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The 2′-Hydroxyl Function Assisted Cleavage of the Internucleotide Phosphotriester Bond of a Ribonucleotide Under Acidic Conditions

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The stability of the internucleotidic phosphotriester of a diribonucleoside monophosphate was studied during the acid-promoted deblocking of a 2′-acid labile group.

It is a common practice in oligoribonucleotide synthesis, using the phosphotriester approach,[1] that the 2′-hydroxyl function is protected by an acid-labile group[2–5] while the internucleotide 3′→5′ phosphotriester is protected with a suitably substituted phenol[6] with a pk_a close to that of *o*-chlorophenol (pK_a 8.47). The 2′-acid-labile group is normally removed in the last step by an acidic reagent, after conversion of the internucleotidic phosphotriester to the diester level. In a strategy of RNA synthesis with two complementary acid-labile groups[3,4] at the 2′- and the 5′-ends, we observed the formation of a small amount (*ca.* 5–10 %) of baseline material on TLC during the selective removal of the 5′-acid-labile group. It was anticipated that the baseline material could be a charged species which might have formed due to the degradation of the internucleotidic phosphotriester bond. In this work, we report the synthesis of a simple dinucleoside monophosphate *6*, as a model compound, and show that the internucleotide phosphotriester bond, with vicinal hydroxyl function, is indeed unstable in the acidic medium.

The synthetic route leading to the preparation of *6*, starting from an easily accessible[8] 2′-protected building block *1*, is outlined in Scheme 1.

A chloroform solution of *6* was then treated with 4-toluenesulfonic acid monohydrate (5 equiv.) at 20 °C; it was consumed within 5 min. TLC revealed the formation of a compound which had the same R_f value as that of 4-*N*-benzoyl-2′,3′-di-*O*-acetylcytidine besides the base-line materials. Excess of acid was neutralized by triethylamine. All the volatile materials were removed *in vacuo*. The reaction mixture was then fractionated on a DEAE Sephadex A25 column using gradients of triethylammonium hydrogen carbonate (pH 7.3) (see experimental section for details). The following compounds were isolated: *7* (35 %), a mixture of 3′→5′ and 2′→5′ dimers, *8* and *9* in ca. 7:3 ratio respectively (31 %), triethylammonium tosylate, and an isomeric mixture of 3′- and 2′-6-*N*-benzoyladenylic acid, *10* and *11* in 3:2 ratio respectively (35 %). It was then considered important to explore the acid hydrolysis of *12B* in order to compare the leaving-group abilities of alkoxides

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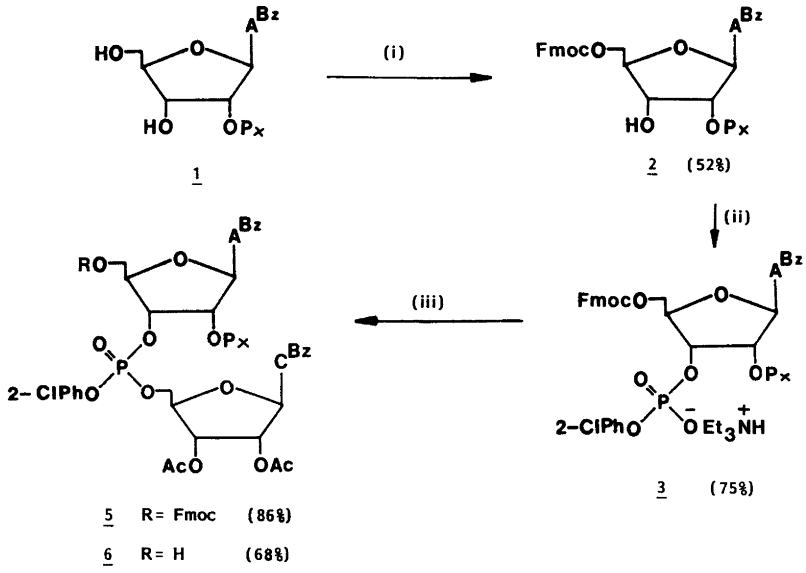


