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ENZYMATIC REMOVAL OF ACYL PROTECTING GROUPS. THE USE OF DIHYDRO-CINNAMOYL GROUP IN OLIGONUCLEOTIDE SYNTHESIS AND ITS CLEAVAGE BY α-CHYMOTRYPSIN

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One of the major problems in oligonucleotide synthesis is the design of suitable protecting groups for the reactive hydroxyl and amino functions of nucleosides and nucleotides. Ideally, these protecting groups should satisfy the following conditions: 1) they should be stable under the reaction conditions; 2) they should be removable under essentially neutral conditions, and 3) they should display selectivity for the one or the other of the hydroxyl or amino functions.[1] Acyl protecting groups usually satisfy the first of these conditions. However, their removal requires relatively drastic alkaline treatment, expecially in the case of di-, tri- and higher oligonucleotides[2] when this treatment is the most destructive. One of the current approaches to this problem consists in exploring new acyl protecting groups such as phenoxyacetyl[3] or p-benzoylpropionyl[4] which are expected to come off under milder conditions than the acetyl or the benzoyl group. In the course of our work on the synthesis of oligoribonucleotides we have explored an alternate way for deprotecting nucleotidic materials under neutral conditions. This method is based on the use of suitable enzymes such as α-chymotrypsin. It is interesting that this enzymic method proved not only to be effective, but also demonstrated a desirable selectivity in its action.

Preliminary experiments on simple nucleosides and nucleotides showed that this enzyme was very sluggish in hydrolyzing acetic acid esters. Better results were obtained with N-acetyl-L-phenylalanyl and cinnamoyl esters[5], but these protecting groups did not satisfy the requirements listed above. However, dihydrocinnamoyl appeared to be a potentially useful protecting group. Dihydrocinnamoyl esters fulfill to a certain extent the steric requirements of α-chymotrypsin;

and, in addition, both dihydrocinnamoyl chloride and anhydride reacted with model nucleosides and nucleotides quite smoothly and completely. A series of compounds, including a dinucleoside monophosphate, protected with the dihydrocinnamoyl group (DHC) at various positions, have been prepared for the purpose of this study.

Preparation of protected compounds.-

N,$0^{2'}$,$0^{3'}$-Triacetyladenosine-5'-phosphate and $0^{2'}$,$0^{5'}$-diacetyluridine-3'-phosphate were prepared according to the literature.[6] A similar procedure was used for the preparation of N,$0^{2'}$,$0^{3'}$-tri-(DHC)adenosine-5'-phosphate.

$0^{3'}$,$0^{5'}$-Diacetyl-2'-deoxyuridine was prepared by the acetylation of 2'-deoxyuridine with an excess of acetic anhydride in pyridine; $0^{3'}$-acetyl-2'-deoxyuridine was prepared by acetylation of $0^{5'}$-dimethoxytrityluridine followed by detritylation with 80% acetic acid. By a similar procedure, using dihydrocinnamoyl (DHC) chloride or anhydride instead of acetic anhydride, $0^{3'}$, $0^{5'}$-di(DHC)-2'-deoxyuridine,$0^{3'}$-(DHC)-2'-deoxyuridine, and $0^{3'}$-(DHC)-thymidine have been obtained.

Condensation of N,$0^{2'}$,$0^{3'}$-tri-(DHC)adenosine-5'-phosphate with $0^{3'}$-(DHC)-thymidine in the presence of dicyclohexylcarbodiimide in pyridine solution,[7] gave the fully protected mixed dinucleoside monophosphate, namely, tetra(DHC)thymidylyl-(3'-5')-adenosine.

The newly prepared compounds were characterized by analysis, nmr spectra, and Rf values in various paper chromatographic and tlc systems.

Enzymic hydrolysis with α-chymotrypsin.-

In a typical experiment, 10 mg of the protected compound was dissolved (or suspended) in a mixture of 0.18 ml of acetonitrile and 0.42 ml of 0.05M potassium phosphate buffer, pH 7.0. The enzyme was added as a solution in 0.2 ml of water. Its amount was varied in different experiments so as to have the enzyme: substrate ratio from 1:1 down to 1:500 by weight. The reaction mixture was incubated at 37°C, and aliquots were examined by tlc at suitable intervals. Silica gel plates (F-254, E. Merck) were used for nucleosides (solvent system: ethyl acetate-ethanol 9:1 v/v), and cellulose F (E. Merck) plates for nucleotides (solvent system: 1 M ammonium acetate pH 7.5: ethanol 3:7 v/v). Ratios of products formed upon hydrolysis were determined on the basis of O.D. measurements at maximum absorption of individual compounds separated by preparative tlc. Blank experiments (excluding the enzyme) were run in all cases: no observable hydrolysis was ever detected. The activity of the enzyme was checked by using N-acetyltyrosine ethyl ester as substrate.

