Substrates for Cytochemical Demonstration of Enzyme Activity.[1] V. Thymidine 3′- and 5′-(5-Bromo-4-chloro-3-indolyl)phosphates

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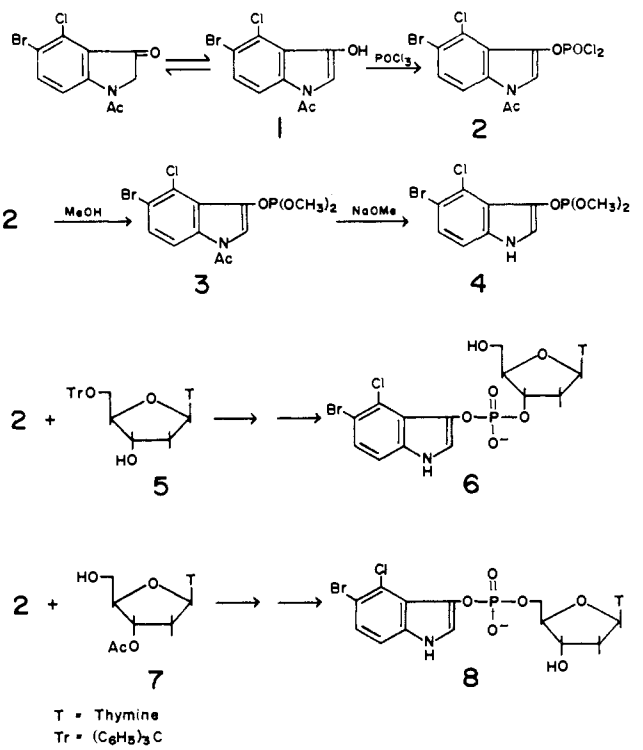
This communication describes the syntheses of thymidine 5′- and 3′-(5-bromo-4-chloro-3-indolyl)-phosphates (**8** and **6**, respectively) which are effective substrates for the intracellular localization of the PDases (I, II) by biochemical[3] and histochemical methods.[4] The esterification of 1-acetyl-5-bromo-4-chloro-3-indolylphosphoryl chloride (**2**) and the selective removal of the protective acetyl group from the product **3**, which provided the guidelines for syntheses of **6** and **8**, are also reported.



Experimental Section

Evaporations were carried out *in vacuo* at a bath temp of 40° unless indicated otherwise. Melting points were determined on a Thomas-Hoover apparatus (capillary method) and are uncorrected. Microanalyses were performed by Micro-Tech Laboratories Inc., Skokie, Ill. Where analyses are indicated only by symbols of the elements, analytical results obtained for these elements were within ±0.4% of the theoretical value. Chromatography, on Whatman No. 1 paper, was carried out using the descending technique in the indicated solvent. Optical rotations were determined in H_2O with a Perkin-Elmer Model 141 polarimeter. Uv data were obtained with a Cary Model 11 spectrometer. Ir and nmr spectra were as expected.

Dimethyl 1-Acetyl-5-bromo-4-chloro-3-indolyl phosphate (3). In a dry 100-ml flask equipped with a serum cap, condenser, and magnetic stirrer, 2.16 g (7.5 mmoles) of 1-acetyl-5-bromo-4-chloro-indol-3-ol (**1**[5] was suspended in 50 ml of dry C_6H_6 containing 1.6 ml (17.5 mmoles) of $POCl_3$. The reaction mixture was heated to just below reflux and maintained at this temp during the introduction of 0.6 ml of pyridine by means of a syringe over a period of *ca.* 0.5 hr. The solution was then refluxed for 0.25 hr and the salts were then quickly removed by filtration from the coolest reaction mixture. The amber filtrate was evapd to dryness and excess dry MeOH was added all at once. The reaction mixture was stirred at 5° for 4–6 hr and the product was collected, wt 2.96 g (74% yield), mp 139–142°. A single recrystallization from MeOH gave 2.2 g of a colorless solid, mp 142–144°. *Anal.* ($C_{12}H_{12}BrClNO_5P$) C, H, Br + Cl, N.

Dimethyl 5-Bromo-4-chloro-3-indolyl phosphate (4).—To 150 ml of dry MeOH containing 14 mmoles of NaOMe, cooled externally by an ice–salt bath, was added all at once 2.2 g (5.6 mmoles) of **3**. The reaction mixture, protected from moisture by a $CaCl_2$ tube, was stirred magnetically at 0° for 2 hr and then stored in a refrigerator at 5° for 16 hr. The solution was carefully neutralized with dilute MeOH–AcOH and treated with Norit and the filtered solution was evapd to dryness. The residue, 1.75 g, crystallized from MeOH as a colorless solid, wt 1.35 g (68% yield), mp 134–135°. An analytical sample, mp 139–141°, was obtained after two additional recrystallizations from the same solvent. *Anal.* ($C_{10}H_{10}ClBrNO_4P$) C, H, Cl + Br, N.