(i) Fluoren-9ylmethoxycarbonyl (Fmoc) chloride in dry pyridine

(ii) 2-Chlorophenylphosphorobis-(1,2,4-triazolide) in dry pyridine

(iii) 1-Mesitylenesulfonyl-3-nitro-1,2,4-trazole and 7 in dry pyridine

Px = 9-Phenylxanthen-9-yl-

C^{Bz} = 4-N-Benzoylcytosin-1-yl-

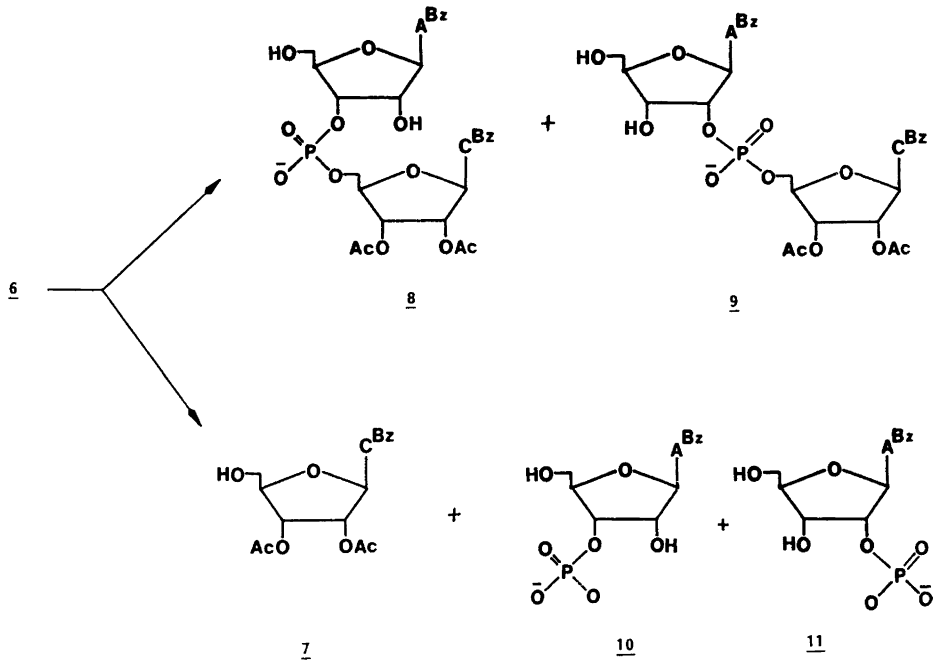
A^{Bz} = 6-N-Benzoyladenin-9-yl-

Scheme 1.

(nucleosides *versus* ethanol) under the above acidic hydrolytic condition. When *12B* was treated, under the above acidic condition, compounds *14A* and *14B* (in *ca.* 45:55 ratio; 39 %) and *10* and *11* (in *ca.* 2:3 ratio; 60 %) were formed through the intermediate *13.* These compounds were isolated in a similar way as described for the reaction products from *6.* It was then interesting to subject the compound *4* to a similar acidic treatment. The sole product that was isolated from this reaction was an isomeric mixture of 3'- and 2'-6-*N*-benzoyladenylic acid (in *ca.* 2:3 ratio) in 82 % yield. Detailed kinetic measurements are in progress to delineate the actual reaction course.

