Oligonucleotide Synthesis. III.[1] Enzymatically Removable Acyl Protecting Groups

ALISON TAUNTON-RIGBY

Chemical Research Laboratories, Collaborative Research, Inc., Waltham, Massachusetts 02154

Received June 15, 1972

This work involves a study of enzymatically removable esters (acyl groups) as a means of protection for the sugar hydroxyl functions of nucleosides and nucleotides. The dihydrocinnamoyl, *o*-phenylenedioxyacetyl, and D-(+)-dihydrocoumariloyl groups were investigated to determine the relative ease of separation of the required blocked nucleoside and nucleotide intermediates and to determine the ease of removal with α-chymotrypsin. Difficulties were encountered in using the *o*-phenylenedioxyacetyl and D-(+)-dihydrocoumariloyl protecting groups in actual synthesis. The acyl chlorides were poor acylating agents, and the nucleotide esters of these acids were too alkali labile for purification of the protected oligonucleotides by column chromatography on diethylaminoethyl cellulose using triethylammonium bicarbonate buffers at pH 7.5 to 9.0. These problems were not observed with the dihydrocinnamoyl group. High yields of nucleosides, protected in the 3′ and 5′ positions, and nucleotide 5′-monophosphates, protected at the 3′ position, were obtained by reaction of the appropriate nucleoside or nucleoside 5′-monophosphate with dihydrocinnamic anhydride. The synthesis of the tetranucleotide d-pTpCpApG was achieved using the dihydrocinnamoyl group for protection of the 3′-hydroxyl functions showing that the use of this protecting group is both useful and practical. Kinetic studies on the enzymatic hydrolysis of nucleoside and nucleotide esters of the three acids showed that the *o*-phenylenedioxyacetyl and D-(+)-dihydrocoumariloyl groups were good substrates for α-chymotrypsin; complete removal of the protecting groups could be achieved in under 1 hr at 37° in neutral conditions. Removal of the dihydrocinnamoyl group was slower but could be achieved at 37° in a reasonable time (8–16 hr).

The general scheme for the synthesis of deoxyribooligonucleotides developed during the past decade is based on the sequential assembly of suitably protected monomeric building blocks.[2] The most commonly used protecting groups for hydroxyl, phosphate, and amino functions are either acid labile, such as the trityl group,[3] or alkali labile, such as the acetyl and cyanoethyl groups.[4] However, during the synthesis of a deoxyribooligonucleotide carrying a 5′-terminal phosphate, the use of these particular protecting groups is unsatisfactory, since the cyanoethyl group protecting the 5′-phosphate is generally removed under the alkaline conditions necessary to hydrolyze the 3′-hydroxyl acyl protecting group. Recently two phosphate protecting groups have been developed which circumvent this difficulty. The phosphorothioate protecting group[5] is removed by mild oxidation and the phosphoramidate group[6] by treatment with isoamyl nitrite. We have approached the problem by developing protecting groups for the 3′-hydroxyl function which are enzymatically removed under neutral conditions leaving the cyanoethyl phosphate protecting group intact.[7] In this paper we describe the use of α-chymotrypsin to hydrolyze nucleoside and nucleotide esters of three different acids, which are used as 3′-hydroxyl protecting groups. The protecting groups studied are the dihydrocinnamoyl, D-(+)-dihydrocoumariloyl, and *o*-phenylenedioxyacetyl groups. A comparison has been made of these acids as protecting groups in oligonucleotide synthesis.

(1) Part II of this series: A. Taunton-Rigby, Y. H. Kim, C. J. Crosscup, and N. A. Starkovsky, *J. Org. Chem.*, **37**, 956 (1972); Part I: H. S. Sachdev and N. A. Starkovsky, *Tetrahedron Lett.*, 733 (1969).

(2) H. G. Khorana, *Fed. Proc., Fed. Amer. Soc. Exp. Biol.*, **19**, 931 (1960); H. Kossel, H. Buchi, T. M. Jacob, A. R. Morgan, S. A. Narang, E. Ohtsuka, R. D. Wells, and H. G. Khorana, *Angew. Chem., Int. Ed. Engl.*, **8**, 387 (1969).

(3) M. Smith, D. H. Rammler, T. H. Goldberg, and H. G. Khorana, *J. Amer. Chem. Soc.*, **84**, 430 (1962).

(4) H. Schaller and H. G. Khorana, *ibid.*, **85**, 3841 (1963); G. Weinmann, H. Schaller, and H. G. Khorana, *ibid.*, **85**, 3835 (1963).

(5) E. Heimer, M. Ahmed, S. Roy, A. Ramel, and A. L. Nussbaum, *ibid.*, **94**, 1707 (1972).

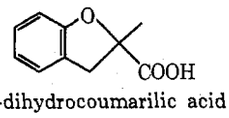
(6) E. Ohtsuka, M. Ubasawa, and M. Ikehara, *ibid.*, **92**, 5507 (1970).

(7) See part I of this series: H. S. Sachdev and N. A. Starkovsky, *Tetrahedron Lett.*, 733 (1969).

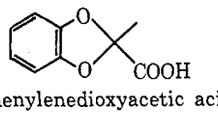
Results

(a) Synthesis of the Protecting Groups

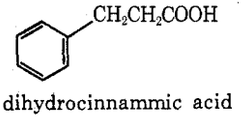
D-(+)-Dihydrocoumarilic acid (DCM acid)[8] was prepared by reduction of coumarilic acid and the resulting racemate was resolved by crystallization with d-amphetamine.[9] *o*-Phenylenedioxyacetic acid (PDA acid) was synthesized by condensation of catechol with ethyl dichloroacetate followed by hydrolysis of the resulting ester.[10] Acid chlorides were prepared without difficulty from these acids, and from dihydrocinnamic acid (DHC acid), by treatment with thionyl chloride. Dihydrocinnamic anhydride was synthesized from the acid by distillation with acetic anhydride, or by treating the acid with an equimolar amount of the acid chloride. However, synthesis of anhydrides from the other acids presented difficulties. *o*-Phenylenedioxyacetic anhydride and D-(+)-dihydrocoumarilic anhydride were prepared by both methods but in extremely low yields as the anhydrides decomposed at the temperature required for distillation (bath temperature 250° at 0.01 mm). In addition, racemization of the D-(+)-dihydrocoumariloyl group occurred at the high distillation temperature. Other milder methods of preparing the anhydrides, such as condensation with dicyclohexylcarbodiimide, were tried but only starting material was recovered. As a result, the acid chlorides



DL-dihydrocoumarilic acid



o-phenylenedioxyacetic acid



dihydrocinnammic acid

(8) DCM refers to the D-(+) enantiomer of dihydrocoumarilic acid unless otherwise stated.

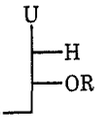
(9) D. M. Bowen, J. I. DeGraw, Jr., V. R. Shah, and W. A. Bonner, *J. Med. Chem.*, **6**, 315 (1963).

(10) H. A. Hartzfeld, R. G. Johnson, and H. Gilman, *J. Org. Chem.*, **22**, 1717 (1957); A. Burger, D. G. Markees, W. R. Nes, and W. L. Yost, *J. Amer. Chem. Soc.*, **71**, 3307 (1949); W. G. Christiansen and M. A. Dolliver, *ibid.*, **71**, 3307 (1949); **66**, 312 (1944).

from D-(+)-dihydrocoumarilic acid and *o*-phenylenedioxyacetic acid and the anhydride of dihydrocinnamic acid were used to esterify the nucleosides and nucleotides.

(b) Synthesis of Protected Nucleosides and Nucleotides

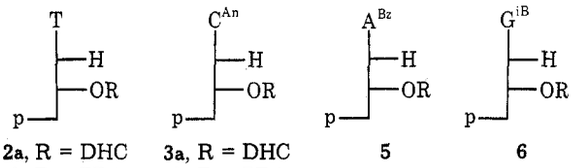
The derivatives of deoxyuridine (**1a–d**),[11] protected at the 3′ and 5′ positions, were prepared by treatment



1a, R = DHC
b, R = PDA
c, R = DCM (racemate)
d, R = DCM[(D-(+) isomer)]

with the corresponding acid chlorides or anhydrides. The yields were generally 80–95%. However, small traces of the monosubstituted nucleosides could be detected by tlc (≈2%) and so further purification was carried out by preparative tlc.

