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Studies on transfer ribonucleic acids and related compounds. VIII[1]. Further studies on aromatic phosphoramidates as a protecting group for phosphomonoesters

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ABSTRACT

Stability of aromatic phosphoramidates was studied using 2',3'-O-dibenzoyluridine 5'-phosphoramidates and N,2',3'-O-tribenzoylcytidine 5'-phosphate. The effect of dicyclohexylcarbodiimide in this mixture was investigated. Decomposition of the anilidate was slower in the presence of DCC.

Substituted anilidates of uridine 5'-phosphate were synthesized and the stability of these amidates in anhydrous pyridine was studied.

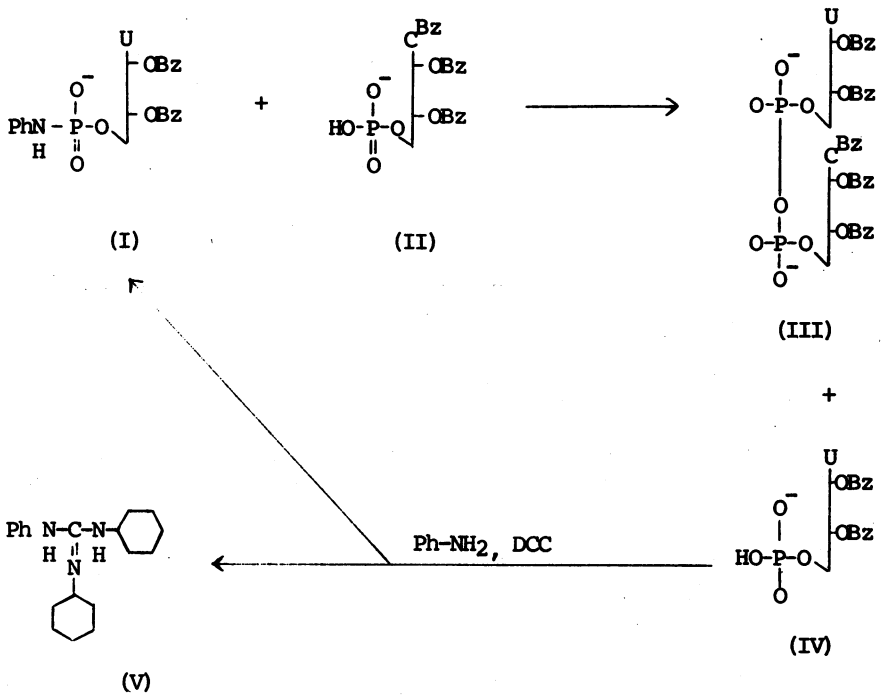
2'-O-Benzoyluridine 3'-phosphoranilidate and the corresponding β-naphthylidate were compared in their stabilities in anhydrous pyridine, 50% aqueous pyridine and 80% acetic acid. 2'-O-Benzoyluridine 3'-phosphoro-β-naphthylidate was used for synthesis of dinucleotides.

INTRODUCTION

Aromatic phosphoramidates of ribonucleosides have recently been shown to serve as protecting groups of phosphomonoesters in the synthesis of oligonucleotides[2,3]. During the condensation reaction, however, a partial decomposition and further activation of phosphoramidates have been observed. To improve the extent of the condensation and minimize side reactions in phosphodiester synthesis further studies on the properties of aromatic phosphoramidates are required. In this paper fate of 2',3'-O-dibenzoyluridine 5'-phosphoranilidate (I) in pyridine was investigated (Chart 1) in the presence of N,2',3'-O-tribenzoyl cytidine 5'-phosphate (II). The effect of dicyclohexylcarbodiimide (DCC) in this reaction was studied to find stability of I during condensation. To investigate the effect of the basicity of the aromatic amine of the phosphoramidates on the stability of the P-N linkage, substituted anilidates of uridine 5'-phosphate were synthesized and their half lives in anhydrous pyridine were measured.