EXPERIMENTAL

^{1}H NMR spectra were measured at 60 MHz with a Perkin-Elmer R 600 and at 90 MHz with a Jeol FX 90Q spectrometer using tetramethylsilane as an internal standard (δ scale). ^{31}P NMR spectra were recorded at 36 MHz in the same solvent mixture as for ^{1}H NMR using phosphoric acid as an external standard (δ scale). UV absorption spectra were recorded with



a Cary 2200 spectrophotometer in methanol. Reactions were monitored by using Merck pre-coated silica gel 60 F_{254} plates using the following solvent systems:

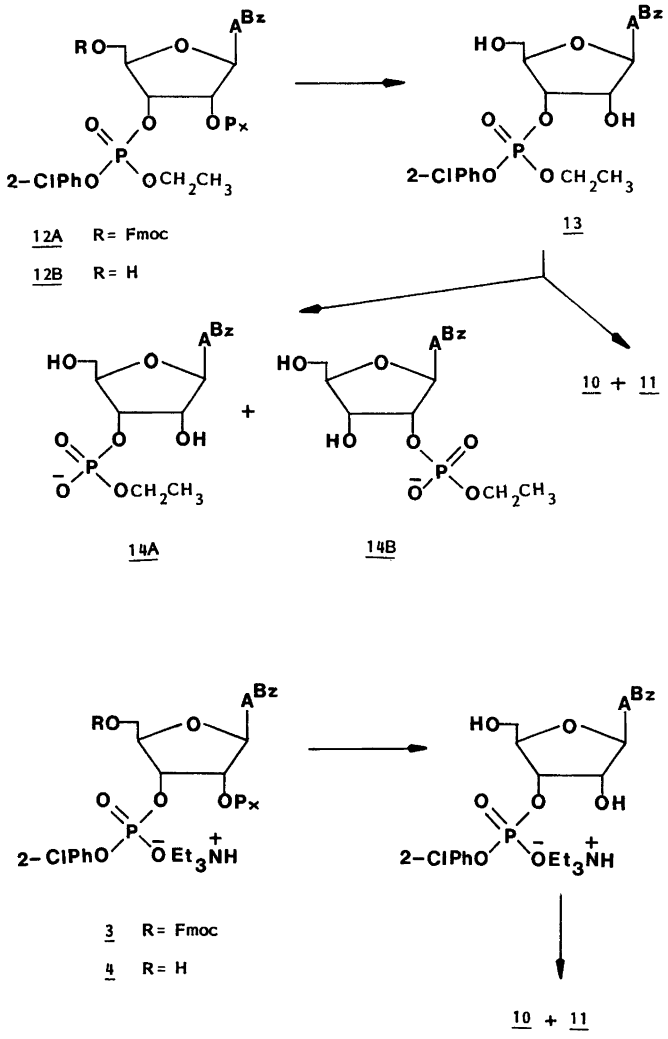
(A) 5 % methanol–chloroform (v/v)

(B) 10 % methanol–chloroform (v/v)

Merck Kieselgel G was used for short column chromatography.[11] Dried solvents were prepared using literature procedures.[3] The key reagents: 9-chloro-9-phenylxanthene (Px–Cl),[7] 1-(mesitylenesulfonyl)-3-nitro-1,2,4-triazole (MS–NT),[6] 2-chlorophenylphosphorobis(1,2,4-triazolide)[10] were prepared using literature procedures.

6-N-Benzoyl-2'-O-(9-phenylxanthen-9-yl)-5'-O-(fluoren-9-ylmethoxycarbonyl)-adenosine (2). 1 (1.88 g, 3 mmol) was dried by co-evaporations with pyridine (3×10 ml). The dry residue was taken up in the same solvent (30 ml), fluoren-9-ylmethoxycarbonyl chloride (1.55 g, 6 mmol) was then added and the mixture was stirred for 4 h at 20 °C. The reaction mixture was then worked up using a literature procedure.[3] The gummy residue was then purified by passage through a short silica gel column using the following sequence of mobile phases for elution: light petroleum–dichloromethane–pyridine (50:50:1 v/v), dichloromethane–pyridine (100:1 v/v) then ethanol–dichloromethane–pyridine (1:99:1 v/v) mixture. Appropriate fractions were collected, concentrated and co-evaporated a few times with toluene to give a glass. Precipitation from dichloromethane–light petroleum mixture gave the title compound as white powder. Yield 1.33g (52 %). R_f: 0.38 (A). ^{1}H NMR ($CDCl_3$+CD_3OD): 8.67 (*s*, 1H), H-8; 8.1–6.39 (*m*, 27H) H-2 and aromatic protons; 6.02 (*d*, 7.4 Hz, 1H) H-1'; 4.79 (*dd*, 7.4 Hz & 4.7 Hz, 1H), H-2'; 4.32 (*m*, 6H) H-4', H-5',5", methylene and methine protons of Fmoc group; 3.26 (*d*, 4.7 Hz, 1H) H-3'.

