The Synthesis of Ribonucleotide-5′-(5-Iodoindol-3-ol) and (4-Methylcoumarin-7-ol) Esters for the Histochemical Demonstration of Nucleases (1a)

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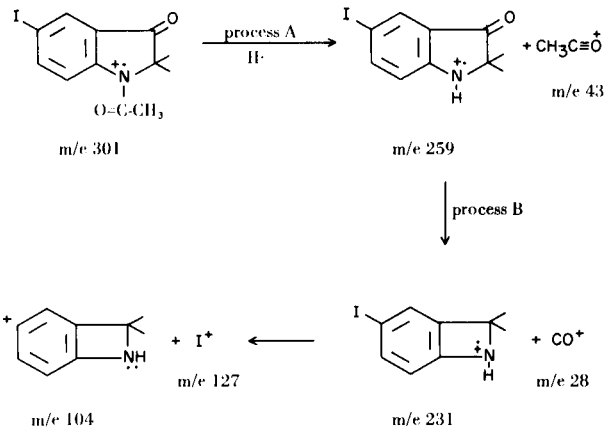
The syntheses of ribonucleotide-5′ monoesters with 5-iodoindol-3-ol and 5′-uridylic acid and 5′-adenylic acid monoesters with 4-methylcoumarin-7-ol has been accomplished by the reaction of phosphorodichloridates with various 2′,3′-*O*-isopropylidene ribonucleosides, followed by hydrolysis and anion exchange chromatography.

Nucleoside-5′ and nucleoside-3′ *p*-nitrophenyl phosphates have been shown to be useful substrates for phosphodiesterases (2-5). In a previous study, 5-iodoindoxyl was chosen as the ester chromogen for the synthesis of esters of deoxyribonucleotides (6). Enzymatic hydrolysis of these compounds results in the formation of an insoluble electron-dense 5,5′-diiodoindigo dye which can be detected by using either the light (7) or the electronmicroscope (8). In the present study, the synthesis of the corresponding ribonucleoside-5′-(5-iodoindol-3-yl phosphates) was undertaken in order to better understand the specificity of phosphodiesterases. In the course of this work, uridine-5′ and adenosine-5′-(4-methylcoumarin-7-yl phosphates) were also prepared. Enzymatic hydrolysis of these substrates could be followed fluorometrically since fluorescent 7-hydroxy-4-methylcoumarin (9) would be liberated.

The phosphorylation of 1-acetyl-5-iodoindoxyl (10,11) was found to occur readily with phosphorus oxychloride in anhydrous pyridine at room temperature. The phosphorodichloridate (1a) was not isolated due to its instability but was reacted directly with the 2′,3′-*O*-isopropylidene derivatives of adenosine, uridine, and guanosine, respectively. 2′,3′-*O*-Isopropylideneadenosine 5′-(1-acetyl-5-iodoindol-3-yl phosphate) (I) was purified by chromatography on silica gel followed by recrystallization from methanol. The structure of this compound was substantiated by ir, uv, nmr, and mass spectral data. The isopropylidene group was removed by treatment with Amberlite IR-120 resin (H^+ form) in ethylene glycol (12). The resulting product was chromatographed on a DEAE-Sephadex anion exchange column (bicarbonate form) using aqueous ammonium bicarbonate as the eluting solvent and gave ammonium 1-acetyl-5-iodoindol-3-yl 5′-adenylate (II). Removal of the acetyl group was accomplished by treatment of II with 0.8 *N* sodium hydroxide and gave ammonium 5-iodoindol-3-yl 5′-adenylate monohydrate (III) after chromatography. It was later found that the basic chromatographic conditions were usually sufficient to remove the *N*-acetyl group of the indole moiety. These conditions involved treatment with ammonium hydroxide until a *pH* of approximately 8 was obtained and elution with ammonium bicarbonate solution. In order to show that phosphorylation had occurred at the 5′ position of adenosine, I was also synthesized from Ia and N^6-dimethylaminomethylene-2′,3′-*O*-isopropylideneadenosine (IV) (13). Removal of the protecting groups followed by ion-exchange chromatography gave a product identical with III as shown by the ir and uv spectra and paper chromatography.