Mononucleotides protected at the 3′ position by the DHC group (**2a**, **3a**, **5**, and **6**) were prepared from the



2a, R = DHC
b, R = PDA
c, R = DCM

3a, R = DHC
b, R = PDA
c, R = DCM

5

6

corresponding mononucleotide, base protected if necessary, and DHC anhydride. Yields were almost quantitative and purification was achieved without difficulty by precipitation with ether. In contrast, the syntheses of d-pT-OR and d-pC^{An}-OR (R = DCM or PDA) using the corresponding acid chlorides were only achieved in low yields. In all cases, the main product had faster mobility on paper chromatography (solvent D) than the parent mononucleotide and was tentatively identified as the symmetrical pyrophosphate by uv spectroscopy, R_f value, and enzymatic degradation. Purification of the protected mononucleotides **2b**, **2c**, **3b**, and **3c** was achieved by preparative paper chromatography (solvent D).

(c) Kinetic Studies

Kinetic studies of the rates of hydrolysis of these esters were carried out. In a typical experiment, the substrate was dissolved or suspended in 0.1 *M* sodium chloride solution at a concentration of 10^{-2} *M* and the pH adjusted to 7.8 with dilute sodium hydroxide solution. The α-chymotrypsin was added, the amount being calculated on the basis of units per micromole of substrate (the specific activity quoted by the supplier was not checked). This gave an enzyme concentration varying from 10^{-4} to 10^{-6} *M* in these experiments. The total volume of the reaction mixture was adjusted to 2.0 ml and the mixture was incubated at 37°. The addition of the enzyme was taken as zero time. The pH of the mixture (measured on a pH meter) was maintained at 7.5 to 7.8 by titration with 0.1 *M* sodium hydroxide solution. The hydrolysis was judged to be complete when the pH of the reaction mixture had been constant for at least 2 hr and tlc showed the absence of any starting material. In addition, calculations were made of the volume of alkali required to neutralize the acid liberated by the enzymatic hydrolysis. Experiments were discounted in which the actual volume of alkali used did not correlate closely with the theoretical amount.

Control experiments excluding the enzyme were always run. Under these conditions no observable hydrolysis of the protected nucleosides or nucleotides occurred. Control experiments excluding the substrate were also run, as it is known that under certain circumstances α-chymotrypsin can function both as a catalyst and as a substrate.[12] It was found that at the high enzyme–substrate concentrations autolysis occurred, and, correspondingly, the kinetic results were corrected for this factor.

The results of the kinetic hydrolyses are summarized in Tables I and II, and some of the plots are shown in

TABLE I

TIME REQUIRED FOR FULL DEPROTECTION BY α-CHYMOTRYPSIN[a]

Compd 1	Enzyme–substrate ratio expressed in units/μmol of substrate: 28	2.8	0.28
a, R = DHC	3 hr	8 hr	48 hr
b, R = PDA	1 hr	2 hr	8 hr
c, R = DCM (racemate)	3 hr		
d, R = DCM [D-(+) isomer]	50 min	1 hr	4 hr

[a] The reaction mixture contained 0.05 mmol of substrate (*i.e.*, 0.1 mmol of ester) suspended in 2 ml of 0.1 *M* NaCl plus the enzyme, pH maintained at 7.8 by addition of 0.1 *M* NaOH at 37°.

TABLE II

TIME REQUIRED FOR FULL DEPROTECTION BY α-CHYMOTRYPSIN[a]

Compd	Enzyme–substrate ratio expressed in units/μmol of substrate: 30	3.0	0.30
2a, d-pT-ODHC	2 hr	5 hr	48 hr
2b, d-pT-OPDA	*b*	15 min	1 hr
2c, d-pT-ODCM	*b*	5 min	45 min
3a, d-pC^{An}-ODHC	2 hr	5.5 hr	48 hr
3b, d-pC^{An}-OPDA	*b*	15 min	1 hr
3c, d-pC^{An}-ODCM	*b*	15 min	1 hr
5, d-pA^{Bz}-ODHC	2.5 hr	5.5 hr	48 hr
6, d-pG^{iB}-ODHC	2 hr	5 hr	48 hr
17, d-CEpTpC^{An}-ODHC	30 min	2.5 hr	
19, d-CEpTpC^{An}pA^{Bz}-ODHC	30 min	4 hr	
21, d-CEpTpC^{An}pA^{Bz}pG^{iB}-ODHC	45 min	4 hr	

[a] The reaction mixture contained 0.1 mmol of substrate in 2 ml of 0.1 *M* NaCl plus the enzyme, pH maintained at 7.8 by addition of 0.1 *M* NaOH at 37°. [b] The reaction was too fast to follow.

(11) For the system of abbreviations used see H. Schaller and H. G. Khorana, *J. Amer. Chem. Soc.*, **85**, 3841 (1963). Thus, d-pT is thymidine 5′-monophosphate, d-pC^{An} is *N*-anisoyldeoxycytidine 5′-monophosphate, and d-pC^{An}pA^{Bz} is 5′-*O*-phosphoryl-*N*-anisoyldeoxycytidylyl-(3′–5′)-*N*-benzoyldeoxyadenosine. Additional abbreviations used in this paper are iB, the isobutyryl group; DHC, the dihydrocinnamoyl group; DCM, D-(+) enantiomer of the dihydrocoumarilloyl group; PDA, the *o*-phenylenedioxyacetyl group.

(12) C. Niemann, *Science*, **143**, 1287 (1964); T. H. Applewhite, R. B. Martin, and C. Niemann, *J. Amer. Chem. Soc.*, **80**, 1457 (1958); A. Yapel, M. Jan, R. Lumry, A. Rosenberg, and D. F. Shiao, *ibid.*, **88**, 2573 (1966).

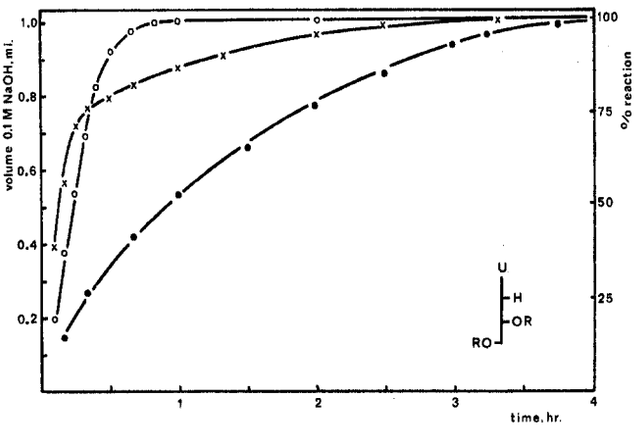


Figure 1.—Kinetics of the enzymatic hydrolysis of nucleosides. (×) R = DCM, racemate **1c**, 28 units/μmol; (○) R = DCM, D-(+) isomer **1d**, 2.8 units/μmol; (●) R = DCM, D-(+) isomer **1d**, 0.28 units/μmol. For reaction conditions see Table I.

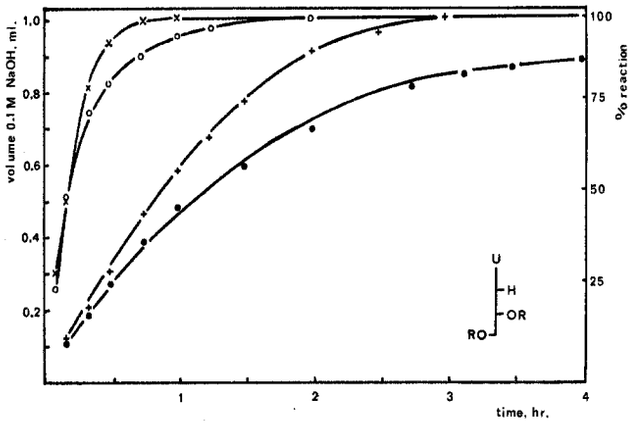


Figure 2.—Kinetics of the enzymatic hydrolysis of nucleosides. (×) R = PDA, **1b**, 28 units/μmol; (○) R = PDA, **1b**, 2.8 units/μmol; (+) R = DHC, **1a**, 28 units/μmol; (●) R = DHC, **1a**, 2.8 units/μmol. For reaction conditions see Table I.