Conversion of 6-N-Benzoyl-2'-O-(9-phenylxanthen-9-yl)-5'-O-(fluoren-9-ylmethoxycarbonyl)adenosine (2) into the triethylammonium salt of its 3'-O-(2-chlorophenyl)-phosphate (3). 2 (0.849 g, 1 mmol) was co-evaporated three times with dry pyridine and then was redissolved in the same solvent (8 ml). Freshly prepared 2-chlorophenylphosphorobis-(1,2,4-triazolide) (0.25 M solution in acetonitrile, 8 ml, 2 mmol) was added. The mixture



was stirred for 1 h at 20 °C and then was worked up in a usual way[3] to give a white powder. Yield: 0.87g. (75 %). R_f: O (B).

^{31}P NMR: −4.76, −5.79.

^{1}H NMR ($CDCl_3$): 8.7 (*s*, 1H) H-8; 8.1−6.99 (*m*, 31H) H-2 and aromatic protons; 6.18 (*d*, 1H) H-1′; 5.1−4.8 (*m*, 2H) H-2′ and H-3′; 4.46−4.35 (*m*, 6H), H-4′, H-5′,5″, methylene and methine protons of Fmoc group; 3.08−2.95 (*q*, 6H) methylene protons of triethylammonium; 1.28 (*t*, 9H), methyl protons of triethylammonium.

Preparation of the fully protected dimer ApC (5). A mixture of *3* (0.23 g, 0.2 mmol) and *7* (0.107 g, 0.25 mmol) were coevaporated three times with dry pyridine. The mixture was redissolved in dry pyridine (3 ml) and 1-mesitylenesulfonyl-3-nitro-1,2,4-triazole (0.74 g, 2.5 mmol) was added. The mixture was then stirred for 1 hour at 20 °C. Then 2-chlorophenylphosphorobis(1,2,4-triazolide) (0.25 M in acetonitrile, 4 ml, 4 equiv. with respect to the cytidine block) was added and stirred for 30 min. It was then worked up following our literature procedure[3] to give a pyridine-free gum. This was purified by passage through a short silica gel column using the following sequence of mobile phases for elution: light petroleum −dichloromethane−pyridine (50:50:1 v/v), dichloromethane−pyridine

(100:1, v/v) and then ethanol–dichloromethane–pyridine (1:99:1, v/v) mixture. The appropriate fractions were collected, concentrated and co-evaporated a few times with toluene to give a glass. It was then precipitated from dichloromethane–light petroleum mixture to give white powder. Yield: 0.25g. (86 %). R_f: 0.638 (B).

^{31}P NMR ($CDCl_3$): −6.96, −7.6.

Removal of fluoren-9-ylmethoxycarbonyl group from the 5'-end of the fully protected dimer ApC (6). 5 (267 mg, 0.184 mmol) was co-evaporated three times with dry pyridine and was then redissolved in dry pyridine (2 ml). Triethylamine (0.25 ml, 1.89 mmol) was added and stirred for 2 h. TLC (system: B) showed the formation of two products with a very close R_f and slightly lower than that of the starting material. Volatile matters were then removed *in vacuo,* co-evaporated with toluene to remove pyridine. The mixture was then applied to five thick layer plates (20×20 cm, 2 mm) and the plates were developed in 10 % methanol–chloroform mixture. Appropriate bands were excised and extracted with 50 % ethanol–chloroform mixture. Volatile matters were removed to give a glass of *6*. Yield: 0.154g (68 %). R_f: 0.619 and 0.553 (B). ^{31}P NMR ($CDCl_3$): −7.69, −8.59.