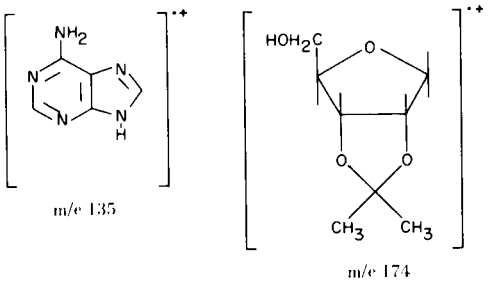
The mass spectrum of I was only of decomposition products since the sample had to be heated to greater than its decomposition temperature (220°) in order to obtain sufficient vapor pressure. The fragmentation could be explained as follows.

SCHEME I



The processes A and B above were further substantiated by the presence of metastable ion peaks at 222.8 and 206 m/e, respectively. The peak at m/e 135 was assigned to the adenine fragment (14) while the 174^+ ion may be due

to the sugar residue. The appearance of a peak at m/e 58 could be due to the elimination of acetone from the isopropylidene group. The m/e 60 peak may result from the



m/e 135 m/e 174

loss of a CH_3^+ group from the isopropylidene group followed by the elimination of acetic acid. The peaks at m/e 77 and 76 may be due to $C_6H_5^+$ and $C_6H_4^{+\cdot}$, respectively.

The syntheses of 2',3'-*O*-isopropylideneuridine 5'-(ammonium-5-iodoindol-3-yl phosphate) (V) and 2',3'-*O*-isopropylideneguanosine 5'-(ammonium 5-iodoindol-3-yl phosphate) (VII) were accomplished by the reaction of the 2',3'-*O*-isopropylidene ribonucleosides with 1-acetyl-5-iodoindol-3-phosphorodichloridate followed by hydrolysis and ion-exchange chromatography. Compounds V and VII were treated first with 80% acetic acid and then 1 *N* ammonium hydroxide to give ammonium 5-iodoindol-3-yl 5'-uridylate (VI) and ammonium 5-iodoindol-3-yl 5'-guanylate (VIII), respectively. Compounds IX-XII were obtained in a similar manner using 4-methylcoumarin-7-phosphorodichloridate as the phosphorylating agent.

EXPERIMENTAL

Fluorescence spectra were obtained on an Aminco-Bowman spectrophotofluorometer. UV absorption spectra were measured on a Beckman Model DB-G recording spectrophotometer. NMR spectra were measured on a Varian A-60A spectrometer with TMS as the internal standard. IR spectra were obtained on Perkin-Elmer Models 137 or 421 spectrophotometers as potassium bromide pellets. Unless otherwise stated, all R_f values were obtained on Whatman No. 1 paper using 2-propanol-concentrated ammonium hydroxide-water, 7:1:2. Pyridine was dried over calcium hydride or sodium hydroxide and was distilled before use. The mass spectrum was obtained from Morgan Schaffer Corp., 5110 Courtrai Ave., Montreal 26, Quebec, Canada. The elemental analyses were performed by Dr. Stephen M. Nagy, 78 Oliver Rd., Belmont, Mass. or Galbraith Laboratories, Knoxville, Tenn.

2',3'-*O*-Isopropylideneadenosine 5'-(1-Acetyl-5-iodoindol-3-yl Phosphate) (I).