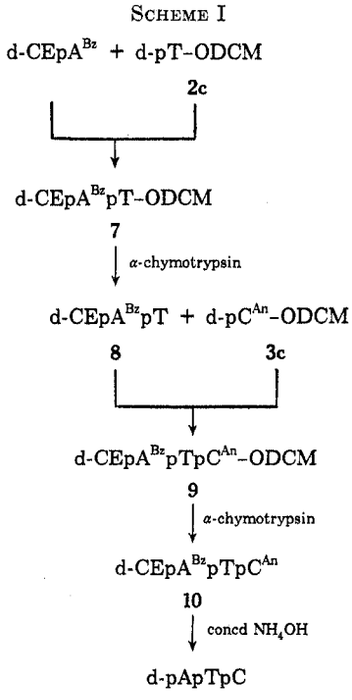
Figures 1–3. It can be seen that the best substrates for α-chymotrypsin are the DCM [D-(+) enantiomer] and PDA groups, and in fact removal of these two protecting groups from nucleotides was too fast to follow except at the lower enzyme–substrate ratios. The DCM esters were hydrolyzed more slowly.

The stability of these esters to alkaline hydrolyses was studied. Esters of DHC acid (**2a**) were stable at pH 9.5 for up to 3 days, whereas esters of the other two acids (**2b** and **2c**) were found to be extremely alkali labile. At pH 9.5 hydrolysis was complete in 4 hr in both cases and at pH 8.0 partial hydrolysis (11–13%) was observed in 24 hr.

(d) Synthesis of Oligonucleotides

The protected mononucleotides (**2a**, **2c**, **3a**, **3c**, **5**, and **6**) were used in the synthesis of various oligonucleotides. Preparation of the trinucleotide d-pApTpC was achieved as shown in Scheme I using mesitylenesulfonyl

SCHEME I



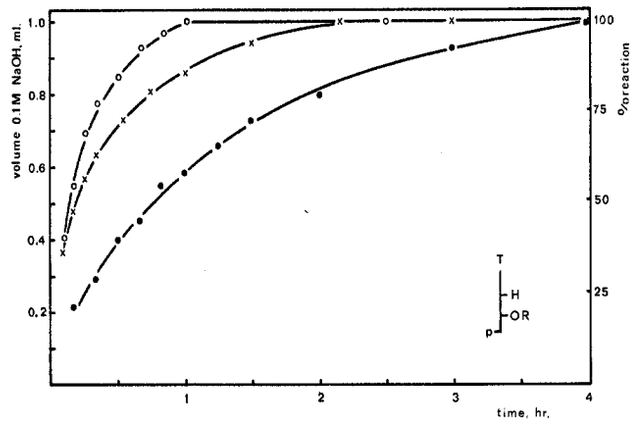


Figure 3.—Kinetics of the enzymatic hydrolysis of nucleotides. (○) R = PDA, **2b**, 0.30 unit/μmol; (×) R = DHC, **2a**, 30 units/μmol; (●) R = DHC, **2a**, 3.0 units/μmol. For reaction conditions see Table II.

chloride (MSC) as the condensing agent. In this sequence, the first condensation to give d-CEpA^{Bz}pT-ODCM (**7**) was worked up by chromatography on DEAE cellulose using a gradient of TEAB. However the R_f value of the dinucleotide isolated by this procedure seemed lower than anticipated (R_f 0.64) suggesting that the DCM protecting group had been lost during the column work-up. This was the first evidence that the DCM group was not entirely suitable for use in synthesis. Loss of the protecting group was confirmed when a second preparation was carried out but the reaction mixture was purified by preparative paper chromatography in solvent D. In this case, the pH could be maintained at 7.5 using the ammonium acetate buffer whereas when running the DEAE column using TEAB buffer at pH 7.5 it was found that after passage through the column the pH had risen to 8.5–9.0 due to loss of CO_2. Attempts to use TEAB at a lower initial pH (6.5) gave identical results. As a result of the prolonged length of time involved in running a DEAE column loss of the DCM protecting group occurred. Preparative paper chromatography at pH 7.5 proved to be a useful alternative method for isolating fully protected intermediates. Separation of materials with close R_f values was achieved by running the chromato-

grams for 24 to 36 hr giving an effective solvent front of 60–80 cm.

Treatment of the dinucleotide d-CEpA^{Bz}pT-ODCM (**7**), R_f 0.74, with α-chymotrypsin for 4 hr at 37° at an enzyme–substrate ratio of 5 units/μmol gave the partially protected dimer d-CEpA^{Bz}pT (**8**) which had R_f 0.64. The structure was also confirmed by preparation of a sample of **8** by cyanoethylation of the dimer d-pA^{Bz}pT.

Synthesis of the trinucleotide d-CEpA^{Bz}pTpC^{An}-ODCM (**9**) from d-pC^{An}-ODCM (**3c**) and **8** was achieved but the yield was extremely low (11%). Deprotection with α-chymotrypsin at a ratio of 1.5 units/μmol gave d-CEpA^{Bz}pTpC^{An} (**10**) which was isolated in 69% yield. The structure of this trimer was confirmed by degradation with snake venom phosphodiesterase after removal of all protecting groups.

Scheme II shows the synthesis of the tetranucleotide

SCHEME II

d-CEpC^{An} + d-pT-ODHC

2a

d-CEpC^{An}pT-ODHC

11

α-chymotrypsin

d-CEpC^{An}pT + d-pC^{An}-ODCM

12 **3c**

d-CEpC^{An}pTpC^{An}-ODCM

13

α-chymotrypsin

d-CEpC^{An}pTpC^{An} + d-pT-ODCM

14 **2c**

d-CEpC^{An}pTpC^{An}pT-ODCM

15

α-chymotrypsin

d-CEpC^{An}pTpC^{An}pT

16

concd NH_4OH

d-pCpTpCpT

d-pCpTpCpT using two protecting groups (DCM and DHC). Condensation of d-CEpC^{An} with d-pT-ODHC (**2a**) using dicyclohexylcarbodiimide (DCC) as the condensing agent gave the dinucleotide d-CEpC^{An}pT-ODHC (**11**) in 53% yield, which was purified by chromatography on a column of DEAE cellulose. Partial deprotection was achieved by treatment with α-chymotrypsin. Separation of the nucleotide from the protein was achieved by preparative paper chromatography.

The tri- and tetranucleotides in this series were prepared by using the DCM protecting group. The fully protected trinucleotide **13** was not isolated as separation of unreacted d-pC^{An}-ODCM and the trimer could not be achieved, but treated directly with α-chymotrypsin to give d-CEpC^{An}pTpC^{An} (**14**) which was purified by paper chromatography. Condensation of **14** with d-pT-ODCM (**2**) gave the tetramer **15** which was isolated by paper chromatography. The base composition of the tri- and tetramer were confirmed by degradation with snake venom phosphodiesterase to give the component nucleotides in the expected ratios.

The tetranucleotide d-pTpCpApG was synthesized by a stepwise procedure utilizing the DHC protecting group as shown in Scheme III. The yields at the di-,

SCHEME III

d-CEpT + d-pC^{An}-ODHC

3a

d-CEpTpC^{An}-ODHC

17

α-chymotrypsin

d-CEpTpC^{An} + d-pA^{Bz}-ODHC

18 **5**

d-CEpTpC^{An}pA^{Bz}-ODHC

19

α-chymotrypsin

d-CEpTpC^{An}pA^{Bz} + d-pG^{iB}-ODHC

20 **6**

d-CEpTpC^{An}pA^{Bz}pG^{iB}-ODHC

21

α-chymotrypsin

d-CEpTpC^{An}pA^{Bz}pG^{iB}

22

concd NH_4OH

d-pTpCpApG

tri-, and tetramer stages were 54, 46, and 36%, respectively. In all cases work-up was by paper chromatography in solvent E. Intermediate deprotection was achieved by treatment with α-chymotrypsin. Confirmation of the assigned structures was obtained by removal of all protecting groups and degradation with snake venom phosphodiesterase and also by removal of the terminal 5′-phosphate by alkaline phosphatase and subsequent degradation with spleen phosphodiesterase.

(e) Enzymatic Hydrolysis of Oligonucleotides

The kinetics of the enzymatic removal of the DHC group from the di-, tri-, and tetranucleotides was studied and compared with the corresponding rates of removal of this group from the mononucleotides (Table II). It was observed that the rate of hydrolysis was faster for the oligonucleotides than for the monomers. The hydrolysis rate was fastest for the di-

nucleotide and decreased slightly with increasing chain length.