Thymidine 3′-(5-Bromo-4-chloro-3-indolyl)phosphate (6). A solution of 5′-*O*-tritylthymidine[6] (3.8 g, 7.7 mmoles) in anhyd dioxane (15 ml) was added dropwise under N_2 to a magnetically stirred dioxane (10 ml) solution at 0° of dry pyridine (0.66 ml) and **2**. The latter had previously been prepared from 2.9 g (0.01 mole) of **1** and 1.83 ml of $POCl_3$ as described above. After completion of addition, which required 0.5 hr, the mixture was allowed to attain ambient temp, after which stirring was maintained for an additional 16 hr. A solution of 1 ml of pyridine in 5 ml of H_2O was rapidly added with stirring and the reaction mixture was evapd to a gum. The residue was partitioned between equal volumes of $CHCl_3$ and H_2O, and the organic layer was drawn off and washed with aq pyridine·HCl (1 *M*). The dried ($MgSO_4$) $CHCl_3$ extract was evapd to dryness, the yellow residue was dissolved in 80% AcOH, and the solution was heated at 100° for 20 min. The reaction mixture was evapd to dryness and the residue was suspended in H_2O. After *ca.* 16 hr at 4°, the triphenylcarbinol was removed by filtration and the filtrate was evapd to dryness at *ca.* 30°. The product was dissolved in water and then converted into the free acid by passing through a column (20 × 3 cm) of Amberlite IR-120 resin (H^+). The effluent was carefully neutralized with aq NH_4OH and evapd to dryness at *ca.* 30°. The residue was dissolved in cold (−10°), satd MeOH–NH_3 (50 ml) and the solution was held at 4° overnight (16 hr). The reaction mixture was evapd to dryness and the residue crystallized from EtOH–Et_2O to give 2.1 g (37% yield) of product as an ammonium salt. An anal. sample was obtained after three additional recrystallizations from the same solvent system. The product, mp 234–235° dec with darkening beginning at *ca.* 215°, was homogeneous (*R*f 0.73 *i*-PrOH–NH_4OH–H_2O, 7:1:2); $[\alpha]^{26}D$ +17° (*c* 1, H_2O); uv max (pH 6) 270 nm (ε 12,820), min 248 nm. *Anal.* ($C_{16}H_{21}BrClN_3O_8P$) C, H, N; P: calcd, 5.46; found, 4.01.

A sample of the ammonium salt was transformed first into the corresponding free acid by passage over a column (20 × 3 cm) of Amberlite IR-120 (H^+) and then to a Ca^{2+} salt by a second passage over IR-120 (Ca^{2+}). Evaporation of the filtrate gave a tan solid, mp 215–217° dec with prior softening and darkening at *ca.* 200°; $[\alpha]^{26}D$ +14.5° (H_2O); uv max 268 nm (ε 10,260), min 248. *Anal.* ($C_{16}H_{17}NBrClN_3O_8P\cdot0.5Ca\cdot2.5H_2O$) C, H, N, P.

Thymidine 5′-(5-Bromo-4-chloro-3-indolyl)phosphate (8).—The preparation of **2** from 3 g (10.4 mmoles) of 1-acetyl-5-bromo-

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4-chloroindol-3-ol (1) 1.7 ml (20 mmoles) of $POCl_3$ in 60 ml of C_6H_6 was carried out as described above. To a magnetically stirred solution of **2** in 60 ml of dry dioxane, cooled externally by an ice bath, was added dropwise and under N_2 a solution of 3.6 g (12.7 mmoles) of 3-*O*-acetylthymidine[6] (**7**) in 40 ml of dioxane containing 1.2 ml (14.9 mmoles) of pyridine. The work-up followed that described above for **6** omitting only the treatment with AcOH to effect detritylation. The residue, 1.98 g, derived from the column conversion (IR-120 H^+) of the product into a free acid, was dissolved in 50 ml of dry MeOH and added dropwise with magnetic stirring to a satd MeOH–NH_3 solution (20 ml) at −10°. The reaction mixture was slowly allowed to attain ambient temp and then stirred for an additional 16 hr. Traces of 5,5′-dibromo-4,4′-dichloroindigo were removed by rapid filtration and the filtrate was evapd to dryness. The greenish brown solid was dissolved in MeOH and treated with Norit and the filtered solution was evapd to dryness to give a tan, chromatographically homogeneous solid (R_f 0.71 *i*-PrOH–NH_3–H_2O, 7:2:1); wt 0.250 g (4% yield based on 1). The latter was converted into a Ca salt in the manner described for **6**. Evaporation of the effluent gave an off-white amorphous solid: mp 229–230° dec with prior darkening at 220°; $[\alpha]^{20}D$ −17° (*c* 0.2, H_2O); uv max (pH 6) 272 nm (ϵ 9120), min 250 nm. *Anal.* ($C_{18}H_{17}ClBrN_3O_8P \cdot 0.5Ca \cdot 2.5H_2O$) C, H, N, P.