1-Acetyl-5-iodoindoxyl (3.64 g., 12 mmoles) was dissolved in 40 ml. of dry pyridine. Freshly distilled phosphorus oxychloride (3.7 ml.) was added, and the mixture was stirred for 15 hours. The reaction mixture was evaporated to dryness at 0.5 mm pressure at 70°. Dry 2',3'-*O*-isopropylideneadenosine (3.72 g., 12 mmoles) was dissolved in 40 ml. of dry pyridine and added to the residue. The reaction mixture was stirred to dissolve the gummy residue, allowed to stand for 23 hours and evaporated to dryness at 0.25 mm pressure and 60°. The residue was triturated with 40 ml. of water, and the insoluble solid was collected by filtration, dissolved in chloroform and applied to a silica gel column (3 x 30 cm.) packed in chloroform. Elution with acetone gave 0.46 g. (6%) of impure product. Elution with methanol afforded 1.81 g. (22%) of the desired compound. Analytical samples were recrystallized from methanol-acetone, m.p. 216-220° dec.; R_f = 0.84 tlc on silica gel using methanol. UV λ max in methanol, mμ (log ε) 250 (4.49), 301 (3.69), 310 (3.69); ν max (potassium bromide), 1710, 1660 cm^{-1}; NMR (DMSO-d_6) δ 1.32, 1.53 (6H, doublet, $(CH_3)_2$–C), 4.50 (5H, broad peak, H_2', H_3', H_4', H_5'), 6.27 (1H, multiplet, H_1'), 7.65, 7.77, 8.00, 8.10, 8.37, 8.47 (6H, multiplet, H_2 and H_8 adenine protons and the 2, 4, 6 and 7 indole protons). The N–CO–CH_3 protons were assumed to be masked by the solvent peak at δ 2.52; mass spectrum m/e (relative intensity) 301 (14), 259 (18), 231 (8), 174 (1), 135 (6), 127 (1), 104 (4), 95 (3), 77 (7), 76 (12), 60 (11), 58 (23), 43 (100).

Anal. Calcd. for $C_{23}H_{24}IN_6O_8P$: C, 41.21; H, 3.61; N, 12.54; P, 4.62; I, 18.93. Found: C, 41.22; H, 3.83; N, 12.45; P, 4.79; I, 18.95.

Ammonium 1-Acetyl-5-iodoindol-3-yl 5'-Adenylate (II).