The use of an insolubilized form of α-chymotrypsin was explored as it was hoped that column procedures could be set up for the partial deprotection step. Correspondingly, removal of the DHC group from these oligonucleotides was studied using the insolubilized form of α-chymotrypsin, Enzite-CHT. The amount of Enzite-CHT used in these experiments was calculated so as to give an equal number of units as that used with the free enzyme. The results are summarized in Table III. These show that, while the rate of hydrolysis was

TABLE III

TIME REQUIRED FOR FULL DEPROTECTION BY FREE α-CHYMOTRYPSIN[a] AND BY ENZITE-CHT[b]

Compd	Enzite-CHT, 34 units/μmol	Free α-chymotrypsin, 31 units/μmol
2a, d-pT-ODHC	8 hr	2 hr
17, d-CEpTpC^{An}-ODHC	3 hr	30 min
19, d-CEpTpC^{An}pA^{Bz}-ODHC	3 hr	30 min
21, d-CEpTpC^{An}pA^{Bz}pG^{iB}-ODHC	3 hr	45 min

[a] The reaction mixture contained 1.6 μmol of substrate in 0.05 *M* phosphate buffer, pH 7.5, plus 1 mg of α-chymotrypsin (50.4 units). [b] The reaction mixture contained 1.6 μmol of substrate in 1.0 ml of 0.05 *M* phosphate buffer, pH 7.5, plus 100 mg of Enzite-CHT (56 units).

faster for an oligonucleotide than for a mononucleotide, the hydrolysis rate in all cases was much slower than the rate obtained when using an equivalent amount of the free enzyme.

Discussion

Three different substrates of α-chymotrypsin have been used as protecting groups for the 3′-hydroxyl function of 2′-deoxynucleotides and the 3′- and 5′-hydroxyl positions of 2′-deoxynucleosides. Esters of dihydrocoumarilic acid and dihydrocinnamic acid have been studied[13,14] previously as substrates of α-chymotrypsin and compared with esters of the best synthetic substrate *N*-acetyl-L-phenylalanine. These studies showed that esters of the D-(+) enantiomer of dihydrocoumarilic acid were hydrolyzed by the enzyme at a much faster rate than those of the L-(−) form and at a rate comparable to esters of *N*-acetyl-L-phenylalanate[13] (k_{cat}/K_m, methyl *N*-acetyl-L-phenylalanate, 6.9×10^4; methyl D-dihydrocoumarilate, 1.4×10^4). Esters of dihydrocinnamic acid are not such good substrates for α-chymotrypsin[14] (k_{cat}/K_m, ethyl dihydrocinnamate, 1.5×10) and esters of *o*-phenylenedioxyacetic acid have not been studied previously as substrates for this enzyme.

The difficulties encountered in esterifying nucleotides with the acid chlorides from PDA and DCM acids are a considerable disadvantage to the use of these groups. It has been noted previously that treatment of monophosphates with low molar ratios (1–5 equiv) of an acid chloride or anhydride can result in the formation of the symmetrical pyrophosphate but use of an excess of these reagents (10–20 equiv) leads to a breakdown of the pyrophosphate bond.[15–17] However, the use of large excesses of the acid chlorides from all three acids still gave what was tentatively identified as the symmetrical pyrophosphate as the major product. The problem was solved in the case of the DHC group by use of the anhydride of this acid, which gave much cleaner reactions, but the anhydrides of the other two acids proved inaccessible. Alternative procedures involving the preparation of the mixed anhydrides with ethyl chloroformate gave no better yields of the protected mononucleotides. These results are probably due to the size and steric shape of these acylating agents, which results in an increase in the rate of pyrophosphate formation as compared to the rate of acylation.[17]

Comparison of these protecting groups as substrates for α-chymotrypsin showed that the PDA and DCM groups were very similar and both better substrates than the DHC group. The amounts of enzyme used in the kinetic experiments were measured on the basis of units per micromole of substrate so that direct comparison could be made of the results with the nucleosides carrying 2 mol of the substrate and the mono- and oligonucleotides which have only 1 mol of substrate.

In all cases hydrolysis from nucleotides was faster than from nucleosides, possibly due to the fact that the protected nucleosides are insoluble in the aqueous media and as a result the enzymatic hydrolyses were carried out on suspensions. Attempts to use a cosolvent such as acetonitrile, to increase the solubility, did not improve the results as the amounts of organic solvent required to keep the nucleoside in solution were sufficient to severely inhibit the α-chymotrypsin. Interestingly, the kinetic curve for the hydrolysis of deoxyuridine protected in the 3′ and 5′ positions by the racemate of the DCM group shows a distinct break in the curve due to the rate of hydrolysis of one enantiomer being much faster than the other.

The DHC and DCM protecting groups were used in the synthesis of several oligonucleotides. The PDA group was not used further as the kinetic results and synthetic work so far showed that no advantage could be achieved using it.

Synthesis of the trinucleotide d-pApTpC and the tetranucleotide d-pCpTpCpT revealed further disadvantages of the DCM protecting group. This group appears to be too alkali sensitive to survive passage through a DEAE cellulose column using TEAB as the eluant due to the change in pH. A more stable buffer system could have been used but this would then raise the problem of desalting the product. A suggested use for this protecting group would be as a 3′-hydroxyl protecting group which could be removed selectively by mild alkaline treatment in the presence of the cyanoethyl group. The DHC group was stable under the conditions required for column chromatography on DEAE cellulose using TEAB as the eluant.

The enzymatic conditions used to remove the protecting groups during synthesis are summarized in Table IV. Later kinetic results on the oligonucleotides **17**, **19**, and **21** showed that, in fact, much milder conditions

(13) W. B. Lawson, *J. Biol. Chem.*, **242**, 3397 (1967).
(14) S. G. Cohen, A. Milovanovic, R. M. Schyltz, and S. Y. Weinstein, *ibid.*, **244**, 2664 (1969).
(15) H. G. Khorana and J. P. Vizsolyi, *J. Amer. Chem. Soc.*, **81**, 4660 (1959).
(16) M.W. Moon and H. G. Khorana, *ibid.*, **88**, 1798 (1966).
(17) M. W. Moon and H. G. Khorana, *ibid.*, **88**, 1805 (1966).

TABLE IV

CONDITIONS USED FOR THE ENZYMATIC REMOVAL OF ACYL PROTECTING GROUPS IN SYNTHESIS

	Time, hr	Enzyme–substrate ratio, units/μmol	Yield, %
d-CEpA^{Bz}pT-ODCM → d-CEpA^{Bz}pT	4	5	90
d-CEpA^{Bz}pTpC^{An}-ODCM → d-CEpA^{Bz}pTpC^{An}	3	1.5	69
d-CEpC^{An}pT-ODHC → d-CEpC^{An}pT	16	5.4	73
d-CEpC^{An}pTpC^{An}-ODCM → d-CEpC^{An}pTpC^{An}	2	1.8	41
d-CEpC^{An}pTpC^{An}pT-ODCM → d-CEpC^{An}pTpC^{An}pT	2	8.7	75
d-CEpTpC^{An}-ODHC → d-CEpTpC^{An}	8	5.4	98
d-CEpTpC^{An}pA^{Bz}-ODHC → d-CEpTpC^{An}pA^{Bz}	24	7.3	91
d-CEpTpC^{An}pA^{Bz}pG^{iB}-ODHC → d-CEpTpC^{An}pA^{Bz}pG^{iB}	24	10.8	82

could have been used. Better results were obtained when the oligonucleotides were purified prior to treatment with α-chymotrypsin than when deprotection was carried out on the crude reaction mixture. This was due to the fact that organic solvents and other insoluble residues had to be removed before enzymatic treatment.

The separation of protein and nucleotide was achieved by preparative paper chromatography. The α-chymotrypsin used in these experiments contained several components but all were easily separated from the oligonucleotides in solvents D or E.

The tetramer d-pTpCpApG was synthesized in good yield using the DHC protecting group, demonstrating its use with all four bases. Under the conditions of enzymatic hydrolysis no loss of any base protecting group was observed. Studies of the kinetics of the enzymatic removal showed that the time required for the hydrolysis decreased considerably from monomer to dimer and then increased slightly with increasing chain length. This is in agreement with the fact that macromolecules are generally better substrates for α-chymotrypsin.