Stability of aromatic amidates of 2'-O-benzoyluridine 3'-phosphate were then measured in anhydrous pyridine, 50% pyridine and 80% acetic acid. Two types of dinucleotides were synthesized using 2'-O-benzoyluridine 3'-phosphoro-β-naphthylidate.

Chart 1



Treatment of 2',3'-O-dibenzoyluridine 5'-phosphoranilidate with N,2',3'-O-tribenzoylcytidine 5'-phosphate in anhydrous pyridine in the absence and presence of DCC.

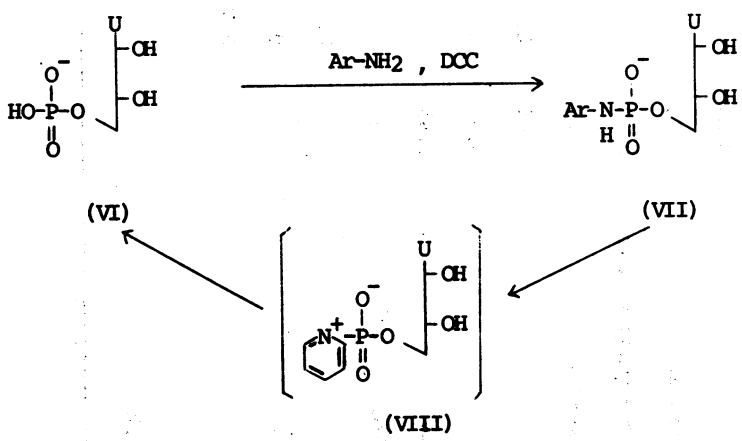
During coevaporation of 2',3'-O-dibenzoyluridine 5'-phosphoranilidate(I) with pyridine in the presence of N,2',3'-O-tribenzoylcytidine 5'-phosphate(II) decomposition of I to the corresponding phosphate (IV) was observed (27%) as detected by paper chromatography (Table I). After 10 hr mixed pyrophosphate (III) was detected together with P^1,P^2-diuridine 5'-pyrophosphate, and the phosphate IV increased to 40%. A trace of P^1,P^2-dicytidine 5'-pyrophosphate was also detected after 30 hr and did not increase appreciablly. After 117 hr the composition was as the following: the amidate I, 24%; the phosphate IV, 36%; P^1,P^2-diuridine 5'-pyrophosphate, 9%; the mixed pyrophosphate, 31%. In the presence of DCC the decomposition of the anilidate I was slower than in the absence of DCC. Fifty-six percent of the anilidate survived after 100 hr. This may be explained by formation of the phosphoramidate from the decomposed phosphate and the amine with DCC[4]. An excess of DCC could react also with the liberated amine to yield the carboxyamidine base (V). This strong

base may stabilize the phosphoranilidate. However, the activation of phosphomonoesters with DCC is inhibited by strong bases[5]. The formation of this guanidine during the condensation using DCC might decrease a yield of phosphodiester bond synthesis. Use of arylsulfonyl chloride as the condensing reagent could give higher yield in condensations. This reagent was used for reaction of the amidate as described later.

Synthesis and properties of some uridine 5'-phosphoraromatic amidates (VIII).

As shown in Chart 2, VII were synthesized from uridine 5'-phosphate and amines. Rate of formation of the phosphoramidate depends upon pKa value of the amine[4]. As summarized in Table II the rate of formation increased as the pKa of the amine became smaller. However, much weaker bases such as m-chloroaniline reacted slower than aniline. In reaction between uridine 5'-phosphate (IV) and weaker bases a side reaction to yield P^1,P^2-diuridine pyrophosphate occured in larger extent. A larger excess of the weak amine increased the yield of the phosphoramidate. Conversion of the pyrophosphate to the phosphoramidate was observed.