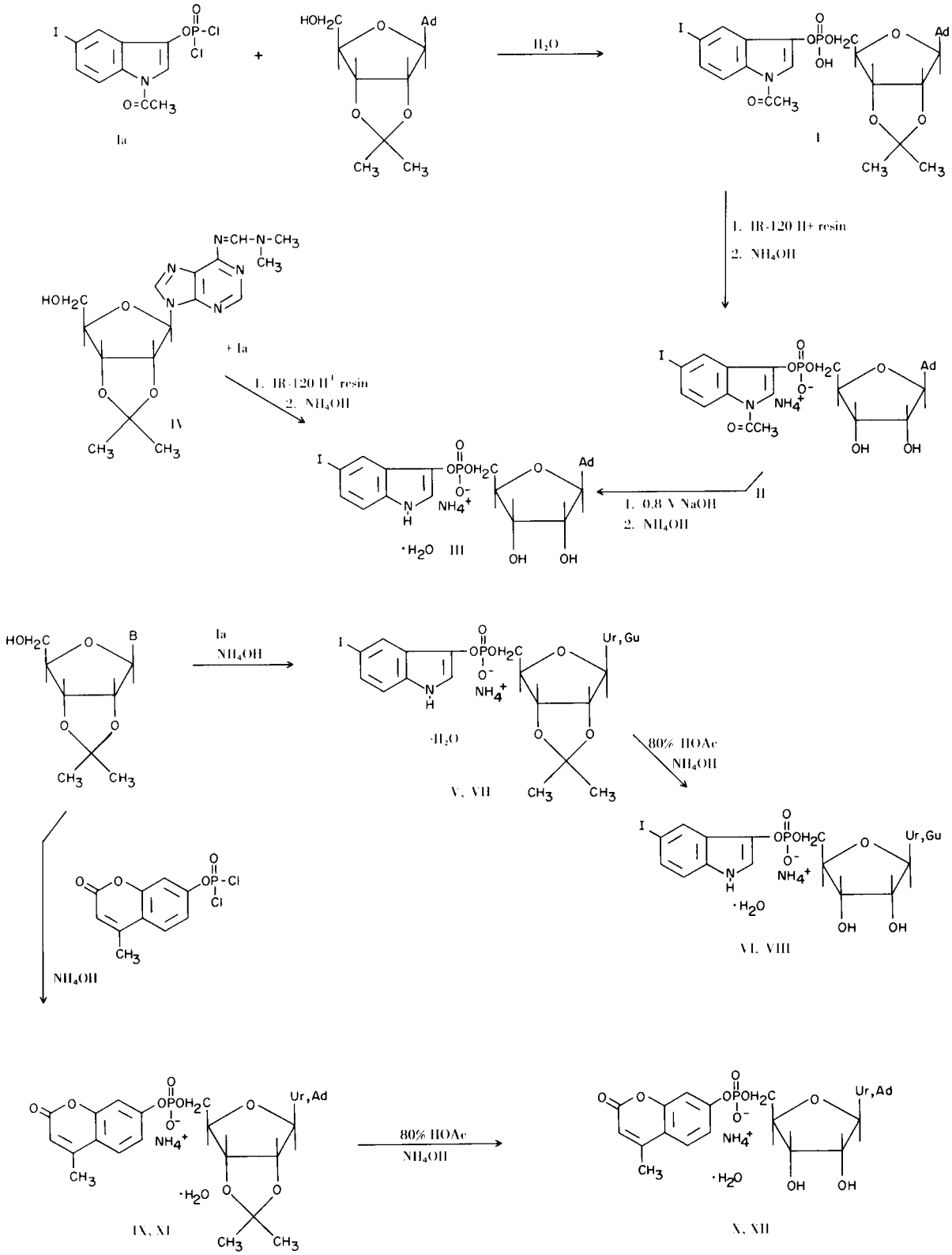
Compound I (6.41 g., 9.6 mmoles) was dissolved in 25 ml. of ethylene glycol by heating. After cooling, excess IR-120 H^+ resin was added, and the mixture was stirred for 66 hours. The resin was removed by filtration, and the filtrate was treated with excess acetone and ether to yield 2.54 g. (42%) of insoluble white solid. After recrystallization from methanol, the m.p. of the cream colored solid was 189-191° dec.; R_f = 0.77 tlc on silica gel using methanol. This compound (0.346 g.) was suspended in water and adjusted to *p*H 7.5 with 1 *N* ammonium hydroxide and applied to a DEAE-Sephadex A-50 column (bicarbonate form, 2.8 x 35 cm). Elution was accomplished using a linear gradient with 0.01 *M* ammonium bicarbonate (2ℓ) in the mixing vessel and 0.35 *M* ammonium bicarbonate (2ℓ) in the reservoir followed by 0.35 *M* ammonium bicarbonate. Fractions of 20 ml. were collected, and those exhibiting the expected uv spectra were combined and freeze-dried to give 0.343 g. (10%) of the desired product, m.p. 192-196° dec.; R_f = 0.71. UV λ max in water mμ (log ε), 240 (shoulder), 253 (4.40), 304 (3.71), 312 (3.69); ν max (potassium bromide), 1702, 1660 cm^{-1}; NMR (DMSO-d_6) of the free acid, δ 4.20 (3H, singlet, H_4', H_5'), 4.52, 4.58 (2H, multiplets, H_2', H_3'), 5.98, 6.06 (1H, doublet, H_1'), 7.23 (2H, broad peak, NH_2), 7.57, 7.67, 7.90, 8,00, 8.13 (3H, complex multiplet, H_4, H_6, H_7 indole), 8.37, 8.63, 8.73, 8.77 (3H, H_2 and H_8 adenine and H_2 indole).

Anal. Calcd. for $C_{20}H_{23}IN_7O_8P$: C, 37.11; H, 3.58; N, 15.15. Found: C, 37.14; H, 3.85; N, 14.92.

Ammonium 5-Indoindol-3-yl 5'-adenylate (III).