The results using the insolubilized form of α-chymotrypsin were disappointing. The insolubilized enzyme does hydrolyze esters of dihydrocinnamic acid but at a slower rate than the free enzyme when amounts containing the same number of units of activity are used. Two reasons for the decrease in rate can be put forward. One is the fact that the support used for the enzyme is carboxymethyl cellulose and as a result the negatively charged oligonucleotide will tend to be repelled by the support. Better results might be obtained if a neutral or positively charged support was used. A second reason could be the stability of the insolubilized enzyme, so that with older samples the activity was probably lower than expected, but this was not checked. It had been hoped that a column procedure could have been set up using a DEAE cellulose column with a layer of insolubilized enzyme on top so that deprotection and separation could be achieved in one step. However, the time required for hydrolysis was too great to be useful.

Conclusions

Of the three protecting groups used in this work the DCM and PDA groups are considerably better substrates for α-chymotrypsin than the DHC group. However, this advantage is offset by the difficulties encountered in synthetic work using these particular groups. Moreover, the alkali sensitivity of the resulting protected nucleotides is such that column chromatography cannot be used for purification. On the other hand the dihydrocinnamoyl group has been shown to be useful in small scale synthesis and further work involving the use of this protecting group in the large scale synthesis of an octanucleotide will be reported at a later date.

Experimental Section

Reagent grade pyridine was purified by distillation over chlorosulfonic acid and potassium hydroxide and stored over 4A molecular sieve beads (Linde Co.). All evaporations were carried out at reduced pressure below 25°. Whenever necessary, reagents and reaction mixtures were rendered anhydrous by repeated evaporation of added dry pyridine *in vacuo*. Qualitative paper chromatography was carried out by the descending technique on Whatman No. 1 paper. Preparative paper chromatography was carried out on Whatman No. 3 MM paper. The solvent systems used were (A) *n*-BuOH–acetic acid–H_2O (5:2:3, v/v); (B) *n*-PrOH–concentrated NH_4OH–H_2O (55:10:35); (C) ethyl acetate–ethanol (9:1); (D) ethanol–1 *M* ammonium acetate (pH 7.5) (7:3), (E) ethanol–0.05 *M* ammonium acetate (pH 7.5) (7:3), (F) isobutyric acid–concentrated NH_4OH–H_2O (66:1:33). Thin layer chromatography was run on silica gel (F-254, E. Merck) and cellulose F (E. Merck). Separation of products with close R_f values was achieved by running the chromatograph for 24 to 36 hr instead of the usual 16 hr to achieve better separation of the bands. The chromatograms were dried and then soaked for 1 hr in absolute ethanol to remove ammonium acetate, washed with diethyl ether and the nucleotides eluted with water or 20% ethanol. Final traces of salts were removed by lyophilization.

The products were characterized by their uv spectra and the treatment of a sample with 1 *N* sodium hydroxide solution for 10 min at room temperature followed by neutralization with Dowex 50X-8 resin (pyridinium form) to remove any phosphate or 3′-hydroxyl protecting groups. Samples were then chromatographed on Whatman No. 1 paper in solvent D. More complete characterization was carried out by treatment with concentrated ammonia to remove all protecting groups. After separation by chromatography, the base composition was determined by enzymatic degradation with snake venom phosphodiesterase, spleen phosphodiesterase, or alkaline phosphatase as described previously.[18]

DL-Dihydrocoumarilic acid was prepared by known procedures[9] and resolved by crystallization with *d*-amphetamine to give D-(+)-dihydrocoumarilic acid, $[\alpha]^{25}D$ 22.7° (*c* 1.5, EtOH). *o*-Phenylenedioxyacetic acid was synthesized by published methods[10] and dihydrocinnamic acid was purchased from the Aldrich Chemical Co. The corresponding acid chlorides were prepared by standard methods using thionyl chloride and had boiling points, DHC chloride, 70–75° (0.3 mm); DCM chloride, 85–88° (0.4 mm); PDA chloride, 90–92° (0.5 mm). Dihydrocinnamic anhydride was prepared from the acid by treatment with acetic anhydride or by treatment with an equimolar amount of the acid chloride and had bp 190–200° (0.3 mm).

Adaptations of known procedures were used to synthesize the pyridinium salts of the base protected nucleotides d-pA^{Bz}, d-pC^{An}, and d-pG^{iB}.[19]

Extinction coefficients at neutral pH were as follows: d-pC^{An}, 17,700 (280 mμ); d-pC, 9,100 (273 mμ); d-pA^{Bz}, 18,300 (280

(18) H. G. Khorana, A. F. Turner, and J. P. Vizsolyi, *J. Amer. Chem. Soc.*, **83**, 686 (1961); H. G. Khorana and J. P. Vizsolyi, *ibid.*, **83**, 675 (1961); W. E. Razzel and H. G. Khorana, *J. Biol. Chem.*, **236**, 1144 (1961); M. Smith, D. H. Rammler, I. H. Goldberg, and H. G. Khorana, *J. Amer. Chem. Soc.*, **84**, 430 (1962).

(19) H. Schaller and H. G. Khorana, *ibid.*, **85**, 3828 (1963).

mμ); d-pA, 14,500 (260 mμ); d-pT, 6,400 (280 mμ) and 9,600 (267 mμ); d-pG^{iB}, 11,500 (280 mμ); d-pG, 13,600 (253 mμ).

α-Chymotrypsin was purchased from Worthington Biochemical Corporation, grades CDI and CDS. The specific activity of the different batches ranged from 47 to 54 units/mg and was not checked. Each batch was checked for lack of nuclease activity. Enzite-CHT is a product of Miles-Serovac and had a specific activity of 0.56 units/mg of dry material.

Preparation of Protected Nucleosides (1a–d). The nucleoside (1 mmol) was suspended in dry pyridine (4 ml) at 0° and the acid chloride or anhydride [dihydrocinnamic anhydride, *o*-phenylenedioxyacetic chloride, or D-(+)-dihydrocoumariloyl chloride (4.0 mmol)] added dropwise. The mixture was allowed to warm to room temperature and stirred for 16 hr, then cooled to −20° and treated with water (5 ml). After stirring 1 hr the mixture was extracted with methylene chloride (3 × 5 ml) and the organic extracts were washed with water, 5% sodium bicarbonate solution, and water again, and dried over sodium sulphate. The methylene chloride solution was concentrated to 3 ml and the protected nucleoside precipitated by addition of the solution to dry ether. The yields were generally 80–90%. Further purification if necessary was by preparative tlc (silica, solvent C). Chromatographic data is summarized in Table V.

TABLE V

CHROMATOGRAPHIC DATA FOR NUCLEOSIDES AND NUCLEOTIDES

Compd	R_f values: Whatman No. 1, solvent A	Whatman No. 1, solvent D	Cellulose tlc, solvent D	Silica tlc, solvent C
1a, R = DHC				0.84
1b, R = PDA				0.89
1c, R = DCM (racemate)				0.85
1d, R = DCM [D-(+) isomer]				0.85
2a, R′ = DHC		0.67		
2b, R′ = PDA		0.63		
2c, R′ = DCM [D-(+)isomer]		0.61		
3a, d-pC^{An}-ODHC		0.59		
3b, d-pC^{An}-OPDA		0.62		
3c, d-pC^{An}-ODCM		0.68		
5, d-pA^{Bz}-ODHC		0.65		
6, d-pG^{iB}-ODHC		0.64		
7, d-CEpA^{Bz}pT-ODCM		0.74	0.75	
8, d-CEpA^{Bz}pT		0.64	0.61	
9, d-CEpA^{Bz}pTpC^{An}-ODCM		0.58	0.56	
10, d-CEpA^{Bz}pTpC^{An}		0.45	0.36	
11, d-CEpC^{An}pT-ODHC		0.78	0.77	
12, d-CEpC^{An}pT		0.56	0.46	
13, d-CEpC^{An}pTpC^{An}-ODCM		0.69	0.61	
14, d-CEpC^{An}pTpC^{An}		0.49	0.37	
15, d-CEpC^{An}pTpC^{An}pT-ODCM		0.27		
16, d-CEpC^{An}pTpC^{An}pT		0.20		
17, d-CEpTpC^{An}-ODHC	0.77	0.72		
18, d-CEpTpC^{An}	0.66	0.62		
19, d-CEpTpC^{An}pA^{Bz}-ODHC	0.69	0.67		
20, d-CEpTpC^{An}pA^{Bz}	0.62	0.65		
21, d-CEpTpC^{An}pA^{Bz}pG^{iB}-ODHC	0.59	0.50		
22, d-CEpTpC^{An}pA^{Bz}pG^{iB}	0.51	0.46		