Chart 2



Stability of these amidates in anhydrous pyridine is also summarized in Table II. Uridine 5'-phosphoranilidate showed the greatest stability. The mechanism of decomposition in pyridine of phosphoramidates is not clear. In 50% aqueous pyridine pyridinium uridine 5'-phosphoranilidate was about 7 times stable than in anhydrous pyridine. Although protonation on the nitrogen of the anilidate could accelerate the liberation of the amine, protonation is not likely to occur in pyridine. The phosphoropyridinium compound (VIII) might

Table I.
Paper Chromatography and Paper Electrophoresis

Compounds	Paper Chromatography Rf in Solvent		Paper Electrophoresis Relative mobility to pU	
	A	B	pH 3.5	pH 7.5
Uridine 5'-phosphate	0.29	0.19	1.00	1.00
Cytidine 5'-phosphate	0.24		0.70	0.96
p^1,p^2-Diuridine 5'-pyrophosphate	0.13		1.15	
p^1-Uridine 5', p^2-cytidine 5'-pyrophosphate	0.09		0.89	
p^1,p^2-Dicytidine 5'-pyrophosphate	0.05		0.70	
pU(OBz)2	0.64	0.53		
PhNH-pU(OBz)2	0.84	0.80		
Uridine 5'-phosphoranilidate	0.45			0.61
Cytidine 5'-phosphoranilidate	0.41			0.57
U(OBz)-p		0.47		0.89
U(OBz)-p-NHPh		0.76		0.43
U(OBz)-p-NH-β-Naph		0.76		0.30
BzO-C^{Bz}(OBz)-p-U(OBz)-p				0.82
C^{Bz}(OBz)-p-U(OBz)-p-NH-β-Naph				0.64

Table II.
Properties of uridine
5'-aromatic phosphoramidates

Phosphoramidates	pKa of the amine	λ max	ε max	λ min	Half-reaction time for the synthesis (hr)	Yield (%)	Half-reaction time for decomposition (hr)
p-Anisidate	5.29	263,236	10.5×10^3	252,225	3	85	13
p-Toluidate	5.07	264,236	10.5×10^3	252	6	73	67
Anilidate	4.58	263,232	10.5×10^3	249	4	90	100
p-Chloroanilidate	3.99	260(s),232	11.5×10^3		2.5	50	27
p-Bromoanilidate	3.72	260(s),243	12.5×10^3		2.5	70	72
p-Iodoanilidate		265(s),247	15.0×10^3		2	79	35
m-Chloroanilidate	3.44	263,228	11.5×10^3	255	13	72	35

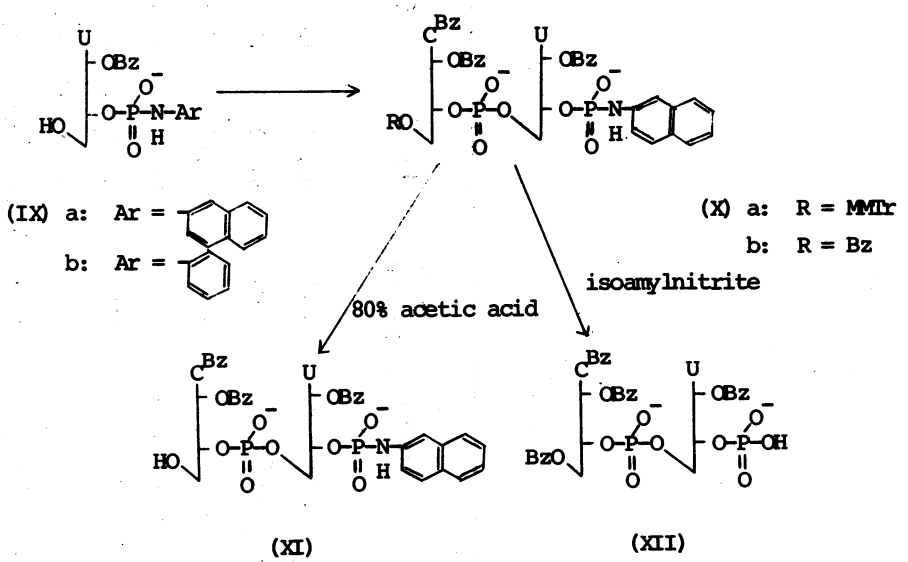
be formed as an intermediate in pyridine solution and further decomposition to the corresponding phosphate (VI) could occur during paper electrophoresis.