Compound II as the free acid (0.503 g., 0.80 mmole) was dissolved in 5.0 ml. of 0.8 *N* sodium hydroxide and stirred for 45 minutes. Excess IR-120 H^+ resin was added to the mixture and a white precipitate formed. Water was added to the mixture until the solid was redissolved. The resin was removed by filtration and the filtrate was freeze-dried. The residue (0.371 g.) was dissolved in water and the solution was adjusted to *p*H 7.5 using 1 *N* ammonium hydroxide. This solution was chromatographed as described for compound II using a 2.8 x 30 cm. column. After freeze-drying, 0.126 g. (26%) of compound III was obtained, m.p. 185-190° dec.; R_f = 0.70. UV λ max in water mμ (log ε), 232.5 (4.44), 260 (4.12), 292 (shoulder), 300 (shoulder); ν max (potassium bromide),

SCHEME 2


Ia
I
II
III
IV
V, VII
VI, VIII
IX, XI
X, XII
H_2O
1. IR-120 H^+ resin
2. NH_4OH
1. 0.8 N NaOH
2. NH_4OH
80% HOAc
NH_4OH

1695, 1650 cm^{-1}; NMR (deuterium oxide), δ 4.40 (broad peak partially masked by the HDO peak), 5.95, 6.00 (1H, doublet, H_1'), 6.87 (2H, singlet, H_4, H_7 indole), 7.10 (1H, singlet, H_6 indole), 7.58 (1H, singlet, H_2 adenine), 8.02, 8.07, 8.15 (2H, multiplet, H_8 adenine (15) and H_2 indole).

Anal. Calcd. for $C_{18}H_{21}IN_7O_7P\cdot H_2O$: C, 34.68; H, 3.72. N, 15.73; P, 4.97; I, 20.36. Found: C, 34.58; H, 3.95; N, 15.86; P, 4.98; I, 19.92.

N^6-Dimethylaminomethylene-2',3'-*O*-isopropylideneadenosine (IV).

To 2',3'-*O*-isopropylideneadenosine (2.65 g., 8.6 mmoles) was added 5.0 ml. of dimethylformamide dimethylacetal and 16 ml. of dimethylformamide. The reaction mixture was stirred for 16 hours and evaporated to dryness at 65° and 0.1 mm pressure. The residue was dissolved in chloroform and was evaporated *in vacuo* to a gum which slowly crystallized. The solid was recrystallized from acetone to give 2.55 g. (81%) of IV as colorless crystals, m.p. 175-175.5°; R_f = 0.85. UV λ max in methanol mμ (log ε), 228 (4.12), 308 (4.53); ν max (potassium bromide), 1635 cm^{-1}; NMR (deuteriochloroform), δ 1.38, 1.64 (6H, doublet, C–CH_3), 3.22, 3.27 (6H, doublet N–CH_3), 3.89 (2H, multiplet, H_5'), 5.07 5.18, 5.26, 5.35 (2H, multiplet, H_2', H_3'), 5.90, 5.96 (1H, doublet, H_1'), 7.98 (1H, singlet, H_2), 8.52 (1H, singlet, H_8), 9.00 (1H, singlet, methine proton of the N=CH–$N(CH_3)_2$ group), the OH proton was not observed.

Anal. Calcd. for $C_{16}H_{22}N_6O_4$: C, 53.03; H, 6.12; N, 23.19. Found: C, 53.33; H, 6.35; N, 23.12.

Preparation of III Using N^6-Dimethylaminomethylene-2',3'-*O*-isopropylideneadenosine.