Acid hydrolysis of the 5'-unprotected dimer (6). *6* (150 mg, 0.122 mmol) was dissolved in chloroform (1 ml), 4-toluenesulfonic acid monohydrate (0.091 g, 0.63 mmol) was added at 20 °C and stirred for 5 min. Excess acid was neutralised by the dropwise addition of triethylamine. All the volatile matters were removed *in vacuo.* The mixture was then subjected to a fractionation on a DEAE sephadex column using the following linear gradients of triethylammonium bicarbonate buffer (pH 7.3):

(a) 0.001 M (400 ml) to 0.15 M (400 ml).

(b) 0.15 M (400 ml) to 0.3 M (400 ml)

(c) 0.3 M (400 ml) to 0.6 M (400 ml)

Appropriate fractions corresponding to each peak were pooled. The volatile matters were removed *in vacuo* and co-evaporated a few times with water. All the fractions were then dried over phosphorous pentoxide *in vacuo.*

UV and NMR absorptions of each compound collected under each peak are as shown below:

4-N-benzoyl-2',3'-di-O-acetylcytidine (7). UV: λ_{max} 261 and 316 nm (pH 2); 261 and 303 nm (pH 7); 275 and 318 nm (pH 13); ^{1}H NMR ($CDCl_3$+CD_3OD): 8.3 (*d,* 1H) H-6; 8.01–7.49 (*m,* 6H) H-5 and benzoyl protons; 6.19 (*d,* 4.8 Hz, 1H) H-1'; 5.55–5.48 (*m,* 2H) H-2' & −3'; 4.27 (*m,* 1H), H-4'; 3.93 (*m,* 2H) H-5',5"; 2.12 (*s,* 3H) and 2.1 (*s,* 3H) two acetyl groups.

(3'→5') and (2'→5') isomeric mixture of adenylyl-cytidylic acid: (8) and (9). UV: λ_{max} 257 (sh) and 288 nm (pH 2); 261 (sh) and 275 nm (pH 7); 313 (pH 13). ^{31}P NMR: 0.53, −0.12. ^{1}H NMR (CD_3OD): 8.7 (*bs,* 2H) H-2, H-8 of adenosine; 8.42 (*d,* 1H) H-6 of cytidine; 8.11–7.54 (*m,* 11H) H-5 of cytidine and aromatic protons from benzoyl groups; 6.25 (*d,* 5.6 Hz) H-1' of adenosine and cytidine moieties from the 2'→5' isomer; 6.17 (*d,* 4.9 Hz) H-1' of adenosine and cytidine moieties from the 3'→5' isomer (3:7 ratio); 2.1 and 2.7 (two *s,* 3H each) acetyl protons.

Triethylammonium tosylate. UV: λ_{max} 255, 261, 267 and 289 nm (sh) (pH 2); 255, 261 and 267 nm (pH 7); 246, 255, 261, 267, 290 and 305 nm (sh) (pH 13). ^{1}H NMR: 7.74–7.19 (*dd,* 4H) aromatic protons; 3.18 (*dd,* 6H), methylene protons of triethylammonium group; 2.35 (*s,* 3H) methyl protons of 4-tolyl- group; 1.25 (*t,* 9H) methyl protons of triethylammonium group.