Synthesis and property of 2'-O-benzoyluridine 3'-phosphoraromatic amidates.

As discussed above aromatic phosphoramidates could have different stability. It would be reasonable to assume that an anisidate of nucleoside 3'-phosphates is more unstable than the corresponding anilidate. Although use of N,2'-O-dibenzoyladenosine 3'-phosphoro-p-anisidate and the anilidate gave the same yield in the synthesis of the trinucleotide[3], side products might be different in these cases.

β-Naphthylamidate of 2'-O-benzoyluridine 3'-phosphate (IXa) was synthesized since lipophilic substitution would facilitate isolation of the product with organic solvent[6]. The reaction to form the naphthylidate IXa was not quantitative and the product was isolated by extraction with organic solvents in 63% yield. Other lipophilic aromatic amines, such as p-triphenylmethyl aniline and α-naphthylamine did not react with 2'-O-benzoyluridine 3'-phosphate probably because of steric hindrance. Stability of the β-naphthylidate IXa was compared with that of 2'-O-benzoyluridine 3'-phosphoranilidate IXb in anhydrous pyridine, in 50% aqueous pyridine and in 80% acetic acid. The results are shown in Fig. 1. The anilidate IXb was more stable than the naphthylidate IXa in anhydrous or aqueous pyridine. Compared with the 5'-isomer