1-Acetyl-5-iodoindoxyl (10) (1.51 g., 5.0 mmoles) was treated with phosphorus oxychloride (3.0 ml.) as described for compound I. To the resultant phosphorodichloridate was added a solution of IV (1.81 g., 5.0 mmoles) in 35 ml. of dry pyridine. The mixture was stirred briefly, allowed to stand for 74 hours and evaporated to dryness at 0.05 mm pressure. The residue was dissolved in 50 ml. of water and freeze-dried. The resulting solid was dissolved in 25 ml. of ethylene glycol, excess IR-120 H^+ resin was added, and the mixture was stirred for 19 hours. The resin was removed by filtration and washed with ethylene glycol and acetone. The filtrate and washings were concentrated to 20 ml. at 0.05 mm pressure and 80°. A small amount of methanol and a large amount of ether were added to the solution. The gummy precipitate which formed was collected and dried to give 0.925 g. of solid which was dissolved in 250 ml. of water by adjusting the *p*H to 7.5 with 1 *N* ammonium hydroxide. This solution was applied to a DEAE-Sephadex A-25 column (bicarbonate form, 2.8 x 32 cm.) and chromatographed as described for compound II. After freeze-drying, 24.5 mg. (1%) of III was obtained. This compound was shown to be identical to that prepared by the previous method.

2',3'-*O*-Isopropylideneuridine 5'-(Ammonium 5-Iodoindol-3-yl Phosphate) (V).

1-Acetyl-5-iodoindoxyl (1.97 g., 6.6 mmoles) was treated with phosphorus oxychloride (4.0 ml.) as previously described. To the resultant phosphorodichloridate was added dried 2',3'-*O*-isopropylideneuridine (1.86 g., 6.6 mmoles) in 30 ml. of dry pyridine. The reaction mixture was stirred briefly and allowed to stand for 68 hours in the dark. The residue which was obtained by evaporation at 0.06 mm pressure and 65° was dissolved in 1700 ml. of water by adjusting the *p*H to 7.5 with 1 *N* ammonium hydroxide. The insoluble solid was removed by filtration and the filtrate was chromatographed as described for compound II using an A-25 column (3 x 36 cm.) and a linear gradient of 0.01 *M* ammonium bicarbonate (4ℓ) in the mixing vessel and 0.4 *M* (ammonium bicarbonate (4ℓ) in the reservoir followed by 0.4 *M* ammonium bicarbonate. After freeze-drying, 0.728 g. (17%) of V was obtained, m.p. 165-169° dec.; R_f = 0.82. UV λ max in water mμ (log ε), 232 (4.41), 256 (4.09), 301 (shoulder), 310 (shoulder); ν max (potassium bromide), 1700 cm^{-1}; NMR (deuterium oxide), δ 1.27, 1.50 (6H, doublet, C–CH_3), 4.20 (3H, broad peak, H_4', H_5'), 4.32 (1H, broad peak, H_3'), 4.54 (1H, peak was partially masked by the HDO peak, H_2'), 5.43, 5.55 (1H, doublet, H_5 uracil), 5.68, 5.71 (1H, doublet, H_1'), 7.17, 7.22, 7.25, 7.30, 7.43 (4H, multiplet, indole protons), 7.88 (1H, singlet, H_6 uracil).

Anal. Calcd. for $C_{20}H_{24}IN_4O_9P\cdot H_2O$: C, 37.51; H, 4.09; N, 8.75; P, 4.84; I, 19.82. Found: C, 37.53; H, 3.92; N, 8.92; P, 4.70; I, 19.87.

Ammonium 5'-Iodoindol-3-yl 5'-Uridylate (VI).

Compound V (0.637 g., 0.99 mmole) was dissolved in 24 ml. of 80% acetic acid and heated at 100° for 90 minutes. The reaction mixture was evaporated to dryness at 0.07 mm pressure and 70°. The residue was dissolved in 100 ml. of water by adjusting the *p*H to 8.0 with 1 *N* ammonium hydroxide. This solution was applied to a Sephadex A-25 column (bicarbonate form, 2.8 x 34 cm.). Elution was accomplished as described for compound V. Fractions exhibiting the expected uv spectra were combined and freeze-dried to give 0.357 g. (60%) of VI, m.p. 174-176° dec.; R_f = 0.52. UV λ max in water mμ (log ε), 232 (4.49), 264 (4.03), 300 (shoulder); ν max (potassium bromide), 1690 cm^{-1}; NMR (deuterium oxide), δ 4.25 (5H, broad peak, H_2', H_3', H_4', H_5'), 5.17, 5.30 (1H, doublet, H_5 uracil), 5.92, 5.97 (1H, doublet, H_1'), 7.23, 7.27, 7.32, 7.38, 7.41, 7.52 (4H, multiplet, indole protons), 8.01 (1H, singlet, H_6 uracil).