Isomeric mixture of 3'- and 2'-6-N-benzoyl adenylic acid: (10) and (11) UV: λ_{max} 287 nm (pH 2); 279 nm (pH 7); 313 nm (pH 13). ^{31}P NMR: 1.5 & 2.17. ^{1}H NMR (CD_3OD): 8.69 (*s,* 2H) H-2 & H-8 adenosine protons; 8.11 (*m,* 2H) benzoyl group; 7.53 (*m,* 3H) benzoyl protons; 6.28 (*d,* 6.8 Hz) H-1' of 3'-adenylic acid and 6.18 (*d,* 3.6 Hz) H-1' of 2'-adenylic acid (2:3 ratio).

A comparison of UV absorption spectra of above compounds with appropriate model compounds are shown in Fig. 1.

Preparation of partially protected adenylyl(3'→5')cytidine (8). To a tetrahydrofuran solution of *6* (50 mg, 0.034 mmol) was added the solution of n-tetrabutylammonium fluoride

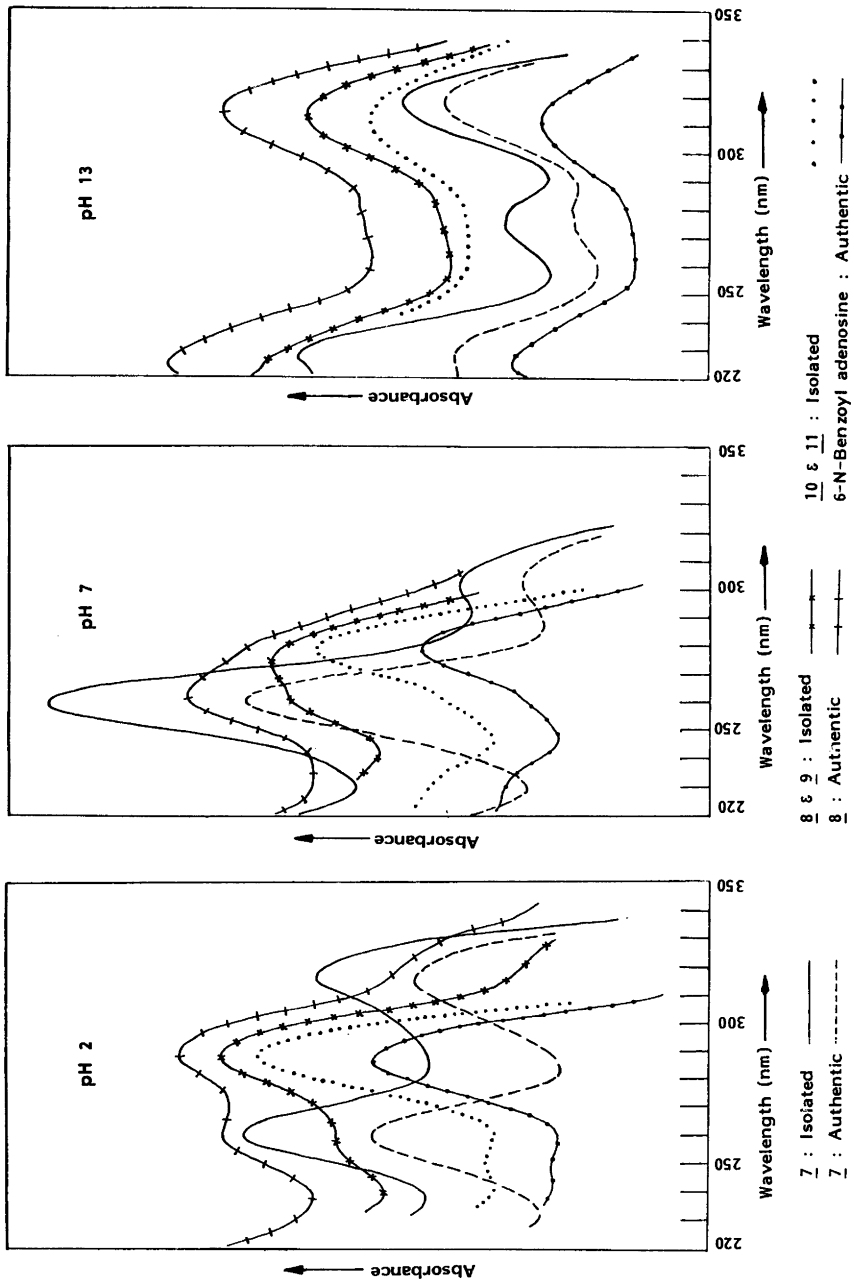


Fig. 1. A comparison of UV absorption spectra of authentic samples at different pH with the purified reaction proucts obtained in the acid hydrolysis of 6.