Chart 3



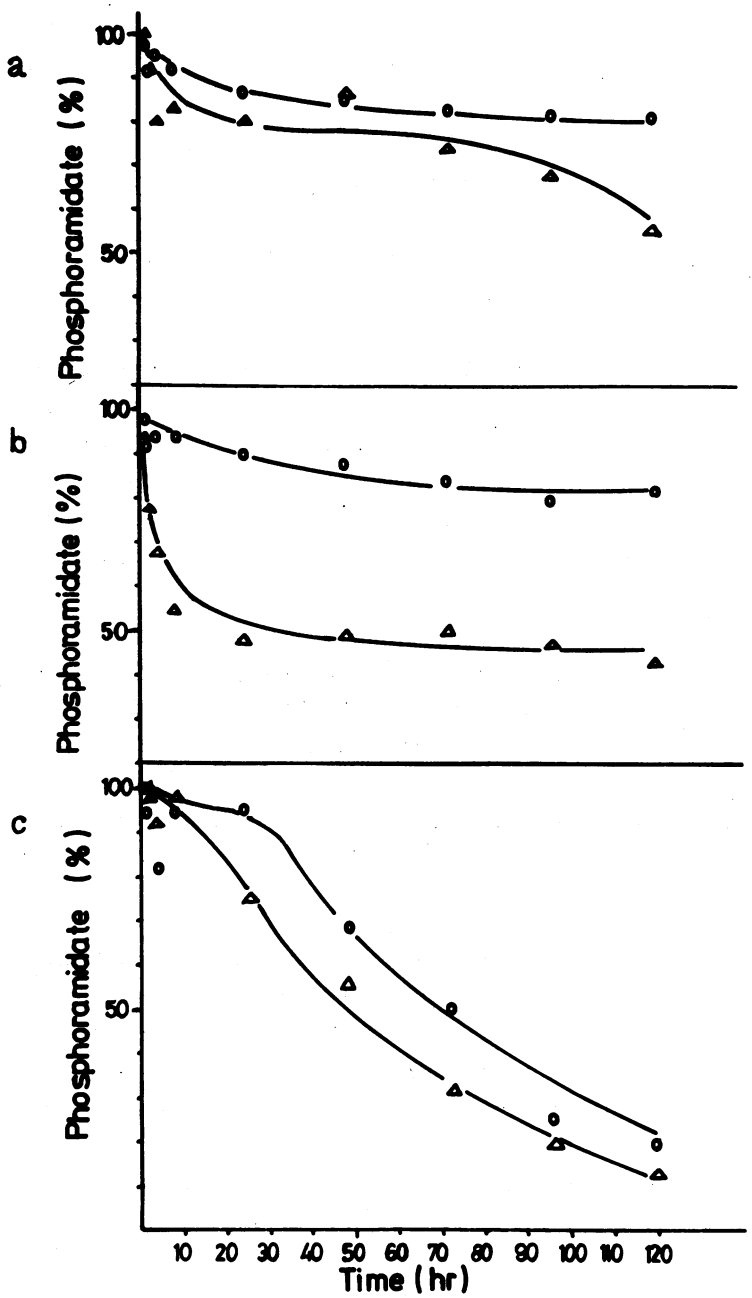


Fig. 1 Stability of the naphthylidate (IXa) (Δ—Δ) and the anilidate (IXb) (o—o) at 23° a, in anhydrous pyridine (0.2 M); b, in 50% pyridine (0.2 M); c, in 80% acetic acid (0.2 M). Aliquots (10 µl) were taken at appropriate time intervals and analyzed by paper electrophoresis (pH 7.5).

(Table II) the 3'-phosphoranilidate IXb was slightly more stable. In 80% acetic acid both of the amidate were essencially unchanged after 2 hr. This means the amidate IX can survive during removal of the 5'-O-monomethoxytrityl group. The naphthylidate IXa was then used for synthesis of dinucleotide.

Synthesis of ribodinucleotides using 2'-O-benzoyluridine 3'-phosphoro-β-naphthylidate (IXa).

The naphthylidate IXa was used for synthesis of two types of dinucleotides XI and XII (Chart 3). The intermediate X was tried to isolate by extraction with organic solvents after condensation using TPS. Partially purified Xa was treated with 80% acetic acid to yield XI which was separated from the starting material IXa by extraction using carbon tetrachloride and chloroform. The dinucleotide XII was obtained by treatment Xb with isoamyl nitrite and further purified by extraction with methylene chloride and n-butanol. The isolated yield was around 20% in both cases. Although the naphthylidate IXa showed reasonable lipophilicity the low yield may be due to loss during extraction.

In another instance, iBuO-G^{iBu}(OiBu)-p-U(OBz)-p$^{7)}$ was obtained in 25% yield after condensation of the naphthylidate IXa followed by isoamyl nitrite treatment and TEAE-cellulose column chromatography[8]. Synthesis of ribodinucleotides using anilidates of N,2'-O-protected nucleoside 3'-phosphates gave 20 to 65% yield[9]. These dinucleotides were used for synthesis of oligonucleotides having sequences of the yeast tyrosine tRNA 5'-end and the result will be published elsewhere.

EXPERIMENTAL

General methods ----- Paper chromatography was performed in solvent systems: A, n-butanol-acetic acid-water (5:2:3); B, ethanol-1 M ammonium acetate, pH 7.5 (7:3). Paper electrophoresis was performed at 900 V/40 cm using 0.05 M ammonium formate, pH 3.5 and 0.05 M triethylammonium bicarbonate, pH 7.5. Rf values and relative mobilities are given in Table I. Other general methods are as described previously[10].

Molar absorptions at pH 1 were: pU, 10 x 10^3 at 260 nm; pC, 13 x 10^3 at 280 nm; p^1,p^2-diuridine 5'-pyrophosphate, 20 x 10^3 at 260 nm; p^1,p^2-dicytidine 5'-pyrophosphate, 26 x 10^3 at 280 nm; p^1-uridine 5', p^2-cytidine 5'-pyrophosphate, 17 x 10^3 at 280nm. Molar absorptions in water were: 2',3'-O-dibenzoyluridine 5'-phosphoranilidate, 12 x 10^3 at 260 nm; 2',3'-O-dibenzoyluridine 5'-phosphoro-β-naphthylidate, 14 x 10^3 at 260 nm; N,2',3'-O-tribenzoylcytidine

5'-phosphate, 12 x 10^3 at 304 nm. Molar absorptions of uridine 5'-phosphoramidates were determined by phosphorus analysis using Allen's method[11].