Anal. Calcd. for $C_{17}H_{20}IN_4O_9P\cdot H_2O$: C, 34.02; H, 3.69; N, 9.33; P, 5.16; I, 21.14. Found: C, 33.79; H, 3.86; N, 9.12; P, 4.99; I, 20.92.

2',3'-*O*-Isopropylideneguanosine 5'-(Ammonium 5-Iodoindol-3-yl Phosphate) (VII).

1-Acetyl-5-indoindoxyl (1.69 g., 5.6 mmoles) was treated with phosphorus oxychloride as previously described. To the resultant phosphorodichloridate was added a suspension of dry 2',3'-*O*-isopropylideneguanosine (1.82 g., 5.6 mmoles) in 30 ml. of dry pyridine. The reaction mixture stood for 23 hours and was worked up and chromatographed as described for compound V using a 3 x 40 cm. column. After freeze-drying, 0.444 g. (12%) of VII was obtained, m.p. 197-203° dec.; R_f = 0.64. UV λ max in water mμ (log ε), 233 (4.49), 255 (shoulder), 272 (3.97); ν max (potassium bromide), 1680, 1630 cm^{-1}; NMR (deuterium oxide), δ 1.25, 1.50 (6H, doublet, C–CH_3), 4.42 (5H, broad peak, H_2', H_3', H_4', H_5'), 5.75 (1H, broad peak, H_1'), 7.12 (3H, broad peak, H_4, H_6, H_7 indole protons), 7.75 (2H, broad peak, H_2 indole and H_8 guanine). Resolution was low due to precipitation.

Anal. Calcd. for $C_{21}H_{25}IN_7O_8P\cdot H_2O$: C, 37.13; H, 4.01; N, 14.42; P, 4.56; I, 18.68. Found: C, 36.95; H, 4.13; N, 14.14; P, 4.59; I, 18.84.

Ammonium 5-Iodoindol-3-yl 5'-Guanylate (VIII).

Compound VII (0.346 g., 0.51 mmole) was dissolved in 10 ml. of 80% acetic acid and heated at 105° for 95 minutes. The reaction mixture was worked up and chromatographed as described for compound V using a 2.8 x 26 cm. column. After freeze-drying, 0.131 g. (40%) of VIII was obtained, m.p. >202° dec.; R_f = 0.41. UV λ max in water mμ (log ε), 233 (4.52), 254 (shoulder), 272 (4.00); ν max (potassium bromide), 1690 cm^{-1}.

Anal. Calcd. for $C_{18}H_{21}IN_7O_8P\cdot H_2O$: C, 33.82; H, 3.63; N, 15.34; P, 4.85; I, 19.85. Found: C, 34.11; H, 3.57; N, 15.07; P, 4.53; I, 19.72.

2′,3′-*O*-Isopropylideneuridine 5′-(Ammonium 4-Methylcoumarin-7-yl Phosphate) (IX).

4-Methylcoumarin-7-ol (1.76 g., 10 mmoles) (Eastman Kodak Co.) was dissolved in 40 ml. of dry pyridine and freshly distilled phosphorus oxychloride (4.0 ml.) was added. The mixture stood for 17.5 hours and was evaporated to dryness at 0.25 mm pressure and 70°. 2′,3′-*O*-Isopropylideneuridine (2.84 g., 10 mmoles) was added to the residue in 50 ml. of dry pyridine. The reaction mixture stood for 3 days and was evaporated to dryness at 0.1 mm pressure and 70°. The residue was dissolved in 2000 ml. of water by adjusting the *p*H to 8.0 with 1 *N* ammonium hydroxide. The insoluble purple solid was removed by filtration and the filtrate was chromatographed as described for compound V using a 3.7 x 34 cm. column and 0.01 *M* ammonium bicarbonate (6ℓ) in the mixing vessel and 0.4 *M* ammonium bicarbonate (6ℓ) in the reservoir. After freeze-drying, 0.612 g. (11%) of IX was obtained, m.p. 164-168° dec.; R_f = 0.60. UV λ max in water mμ (log ε), 267 (4.12), 315 (4.04); ν max (potassium bromide), 1700 cm^{-1}.