Preparation of Protected Nucleotides (2a–c, 3a–c, and 4–6). The pyridinium salt of the nucleotide (1.0 mmol), base protected if necessary, was azeotroped with dry pyridine (2 × 5 ml) and dissolved in dry pyridine (5 ml). After cooling to −30° the solution was treated with the acid chloride or anhydride (10.0–20.0 mmol). The mixture was warmed to room temperature and stirred in the dark for 4–20 hr. The reaction was stopped by cooling to −30° and adding dry methanol (10 ml). After standing at room temperature for 3 hr, pyridine (10 ml) was added and the mixture stored overnight at room temperature. Solvents were removed *in vacuo*; the resulting oil was triturated with dry ether (5 × 20 ml). The residue was dissolved in dry pyridine and then added dropwise to dry ether and the resulting off-white precipitate collected. Yields of nucleotides protected by the DHC group were almost quantitative (95–100%). Mononucleotides carrying the DCM or PDA protecting groups required further purification by paper chromatography (Whatman No. 3 MM, solvent D). The band of protected nucleotide (R_f 0.60 to 0.63) was desalted by washing with absolute ethanol and then eluted with water. The yields were poor (40–45%).

Kinetic Studies. The protected nucleoside or nucleotide (0.05 mmol nucleoside or 0.1 mmol nucleotide, *i.e.*, 0.1 mmol substrate) was suspended or dissolved in 0.1 *N* NaCl solution (0.5 ml) and the pH adjusted to 7.8 (using a pH meter) with dilute NaOH solution. α-Chymotrypsin was added as a solution in 0.1 *N* NaCl and the volume of the mixture adjusted to 2.0 ml. The solution was incubated at 37° and the pH maintained at between 7.6 and 8.0 by the constant addition of 0.1 *N* NaOH solution. For the separation and identification of the reaction products silica gel plates were used for the nucleosides (solvent C) and cellulose plates for the nucleotides (solvent D). Control experiments excluding the enzyme were run in all cases; no observable hydrolysis was ever detected. Control experiments were also run excluding the substrate. It was observed that at high enzyme concentrations (5×10^{-4} *M*) autolysis occurred. Correspondingly, the results of the kinetic runs were corrected for this factor.

In order to economize on materials, enzymatic degradations of oligonucleotides were carried out on a 25-μmol scale. The volume of the mixture was adjusted to 0.5 ml. The results are summarized in Tables I and II.

Stability Studies. Samples of the protected nucleosides and nucleotides (1–5 μmol) were dissolved in 0.5 *M* TEAB solution at pH 8.0 and 9.5 (0.2 ml). The solutions were analyzed at time intervals by paper chromatography; the percentage of hydrolysis was measured by elution of the spots from the chromatograms and measurement of the absorbance at 260 mμ. The results are summarized in Table VI.

TABLE VI

STABILITY STUDIES ON THE PROTECTED NUCLEOSIDES AND NUCLEOTIDES[a]

Compd	Time, hr	Hydrolysis, %: pH 8.0	pH 9.5
1b	4	0	0
	24	0	2
1d	4	0	0
	24	0	3
2b	4	2	100
	24	11	100
2c	4	4	100
	24	13	100

[a] See text for experimental details. No hydrolysis was observed with **1a** and **2a**.

Degradations with Enzite-CHT. Enzite-CHT (100 mg, 56 units) was rehydrated by stirring in 0.5 *M* phosphate buffer at pH 8.0 for 1 hr and then collected by centrifugation. The Enzite-CHT was washed twice more and then suspended in 0.05 *M* phosphate buffer (pH 8.0, 0.8 ml) and incubated at 37° with a solution of the nucleotide (1.6 μmol in 0.2 ml of buffer). The reactions were followed by tlc (cellulose, solvent D).

The Enzite-CHT was recovered for reuse by centrifugation and washed with buffer. The recovered material was stored as a slurry at 0°.

Comparison degradations were run using the same amounts of nucleotides with free α-chymotrypsin (1 mg, 50.4 units) in 0.1 ml of 0.05 *M* phosphate buffer (pH 7.8). The results are summarized in Table III.

Synthesis of Oligonucleotides

d-CEpA^{Bz}pT-ODCM, 7. (a) The mononucleotides of d-CEpA^{Bz} (1.50 g, 2.64 mmol) and d-pT-ODCM (**2c**, 1.0 g, 1.60 mmol) were azeotroped with dry pyridine (3 × 10 ml), taken up in dry pyridine (15 ml), and treated with mesitylene sulfonyl chloride (MSC) (900 mg, 4.1 mmol). After 3 hr the reaction was terminated by cooling and adding water (15 ml). The solution was chromatographed on a diethylaminoethyl (DEAE) cellulose column (4 × 51 cm, gradient of triethylammonium bicarbonate (TEAB), 20% EtOH, 2 l. each 0.1 and 0.7 *M*). The main peak of dinucleotide was pooled and lyophilized. However, this product had R_f 0.64 (Whatman No. 1, solvent D) and was

thought to be d-CEpA^{Bz}pT, the D-(+)-dihydrocoumariloyl group being lost during chromatography. The yield was 830 mg, 60%.

(b) The reaction was repeated using d-CEpA^{Bz} (280 mg, 0.49 mmol) and d-pT-ODCM (200 mg, 0.32 mmol) and worked up by chromatography on 8 sheets of Whatman 3 MM paper, solvent D for 30 hr. The product, 165 mg, 51%, had R_f 0.74 (Whatman No. 1 solvent D). Starting material and d-CEpA^{Bz}pT (R_f 0.62), was also obtained (24 mg, 7.5%).

d-CEpA^{Bz}pT (8). The above dinucleotide (from b) (150 mg, 149 μmol) was dissolved in 0.05 *M* phosphate buffer pH 7.5 (10 ml) and treated with a solution of α-chymotrypsin in buffer (15 mg, 5 ml, enzyme–substrate ratio 5 units/μmol), and the mixture incubated at 37° for 4 hr. The product was isolated by preparative paper chromatography (solvent D) on 4 sheets of Whatman 3 MM paper for 28 hr. This dinucleotide, 115 mg, 90%, had R_f 0.64 (Whatman No. 1, solvent D) and was identified as d-CEpA^{Bz}pT by comparison of its R_f and uv spectrum with those of a sample prepared by cyanoethylation of d-pA^{Bz}pT (R_f 0.29, solvent D). The protecting groups were removed by treatment with concentrated ammonia to give d-pApT (R_f 0.13, solvent D; 0.31, solvent B; 0.35, solvent F). Degradation with snake venom phosphodiesterase gave dpA–dpT, 1.00:0.96.

d-CEpA^{Bz}pTpC^{An}-ODCM (9). The dinucleotide **8** (100 mg, 116 μmol) was condensed in the usual way with d-pC^{An}-ODCM (**3c**) (100 mg, 134 μmol) using MSC (100 mg, 460 μmol) for 3.5 hr. The product 19 mg (11%) was isolated by chromatography on 4 sheets of Whatman 3 MM paper in solvent D for 32 hr. Characterization was by removal of all the protecting groups with concentrated ammonia to give d-pApTpC (R_f 0.05, solvent D). Degradation with snake venom phosphodiesterase gave d-pA–d-pT–d-pC, 1.00:1.01:1.12.

d-CEpA^{Bz}pTpC^{An} (10). Treatment of the trinucleotide **9** (5 mg, 3.3 μmol) with α-chymotrypsin (0.1 mg, enzyme–substrate ratio 1.5 units/μmol) in 0.05 *M* phosphate buffer, pH 7.5 (0.5 ml), at 37° for 3 hr and work-up by paper chromatography gave **10** (3.1 mg, 69%).