Synthesis of 2',3'-O-dibenzoyluridine 5'-phosphate ----- Pyridinium uridine 5'-phosphate was benzoylated by a similar method used for benzoylation of deoxyadenosine 5'-phosphate[12]. Acetolysis of the mixed benzoic anhydride with 2',3'-O-dibenzoyluridine 5'-phosphate was performed using acetic anhydride in pyridine as described for N,2',5'-O-tribenzoyladenosine 3'-phosphate[13]. The yield was nearly quantitative and the product was contaminated with a trace of N-acylated compound.

Synthesis of N,2',3'-O-tribenzoylcytidine 5'-phosphate ----- The compound was prepared by the same procedure used for 2',3'-O-dibenzoyluridine 5'-phosphate. The yield was nearly quantitative.

Synthesis of pyridinium 2',3'-O-dibenzoyluridine 5'-phosphoranilidate ----- Pyridinium 2',3'-O-dibenzoyluridine 5'-phosphate (0.4 mmole) was dissolved in a mixture of t-butanol (4 ml), water (0.8 ml) and aniline (2.8 mmoles, 0.255 ml) with warming. To the clear solution was added DCC (412 mg, 2.0 mmoles) and kept at room temperature for 48 hr. Paper electrophoresis at pH 7.5 showed no starting materials. The solution was evaporated and the residue was dissolved in 30% pyridine. The solution was filtered and extracted with n-hexane. 4-Aniline N,N'-dicyclohexylcarboxamidine was removed by passing the solution through a column of pyridinium Dowex 50X2 (1 x 15 cm). The eluent and washings of the column were evaporated and the residue was precipitated with ether from its solution in pyridine (3 ml). The product was contaminated with pyrophosphate of 2',3'-O-dibenzoyluridine 5'-phosphate(ca.10%).

The reaction of 2',3'-O-dibenzoyluridine 5'-phosphoranilidate with N,2',3'-O-tribenzoylcytidine 5'-phosphate in pyridine ----- Pyridinium 2',3'-O-dibenzoyluridine 5'-phosphoranilidate (890 A_{260} units) and pyridinium N,2',3'-O-tribenzoylcytidine 5'-phosphate (1000 A_{304} units) were evaporated with dry pyridine repeatedly. The residue was dissolved in dry pyridine (1 ml) and aliquots (10 µl) were taken at time intervals at 23°. The samples were treated with 15 N methanolic ammonia for 12 hr and applied to paper chromatography in solvent B. The pyrophosphate fraction was further separated in paper electrophoresis at pH 3.5.

The reaction of 2',3'-O-dibenzoyluridine 5'-phosphoranilidate with N,2',3'-O-tribenzoylcytidine 5'-phosphate in the presence of DCC ----- DCC (10 equivalents to the phosphomonoester component) was added to the above reaction

mixture unless otherwise specified.

Synthesis of uridine 5'-phosphoramidates ----- Substituted aniline derivatives (7 mmoles) were allowed to react with uridine 5'-phosphate (1 mmole) using DCC (5 mmoles) in a mixture of t-butanol (10 ml) and water (2 ml) at 25°. The condition was similar to that used for the synthesis of adenosine 5'-phosphoramidate[4]. The extent of the reaction was checked by paper electrophoresis at pH 7.5. The carboxamidinium salt of uridine 5'-phosphoranilidate was stable in pyridine at least 48 hr at 25°. The product was converted to pyridinium salt as described in the case of 2',3'-O-dibenzoyluridine 5'-phosphoranilidate.

2'-O-Benzoyluridine 3'-phosphoranilidate ----- Pyridinium 2'-O-benzoyluridine[10] (12720 A_{260} units) was dissolved in water (1.3 ml) and t-butanol (13 ml). Aniline (0.65 ml) and DCC (1.1 g) were added and the mixture was reflux for 3 hr. Paper electrophoresis showed no starting material at this stage. Solvents were evaporated and 50% pyridine (20 ml) was added. The urea was removed by filtration and the filtrate was extracted with n-pentane (3 ml). The nucleotides were precipitated with ether-pentane (3:2) from its solution in pyridine. The precipitate was dissolved in 50% pyridine and applied to a column (1.2 x 22 cm) of Dowex 50X2 (pyridinium form). The effluent and washings were evaporated and dissolved in 50% pyridine (12 ml). The product was extracted with methylene chloride (4 ml, 3 portions) to separate from the pyrophosphate. The amidate was precipitated with ether-pentane (3:2) from its solution in anhydrous pyridine. The yield was 7500 A_{260} units 59%.