Anal. Calcd. for $C_{22}H_{26}N_3O_{11}P\cdot H_2O$: C, 47.40; H, 5.06; N, 7.54; P, 5.56. Found: C, 47.43; H, 5.16; N, 7.54; P, 5.62.

Ammonium 4-Methylcoumarin-7-yl 5′-Uridylate (X).

Compound IX (0.452 g., 0.81 mmole) was dissolved in 10 ml. of 80% acetic acid and heated at 100° for 105 minutes. The reaction mixture was worked up and chromatographed as described for compound V using a 3.1 x 30 cm. column. After freeze-drying, 0.349 g. (83%) of X was obtained, m.p. >155° dec.; R_f = 0.42. UV λ max in water mμ (log ε), 268 (4.13), 314 (4.02); ν max (potassium bromide), 1680 cm^{-1}; Fluorescence spectrum in water, λ max (excitation) = 320 mμ, λ max (emission) = 376 mμ; molar intensity = 0.0235 relative to quinine sulfate [1 ug./ml. of $(C_{20}H_{24}N_2O_2)_2\cdot H_2SO_4\cdot 2H_2O$ in 0.1 *N* sulfuric acid] which is taken as 1. Fluorescence spectrum of 4-methylcoumarin-7-ol in water, λ max (excitation) = 326 mμ, λ max (emission) = 451 mμ; molar intensity relative to quinine sulfate = 1.58. Molar intensity of 4-methylcoumarin-7-ol = 67.2 relative to X.

Anal. Calcd. for $C_{19}H_{22}N_3O_{11}P\cdot H_2O$: C, 44.11; H, 4.68; N, 8.12; P, 5.99. Found: C, 44.23; H, 4.76; N, 8.34; P, 5.84.

Ammonium 4-Methylcoumarin-7-yl 5′-Adenylate (XII).

4-Methylcoumarin-7-ol (1.76 g., 10 mmoles) was treated with phosphorus oxychloride as described in the preparation of compound IX. To the resultant phosphorodichloridate were added 2′,3′-*O*-isopropylideneadenosine (3.07 g., 10 mmoles) and 50 ml. of dried redistilled pyridine. The reaction mixture stood for 6 days and was worked up and chromatographed as described for compound IX using a 3.7 x 39 cm. column. After freeze-drying, 0.743 g. (13%) of 2′,3′-*O*-isopropylideneadenosine 5′-(ammonium 4-methylcoumarin-7-yl phosphate) (XI) which was assumed to be the monohydrate was obtained. This compound gave an R_f of 0.52 but was contaminated with 4-methylcoumarin-7-ol (R_f = 0.69). Compound XI (0.358 g., 0.62 mmole) was treated with 80% acetic acid (20 ml.) at 100° for 1 hour and 45 minutes and the reaction mixture was worked up and chromatographed as described for compound V using a 2.5 x 40 cm. column and collecting 23 ml. fractions at 10°. Freeze-drying gave 0.110 g. (33%) of XII, m.p. 181-183° dec.; R_f = 0.36. UV λ max in water mμ (log ε), 265 (4.19), 314 (4.11); ν max (potassium bromide), 1701, 1609 cm^{-1}; Fluorescence spectrum in water, λ max (excitation) = 320 mμ, λ max (emission) = 384 mμ; molar intensity = 0.0146 relative to quinine sulfate. Molar intensity of 4-methylcoumarin-7-ol = 108 relative to XII.

Anal. Calcd. for $C_{20}H_{23}N_6O_9P\cdot H_2O$: C, 44.45; H, 4.66; N, 15.55; P, 5.73. Found: C, 44.39; H, 4.27; N, 15.24; P, 5.52.

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