The results of enzymic hydrolysis of a series of acyl nucleosides and nucleotides are presented in Table I.

TABLE I

Protected compound*)	enzyme/ substrate ratio	time $37^{o}C$ (hr)	Product(s)
$O^{3'},O^{5'}$-diacetyl-dUrd	1:1	48	Starting material, intermediates, and dUrd in 4:4:1 ratio.
$O^{3'}$-acetyl-dUrd	1:1	48	Starting material and dUrd 5:1.
$N,O^{2'},O^{3'}$-triacetyl-5'-AMP	1:1	24	Starting material and miscellaneous products approx. 2:1.
$O^{3'},O^{5'}$-di(DHC)-dUrd	1:50	4	mixture of $O^{3'}$-(DHC)-dUrd and dUrd in a 1:1 ratio.
$O^{2'},O^{3'},O^{5'}$-tri(DHC)-Urd**)	1:50	4	mixture of $O^{2'},O^{3'}$-di(DHC)-Urd and the starting material in 1:5 ratio.
$N,O^{3'},O^{5'}$-tri(DHC)-dAdo	1:10	2	d-Ado only.
$N,O^{2'},O^{3'}$-tri(DHC)-5'-AMP	1:10	2	5'-AMP-only.
Tetra(DHC)adenosyl-(3'-5')-thymidine	1:10	12	Ap(3'-5')T only.***

*) dUrd stands for 2'-deoxyuridine; dAdo for 2'-deoxyadenosine, and (DHC) for dihydrocinnamoyl.
**) The derivatives of uridine were prepared in the same way as the corresponding derivatives of 2'-deoxyuridine.
***) The enzyme used in these experiments was checked for lack of nuclease activity.

Discussion.-

In these preliminary experiments, high enzyme/substrate ratios were used on purpose as complete deprotection was the desired feature. In the course of this work, however, a definite selectivity of the enzyme action at position 5' versus the other positions has been observed.

When 2'deoxyuridine was treated with one equivalent of dihydrocinnamoyl anhydride, the main reaction product was the 5'-substituted compound (Rf 0.40) and the chief byproduct was the 3',5'-disubstituted compound (Rf 0.60). On the other hand, treatment of the 3',5'-disubstituted compound with α-chymotrypsin gave only the 3'-substituted compound (RF 0.45) and 2'-deoxyuridine as the final product. The selectivity of the enzyme action is thereby clearly demonstrated. For further comparison, one can mention that treatment of the 3',5'-disubstituted compound with ammonia (methanol satd. with ammonia at $0^{o}C$ diluted tenfold with methanol, for 6 hr at room temperature) gave a 1:1 mixture of the two monosubstituted compounds as intermediates to 2'-deoxyuridine.

Practical applications of selective deprotection with α-chymotrypsin in oligonucleotide synthesis are now being studied and will be reported later.

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References.-

1) Comp. K.K. Ogilvie and R.L. Letsinger, J. Org. Chem., 32, 2365 (1967).

2) D. Söll and H.G. Khorana, ibid., 87, 360 (1965)

3) C.B. Reese and J.C.M. Stewart, Tetrahedron Letters, 4273 (1968)

4) R.L. Letsinger, M.H. Caruthers, P.S. Miller, and K.K. Ogilvie, J. Amer. Chem. Soc., 89 7146 (1967).

5) The related $O^{2'}(O^{3'})$-(DL-phenylalanyl)adenosine and $O^{2'}(O^{3'})$-(DL-phenylalanyl)uridine have been synthesized by D.H. Rammler and H.G. Khorana, J. Amer. Chem. Soc., 85, 1997 (1963). The carbobenzoxyphenylalanyl groups of $O^{5'}O^{3'}(O^{2'})$-di(N-carbobenzoxy-DL-phenylalanyl) adenosine (Z.A. Shabarova, N.I. Sokolova, and M.A. Prokof'ev, Doklady Akad. Nauk SSSR, 128, 740 (1959)) and N^6-(N'-carbobenzoxy-DL-phenylalanyl)cytidine (Z.A. Shabarova, N.I. Sokolova, L.A. Boikova, and M.A. Prokof'ev, Zhur. Obshchei Khim., 29, 2917 (1959)) can be transferred from the nucleosides onto glycine ethyl ester under the influence of α-chymotrypsin, dipeptides being thus produced.

6) D.H. Rammler and H.G. Khorana, J. Amer. Chem. Soc., 84, 3112 (1962).

7) Y. Lapidot and H.G. Khorana, ibid., 85, 3852 (1963).