2'-O-Benzoyl 3'-phosphoro-β-naphthylidate (IXa) ----- Pyridinium 2'-O-benzoyluridine 3'-phosphate (2 mmoles) was dissolved in t-butanol (50 ml) and water (10 ml) at 60°. DCC (2.12 g) was added and the mixture was heated at 60° for 12 hr. The urea was removed by filtration and the solution was evaporated. The residue was dissolved in 10% pyridine (40 ml) and chloroform (50 ml). The aqueous phase was extracted with chloroform (50 ml) three times and the combined chloroform solution was concentrated. The naphthylidate was precipitated with ether. The yield was 1.3 g (1.8×10^4 A_{260}, 1.24 mmoles, 62%). $\lambda_{max}^{H_2O}$ 235, 260, 290(sh), 340(sh).

C^{Bz}(OBz)-p-U(OBz)-p-NH-β-Naph (XI)----- Pyridinium 5'-O-trityl-N,2'-O-dibenzoylcytidine 3'-phosphate (0.75 mmole) and carboxamidinium salt of the naphthylidate (IX) (0.4 mmole) were treated with TPS (2.4 mmoles) for 8 hr at 10°. Tri-n-butylamine (1.14 ml), pyridine (4.8 ml) and water (4.8 ml) were added in an ice bath. The mixture was kept at room temperature for overnight and

concentrated. The residue was dissolved in chloroform (25 ml) and washed with water (10 ml). Chloroform layer was then washed with 10% pyridine and evaporated to dryness. The residue was treated with 80% acetic acid at room temperature for 1 hr. Acetic acid was removed and the residue was dissolved in 20% aqueous pyridine. The starting material (IXa) was extracted with carbon tetrachloride and the dinucleotide (XI) was extracted with carbon tetrachloride-chloroform (1:1). The organic layer was washed with 20% aqueous pyridine to remove N,2'-O-dibenzoylcytidine 3'-phosphate and evaporated to dryness. The residue was dissolved in pyridine and precipitated in ether-pentane 1:1. The yield was 116 mg, 900 A305 (0.073 mmole) (18%).

BzO-C^{Bz}(OBz)-p-U(OBz)-p (XII)----- Pyridinium N,2',5'-O-tribenzoylcytidine 3'-phosphate (1.44×10^4 A262, 0.8 mmole) and the naphthylidate (IXb) (1.63×10^4 A260, 0.79 mmole) were treated with TPS (909 mg) in anhydrous pyridine (5 ml) for 8 hr at 18°. Tri-n-butylamine (1.5 ml) in pyridine (6 ml) and water (6 ml) were added. The mixture was kept at room temperature for overnight and concentrated. The residue was dissolved in methylene dichloride (20 ml) and washed with water (5 ml, 2 portions). The solvent was evaporated and the residue was dissolved in 50% pyridine (20 ml). The naphthylidate (IXb) was extracted with carbon tetrachloride (10 ml, 3 portions). The dinucleotide intermediate (Xa) was extracted with chloroform and precipitated with ether-pentane (3:2) from its solution in small amount of pyridine. The precipitate was treated with isoamyl nitrite (2 ml) in pyridine-acetic acid (1:1) (16 ml) at room temperature for 4 hr. Volatile materials were removed by evaporation and the residue was dissolved in 10% pyridine (15 ml). BzO-C^{Bz}(OBz)-p was extracted with chloroform (4 ml, 3 portions) and the product was extracted with methylene chloride-n-butanol (2:1) (5 ml, 3 portions). The organic phase was washed with small amount of water and evaporated. The dinucleotide (XII) was precipitated in ether (100 ml) with its solution in pyridine. The yield was 1.37×10^3 A305, 0.15 mmole (19%).

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