d-CEpC^{An}pT-ODHC (11). Condensation of d-CEpC^{An} (230 mg, 0.40 mmol) and d-pT-ODHC (**2a**, 395 mg, 0.65 mmol) using dicyclohexylcarbodiimide (DCC) (1.60 g, 7.60 mmol) in the presence of dry Dowex 50W-X8 pyridinium form for 5 days and work-up by chromatography on a column of DEAE cellulose (4 × 34 cm), TEAB gradient (1.5 l. each of 0.05 and 0.50 *M*) gave **11** (230 mg, 53%). Treatment with ammonia gave the dinucleotide d-pCpT (R_f 0.13, solvent D; R_f 0.33, solvent B; and R_f 0.41, solvent F) which was identified by degradation with snake venom phosphodiesterase (d-pC–d-pT, 1.00:1.08).

d-CEpC^{An}pT (12). The above dinucleotide **11** (100 mg, 92 μmol) was incubated with α-chymotrypsin (10 mg, enzyme–substrate ratio 5.4 units/μmol) in 0.05 *M* phosphate buffer, pH 7.5, (10 ml) for 16 hr. Isolation was by paper chromatography (Whatman 3 MM, solvent D) (nucleotide R_f 0.56, α-chymotrypsin 0.00) and lyophilization (63 mg, 73%).

d-CEpC^{An}pTpC^{An}-ODCM (13). The dinucleotide **12**, (60 mg, 63 μmol) was condensed with d-pC^{An}-ODCM (**3**, 120 mg, 161 μmol) using MSC (90 mg, 410 μmol) in dry pyridine (6 ml) for 7 hr. The reaction was terminated by cooling and treating with water (6 ml). Chromatography of a small sample of the reaction mixture (2 ml) on Whatman No. 3 MM paper, solvent D, gave a main band, R_f 0.69 (13.1 mg). Treatment of this band with alkali, to remove the phosphate and hydroxyl protecting groups, gave a mixture of the trinucleotide d-pC^{An}pTpC^{An} and the mononucleotide d-pC^{An} in equal amounts. This showed that separation of the excess d-pC^{An}-ODCM and d-CEpC^{An}pTpC^{An}-ODCM could not be achieved by paper chromatography. The yield of the protected trinucleotide was estimated from these results to be approximately 41%.

d-CEpC^{An}pTpC^{An} (14). The remaining solution from the above reaction (10 ml) was concentrated *in vacuo* and the residue azeotroped with water (2 × 10 ml), ethanol–water (10 ml), and water again (3 × 10 ml) to remove all traces of pyridine. The residue was dissolved in 0.05 *M* phosphate buffer, pH 7.5 (10 ml), filtered to remove some insoluble material and incubated with α-chymotrypsin (5 mg, enzyme–substrate ratio 1.8 units/μmol) at 37° for 2 hr. The partially protected trinucleotide, **14**, was isolated by chromatography (Whatman 3 MM, solvent D) followed by lyophilization, 10 mg, 13%. The trinucleotide was characterized by removal of all the protecting groups with concentrated ammonia to give d-pCpTpC (R_f 0.06, solvent D; 0.27, solvent B; 0.33, solvent G). Degradation with snake venom phosphodiesterase gave d-pC–d-pT, 1.98:1.00.

d-CEpC^{An}pTpC^{An}pT-ODCM (15). The mononucleotide d-pT-ODCM (**2c**, 20 mg, 32 μmol) was condensed with the trinucleotide **14** (239 OD_{260}, 6.9 μmol) using MSC (20 mg, 91 μmol) in dry pyridine (1 ml) for 15 hr. The tetranucleotide **15** was isolated by paper chromatography (Whatman 3 MM, solvent D) and lyophilization, 29 OD_{260}, 10%. The product was characterized by treatment with ammonia to remove the protecting groups to give d-pCpTpCpT (R_f 0.02, solvent D; 0.20, solvent B; 0.23, solvent F). Degradation with snake venom phosphodiesterase gave d-pC–d-pT, 1.00:1.01.

d-CEpC^{An}pTpC^{An}pT (16). A sample of the tetramer **15** (10 OD_{260}, 0.23 μmol) was dissolved in 0.05 *M* phosphate buffer, pH 7.5 (0.2 ml), and heated with α-chymotrypsin (2 units, enzyme–substrate ratio 8.7 units/μmol) at 37° for 2 hr. Paper chromatography (Whatman 3 MM, solvent D) gave **16**, 7.5 OD_{260}, 75%.

d-CEpTpC^{An}-ODHC (17). d-pC^{An}-ODHC (**4**, 1.0 g, 1.37 mmol) and d-CEpT (1.4 g, 3.1 mmol) both as pyridinium salts were condensed in dry pyridine (10 ml) using MSC (2.1 g, 9.6 mmol) as condensing agent. After 2.5 hr the reaction was stopped by the addition of water (10 ml) and after dilution the solution was chromatographed on a DEAE cellulose column (4 × 90 cm). Elution was with a gradient of TEAB containing 20% EtOH (0.05 to 0.2 *M*, 4 l. of each). The main peak was pooled and lyophilized to give the dinucleotide d-CEpTpC^{An}-ODHC, 806 mg, 54%.

d-CEpTpC^{An} (18). d-CEpTpC^{An}-ODHC (**17**, 11,100 OD_{280}, 0.46 mmol) was dissolved in 0.5 *M* phosphate buffer, pH 7.5 (100 ml), and treated with α-chymotrypsin (50 mg, enzyme–substrate ratio 5.4 units/μmol) for 8 hr at 37°. The solution was streaked onto 6 sheets of Whatman 3 MM paper and chromatographed in solvent E. The main band was eluted and lyophilized to give the partially protected dinucleotide **18**, 10,890 OD_{280}, 98%. The recovery of **17** was 67 OD_{280}, 0.6%.

d-pTpC^{An}. A sample of d-CEpTpC^{An} (**18**, 3 mg) was treated with 1 *N* sodium hydroxide solution (0.5 ml) for 10 min. Chromatography on Whatman No. 1 in solvents D and A gave a single spot of d-pTpC^{An}, R_f 0.27, solvent D; R_f 0.42, solvent A.

d-pTpC. The solution from the above reaction was concentrated to dryness and the residue taken up in concentrated ammonia (1 ml) for 2 days at room temperature. The solution was streaked onto Whatman 3 MM paper and chromatographed in solvent B. A single band of d-pTpC was eluted, R_f 0.29 (R_f 0.38, solvent F). Degradation with snake venom phosphodiesterase gave d-pT–d-pC, 1.00:1.02. Treatment with bacterial alkaline phosphatase gave a single spot of d-TpC, R_f 0.59 (solvent B), which on degradation with spleen phosphodiesterase gave the monomers d-Tp and d-C in the ratio 1.00:0.95.

d-CEpTpC^{An}pA^{Bz}-ODHC (19). d-CEpTpC^{An} (10,800 OD_{280}, 0.45 mmol) and d-pA^{Bz}-ODHC (930 mg, 1.28 mmol) were dried by repeated evaporation with dry pyridine, and MSC (790 mg, 3.60 mmol) was added as a solution in dry pyridine (5 ml). After 2.5 hr at room temperature the reaction was stopped by cooling and adding water (5 ml). The solution was streaked onto Whatman 3 MM paper and chromatographed in solvent E. The main band (R_f 0.69) was eluted and lyophilized to give **19**, 8,700 OD_{280}, 46%.

d-CEpTpC^{An}pA^{Bz} (20). d-CEpTpC^{An}pA^{Bz}-ODHC (7,250 OD_{280}, 170 μmol) was dissolved in 0.5 *M* phosphate buffer, pH 7.5, (90 ml) and treated with α-chymotrypsin (25 mg, enzyme–substrate ratio 7.3 units/μmol) for 24 hr. Work-up by paper chromatography on Whatman 3 MM paper, solvent E, gave the partially protected trinucleotide **20**, 6,600 OD_{280}, 91%.

d-pTpC^{An}pA^{Bz}. d-CEpTpC^{An}pA^{Bz} (**19**, 2 mg) and also d-CEpTpC^{An}pA^{Bz}-ODHC (**20**, 2 mg) were each treated with 1 *N* sodium hydroxide solution (0.5 ml) for 10 min. Chromatography on Whatman No. 1 paper in solvents A (R_f 0.29) and D (R_f 0.25) gave a single spot of d-pTpC^{An}pA^{Bz}.