(TBAF) in tetrahydrofuran–pyridine–water (8:1:1, v/v/v; 3 equiv.) and was stirred for 5 h at 20 °C. Volatile matters were removed *in vacuo* and was co-evaporated few times with toluene. The product was then purified through a silica gel column. This was subjected to acid hydrolysis using 4-toluenesulfonic acid monohydrate for 5 min at 20 °C followed by neutralization with triethylamine. Volatile materials were removed *in vacuo* and the reaction mixture was then applied on a DEAE sephadex column using following linear gradients of triethylammonium hydrogencarbonate (pH 7.3) buffer.

(a) 0.001 M (300 ml) to 0.15 M (300 ml)

(b) 0.15 M (300 ml) to 0.3 M (300 ml)

Compound that was eluted under the main peak was collected (88 %; A_{279} units). The volatile matters were removed *in vacuo* and co-evaporated a few times with water to give a glass.

UV: λ_{max} 258 and 288 nm (pH 2); 262 nm (pH 7); 315 nm (pH 13).

^{31}P NMR: 0.2, 0.21, 0.09. ^{1}H NMR (CD_3OD): 8.67 (2H) H-8 and H-2 of adenosine; 8.42 (*d*, 4.8 Hz, 1H) H-6 of cytidine; 8.1–7.46 (*m*, 11H) H-5 of cytidine and benzoyl protons; 6.17 (*d*, 4.9 Hz, 2H) H-1′ of adenosine and cytidine; 2.1 and 2.07 (two *s*, 3H each) acetyl protons.

*Preparation of the fully protected dimer (*12 A*). 3* (0.231 g, 0.2 mmol) was co-evaporated three times with dry pyridine and then was redissolved in the same solvent (3 ml). Dry ethanol (0.014 ml, 0.25 mmol) and 1-mesitylenesulfonyl-3-nitro-1,2,4-triazole (0.74 g, 2.5 mmol) were added to it. The mixture was then stirred for 90 min at 20 °C and then was worked up in a usual way [3] to give a pyridine-free residue. It was purified by passage through a short silica gel column using the following sequence of mobile phases for elution: light petroleum–dichloromethane–pyridine (50:50:1 v/v), dichloromethane–pyridine (100:1 v/v) and then ethanol–dichloromethane–pyridine (1:99:1 v/v). The appropriate fractions were collected, concentrated and co-evaporated a few times with toluene to give a glass. It was then precipitated from dichloromethane–light petroleum mixture to give a white powder. Yield: 0.1 g (50 %). R_f: 0.67 (B). ^{31}P NMR: −7.61, −7.69.

*Removal of fluoren-9-ylmethoxycarbonyl (Fmoc) group from the 5′-end of the fully protected dimer (*12A*).* FMOC group was removed from the 5′-end of *12A* (0.18 g, 0.17 mmol) to give *12B* as described in the preparation of *6*. Yield: 0.1 g (70.5 %) ^{31}P NMR: −7.83, −8.03.

Acid hydrolysis of 12B. The dimer *12B* (0.1 g, 0.12 mmol) was hydrolysed using 4-toluenesulfonic acid monohydrate (5 eq.) for 5 min at 20 °C followed by neutralisation with triethylamine. Volatile matters were removed *in vacuo* and then the reaction mixture was subjected to DEAE sephadex column chromatography using following linear gradients of triethylammonium bicarbonate buffer (pH 7.3):

(a) 0.001 M (400 ml) to 0.15 M (400 ml).

