUPAC-IUB, *J. Mol. Biol.* **55**, 299 (1971).
23. H. SCHALLER AND H. G. KHORANA, *J. Amer. Chem. Soc.* **85**, 3828 (1963).
24. G. KLOPMAN (ed.), "Chemical Reactivity and Reaction Paths," Chap. 4, Wiley, New York, 1974.
25. R. A. HOLTON, D. M. SPATZ, E. E. VAN TAMELEN, AND W. WIERENGA, *Biochem. Biophys. Res. Commun.* **58**(3), 605 (1974).
26. H. J. FRITZ, R. BELAGAJE, E. L. BROWN, R. H. FRITZ, R. A. JONES, R. G. LEES, AND H. G. KHORANA, *Biochemistry* **17**, 1257 (1978).
27. E. OHTSUKA, M. W. MOON, AND H. G. KHORANA, *J. Amer. Chem. Soc.* **87**, 2956 (1965).
28. G. WEIMANN, H. SCHALLER, AND H. G. KHORANA, *J. Amer. Chem. Soc.* **85**, 3835 (1963).
29. G. M. TENER, *J. Amer. Chem. Soc.* **83**, 159 (1961).
30. H. BÜCHI AND H. G. KHORANA, *J. Mol. Biol.* **72**, 251 (1972).
31. T. F. GABRIEL, *J. Chromatogr.* **36**, 518 (1968).
32. F. CRAMER, H. P. BAR, H. J. RHAESE, W. SAENGER, K. H. SCHEIT, G. SCHNEIDER, AND J. TENNIGKEIT, *Tetrahedron Lett.* **16**, 1039 (1963).
33. L. A. VASNYUK AND V. L. FLORENTIEV, *Bioorg. Khim.* **3**, 1346 (1977).