d-pTpCpA. The solutions from the above reactions were treated with concentrated ammonia (2 ml) for 2 days at room temperature and then chromatographed on Whatman 3 MM paper in solvent B. A single band of d-pTpCpA, R_f 0.27, was observed (R_f 0.31, solvent F). This band was eluted and part of it treated with phosphatase to give d-TpCpA (single spot, R_f 0.53 in solvent B) followed by degradation with spleen phosphodiesterase to give d-Tp, d-Cp, and d-A in the ratio 1.00:0.97:0.96. The second part of d-pTpCpA was degraded with snake venom phosphodiesterase to give d-pT, d-pC, and d-pA in the ratio 1.00:0.98:1.03.

d-CEpTpC^{An}pA^{Bz}pG^{iB}-ODHC (21). d-CEpTpC^{An}-pA^{Bz} (**20**, 6000 OD_{280}, 140 μmol) was condensed with d-pG^{iB}-ODHC (440

mg, 625 μmol) using MSC (330 mg, 1.50 mmol) in dry pyridine (5 ml). The reaction was stopped after 4 hr by cooling and adding water (5 ml). Work-up by paper chromatography (solvent E) gave the fully protected tetranucleotide **21**, 7500 OD_{280}, 36%.

d-CEpTpC^{An}pA^{Bz}pG^{iB} (22). Treatment of the preceding compound (1000 OD_{280}, 18.5 μmol) with α-chymotrypsin (4.0 mg, enzyme–substrate ratio 10.8 units/μmol) in phosphate buffer, pH 7.5 (40 ml), at 37° for 24 hr gave the partially protected tetranucleotide d-CEpTpC^{An}pA^{Bz}pG^{iB} (**22**), 615 OD_{280}, 82%, isolated by paper chromatography in solvent E.

d-pTpC^{An}pA^{Bz}pG^{iB}. d-CEpTpC^{An}pA^{Bz}pG^{iB} (**22**, 50 OD_{280}) was hydrolyzed with 1 *N* sodium hydroxide solution (0.5 ml) for 10 min to give d-pTpC^{An}pA^{Bz}pG^{iB}, and isolated by chromatography in solvent D, R_f 0.19.

d-pTpCpApG. Treatment of d-pTpC^{An}pA^{Bz}pG^{iB} with concentrated ammonia for 2 days gave the tetranucleotide d-pTpCpApG, R_f 0.23, solvent B (R_f 0.20, solvent F). The tetranucleotide was characterized by degradation with snake venom phosphodiesterase to give the mononucleotides in the ratio d-pT–d-pC–d-pA–d-pG, 1.00:1.01:1.01:0.96. Further characterization was by removal of the terminal phosphate to give d-TpCpApG (R_f 0.45, solvent B) followed by degradation with spleen phosphodiesterase to give d-Tp–d-Cp–d-Ap–d-G, 1.00:1.10:1.05:1.08.

Registry No.—**1a**, 23706-27-6; **1b**, 37731-23-0; **1c**, 37731-24-1; **1d**, 37731-25-2; **2a**, 37731-26-3; **2b**, 37731-27-4; **2c**, 37731-28-5; **3a**, 37731-29-6; **3b**, 37731-30-9; **3c**, 37731-31-0; **5**, 37731-32-1; **6**, 37731-33-2; **7**, 37731-34-3; **8**, 37731-35-4; **9**, 37731-36-5; **10**, 37731-37-6; **11**, 37731-38-7; **12**, 37731-39-8; **13**, 37731-40-1; **14**, 37731-41-2; **15**, 37731-42-3; **16**, 37731-43-4; **17**, 37731-44-5; **18**, 37731-45-6; **19**, 37731-46-7; **20**, 37731-47-8; **21**, 37818-77-2; **22**, 37731-48-9; D-(+)-DCM, 17332-01-3; D-(+)-DCM (acid chloride), 37731-49-0; PDA, 37731-50-3; PDA (acid chloride) 37731-51-4; DHC, 501-52-0; DHC (acid chloride), 645-45-4; DHC (anhydride), 15781-96-1; d-pC^{An}, 32909-08-3; d-pC, 1032-65-1; d-pA^{Bz}, 4546-64-9; d-pA, 653-63-4; d-pT, 365-07-1; d-pG^{iB}, 32909-09-4; d-pG, 902-04-5; d-CEpA^{Bz}, 37740-89-9; d-CEpC^{An}, 37740-90-2; d-pTpC^{An}, 37731-45-6; pTpC, 2147-10-6; d-pTpC^{An}pA^{Bz}, 37740-92-4; d-pTpCpA, 37740-93-5; d-pTpC^{An}pA^{Bz}pG^{iB}, 37740-94-6; d-pTpCpApG, 37740-95-7.

Acknowledgment.—This work was supported by the National Institute of Medical Sciences, National Institutes of Health, Bethesda, Md. (Contract No. PH-67-1157). Valuable discussions with Drs. O. M. Friedman, D. Söll, J. B. Hendrickson, N. A. Starkovsky, and J. Hachmann are gratefully acknowledged. The technical assistance of Mrs. C. J. Crosscup and Mr. P. B. Szabady is appreciated.

Intermediates in the Ozonation of Simple Alkynes

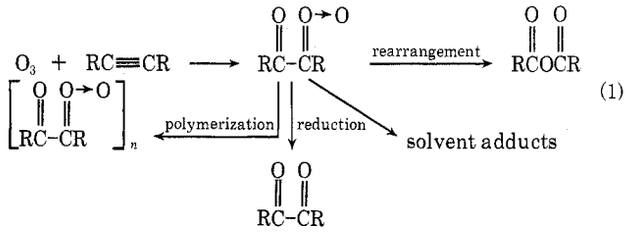
WILLIAM B. DEMORE* AND CHORNG-LIEH LIN

Jet Propulsion Laboratory, California Institute of Technology, Pasadena, California 91103

Received August 24, 1972

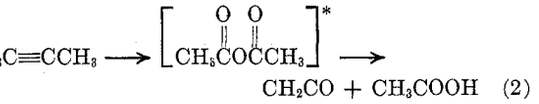
The reactions of O_3 with HC≡CH, $CH_3C{\equiv}CH$, $CH_3C{\equiv}CCH_3$, and $C_2H_5C{\equiv}CH$ have been studied in liquid CO_2 at −45°. The initial products were observed by *in situ* infrared spectroscopy, and subsequent changes occurring upon warm-up or flash vaporization of the mixture were followed by ir or gc analysis. The principal new spectral feature for all alkynes except acetylene was a strong carbonyl absorption near 1740 cm^{-1}, and all alkynes gave relatively weak absorption bands in the carbonyl region which are attributed to the corresponding acid anhydrides. The 1740-cm^{-1} band was shown to be an unstable precursor of the acid anhydrides and other products. The overall mechanism, the identity of the precursor, and factors influencing the final product distribution are discussed.

Relatively little is known about the detailed mechanism of alkyne ozonation. According to the Criegee–Lederer mechanism,[1] an acylcarbonyl oxide is produced which may react in a variety of ways.



(1)

Anhydride formation, first proposed by Paillard and Wieland[2] to explain product ratios in the ozonation of heptyne-1, has been observed only in the ozonation of diphenylacetylene.[3] For the gas-phase ozonation of simple alkynes, it has been suggested[4] that products corresponding to fission of the triple bond arise from the decomposition of an excited anhydride intermediate. For example, ketene and acetic acid produced by the gas-phase ozonation of dimethylacetylene can be explained as follows.



(2)

According to this interpretation, the excited anhydride is short-lived and decomposes completely at 1-atm pressure. However, at higher pressures or in the condensed phase the excited intermediate should be collisionally stabilized. The present work was carried out in an effort to isolate the anhydride intermediate, and to provide additional information on the anhydride precursor. The alkynes studied include acetylene, methylacetylene, dimethylacetylene, and ethylacetylene.

Apparatus and Methods

The experiments involved ozonation of the alkynes in liquid CO_2 at about −45° with *in situ* ir analysis of the products. The reaction cell (Figure 1) was a vac-

(1) R. Criegee and M. Lederer, *Justus Liebigs Ann. Chem.*, **583**, 29 (1953).
(2) H. Paillard and C. Wieland, *Helv. Chim. Acta*, **21**, 1356 (1938).
(3) E. Dallwigk, H. Paillard, and E. Briner, *ibid.*, **35**, 1377 (1952).
(4) W. B. DeMore, *Int. J. Chem. Kinet. III*, 161 (1971).