(b) 0.15 M (400 ml) to 0.3 M (400 ml).

Appropriate fractions corresponding to each peak were pooled. The volatile matters were removed *in vacuo* and co-evaporated a few times with water. UV and NMR absortions of each compound collected under each peak are as follows:

Isomeric (2′→5′) and (3′→5′) mixture of 6-N-*benzoyladenosine ethyl phosphate:* 14A *and* 14B. UV: λ_{max} 288 nm (pH 2); 279 nm (pH 7), 311 nm (pH 13). ^{31}P NMR: 0.0 and −0.7 (in *ca.* 55:45 ratio). ^{1}H NMR (CD_3OD): 8.7 (*s*, 2H) H-2 and H-8 of adenine moiety; 8.11 (*m*, 2H) 1- & 5-benzoyl protons; 7.61 (*m*, 3H) 2-, 3- & 4-benzoyl protons; 6.3 (*d*, 5.8 Hz) H-1′ of adenosine from the 2′→5′ isomer; 6.17 (*d*, 7.4 Hz) H-1′ of adenosine of 3′→5′ isomer (45:55 ratio); 3.64 (*m*, 2H), methylene protons of ethyl group; 1.09 (*m*, 3H), methyl protons of ethyl group.

*Isomeric mixture of 3′- and 2′-6-*N-*benzoyladenylic acid: 10* and *11* UV: λ_{max} *288* (pH 2); 279 nm (pH 7); 310 nm (pH 13). ^{31}P NMR: 1.19, 1.97 (in *ca.* 3:2 ratio); ^{1}H NMR (CD_3OD): 8.69 (*m*, 2H) H-2 and H-8 adenine moiety; 8.03–8.12 (*m*, 2H) 1- & 5-H of benzoyl group;

7.53–7.61 (*m*, 3H) 2-, 3- & 4- of benzoyl protons; 6.28 (*d*, 6.1 Hz) H-1′ of 3′-adenylic acid and 6.18 (*d*, 4.88 Hz) H-1′ of 2′-adenylic acid (2:3 ratio).

Acid hydrolysis of 4. *3* (0.2 g, 0.17 mmol) was co-evaporated three times with dry pyridine and then dissolved in the same solvent (2 ml). Dry triethylamine (10 eq.) was added and stirred for 2 h. Volatile matters were then removed *in vacuo* and the mixture was co-evaporated three times with toluene. Then it was taken up in chloroform (2 ml) and 4-toluenesulfonic acid monohydrate (5 eq.) was added at 20 °C and stirred for 5 min. Excess acid was neutralized with triethylamine. Volatile matters were then evaporated and the residue was subjected to a fractionation on a DEAE sephadex column using the following linear gradients of triethyl ammonium hydrogencarbonate buffer (pH 7.3):

(a) 0.001 M (400 ml) to 0.15 M (400 ml)

(b) 0.15 M (400 ml) to 0.3 M (400 ml)

(c) 0.3 M (400 ml) to 0.6 M (400 ml).

Appropriate fractions corresponding to the main peak were pooled. The volatile matters were removed *in vacuo* and co-evaporated a few times with water to give a glass. Yield: 82 % (A_{279} units).

UV: λ_{max} 288 nm (pH 2); 280 nm (pH 7); 311 nm (pH 13). ^{31}P NMR: 1.26, 2.05. ^{1}H NMR (CD_3OD): 8.72 (*s*, 1H) H-8; 8.64 (*s*, 1H) H-2; 8.06–7.98 (*m*, 2H) benzoyl protons; 7.55–7.47 (*m*, 3H) benzoyl protons; 6.26 (*d*, 6.6 Hz) H-1′ of 3′-adenylic acid and 6.14 (*d*, 4.9 Hz) H-1′ of 2′-adenylic acid (2:3 ratio).

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