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Cyclohexyl Alkylphosphonofluoridates

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Ooms has shown that cyclohexyl ethyl and methylphosphonofluoridates[1] are active inhibitors of serine esterases, particularly chymotrypsin. Our continuing interest in phosphonate esters as inhibitors of serine esterases[2] prompted us to prepare a number of cyclohexyl alkyl and phenylalkylphosphonofluoridates analogous to the previously synthesized *p*-nitrophenyl alkyl and phenylalkylphosphonates.[3]

These compounds proved to be good inhibitors of serine esterases[4a] and two of the cyclohexylphosphonofluoridates, the benzyl and the phenethyl, were very potent inhibitors of chymotrypsin.[4b]

The compounds were prepared by a five-step sequence of standard reactions. Only the conversion of the alkylphosphonodichloridates into the cyclohexyl alkylphosphonochloridates proved to be more difficult and gave lower yields than anticipated.

Experimental Section

Caution.—The phosphonofluoridates are cholinesterase inhibitors and should be prepared and handled with extreme care.

Diethyl Alkylphosphonates.—The diesters were prepared by the Arbuzov–Michaelis or the Michaelis–Becker reactions.[5]

Alkylphosphonic Acids.—The esters were hydrolyzed by refluxing overnight in excess concd HCl and purified by crystallization from AcOH.

Alkylphosphonodichloridates.—The method of Doak and Freedman[6] was modified in that PCl_5 was added cautiously from an addition tube to the solid acid.

Cyclohexyl Alkylphosphonochloridate. Cyclohexyl Butylphosphonochloridate.—To a solution of butylphosphonodichloridate (63.6 g, 0.36 mole) in *ca.* 500 ml of abs Et_2O, was added a solution of cyclohexanol (36.8, 0.36 mole) in Et_3N (36.4 g, 0.36 mole), dropwise over a period of *ca.* 4 hr. with stirring. After addition, the mixture was stirred an additional 6–8 hr (in some cases the mixture, after the addition, was warmed to refluxing temperature *ca.* 2 hr and worked up). The $Et_3N \cdot HCl$ was filtered off and the Et_2O removed by distillation at a final temp of *ca.* 70° and water aspirator pressure. The residue was distilled using a still head only: yield 47%; bp 90° (0.1 mm); $n^{25}D$ 1.4680. These cyclohexyl alkylphosphonochloridates are prone to decomposition during distillation and those boiling above 100° were purified by distillation through a falling film molecular still. Constants of once distilled cyclohexyl phosphonochloridates are given in the order bp °C (mm), $n^{25}D$, yield %: Pr, 79 (0.05), 1.4688, 41; Bu, 90 (0.1), 1.4680, 47; Pent, 99 (0.1), 1.4688, 54; Hex, toluene,[7] 1.4678, 30; Ph, toluene,[7] 1.5328, 48; benzyl, BuOH,[7] 1.5309, 20; phenethyl, BuOH,[7] 1.5240, 39; phenylpropyl, xylene,[7] 1.5244, 42; phenylbutyl, xylene,[7] 1.5192, 45.

Cyclohexyl Alkylphosphonofluoridates. Cyclohexyl Phenylpropylphosphonofluoridate.—Cyclohexyl phenylpropylphosphonochloridate (35.6 g, 0.118 mole) and KF (10.4 g, 0.177 mole, 50% excess) were mixed; usually the flask became warm and the mixture was then stirred. After the flask was placed under reduced pressure (*ca.* 0.005 mm) the mixture was heated gradually in an oil bath for about 4 hr to a final temperature of 80–100°. In cases where the fluoridate distilled below 110°, it was distilled out of the reaction mixture as it was formed. The fluoridate is more stable to heat than the chloridate so higher temperature can be applied to the flask to drive out the remaining fluoridate and unreacted chloridate from the KF–KCl reaction mixture. Repeat using less KF until a constant index of refraction is obtained.

Table I

$RP(O)(OC_6H_{11})F$

No.	R	Formula[a]	Bp, °C (mm)	$n^{25}D$	Yield, %
1	Pr	$C_9H_{18}FO_2P$	43 (0.03)	1.4378	45
2	Bu	$C_{10}H_{20}FO_2P$	77 (0.15)	1.4391	58
3	Pent	$C_{11}H_{22}FO_2P$	60 (0.005)	1.4409	56
4	Hex	$C_{12}H_{24}FO_2P$	93 (0.005)	1.4424	68
5	Ph	$C_{12}H_{16}FO_2P$	94 (0.05)	1.5021	61
6	$PhCH_2$	$C_{13}H_{18}FO_2P$	96 (0.03)	1.5041	68
7	$Ph(CH_2)_2$	$C_{14}H_{20}FO_2P$	113 (0.03)	1.5001	44
8	$Ph(CH_2)_3$	$C_{15}H_{22}FO_2P$	132 (0.005)	1.4988	67
9	$Ph(CH_2)_4$	$C_{16}H_{24}FO_2P$	159 (0.03)	1.4972	33

[a] All compounds were analyzed for C, F, H, and P and were within ±0.4% of theoretical value except for 1 (F: calcd 9.12; found 8.57) and **6** (F: calcd 7.41; found 6.65).

For cyclohexyl alkylphosphonofluoridates boiling over 110°, the reaction mixture of the cyclohexyl phenylpropylphosphonofluoridate was cooled. About 60 ml of abs Et_2O was added, stirred for *ca.* 10 min, and filtered. After washing with abs Et_2O, Et_2O was removed at a water aspirator and final bath temperature of 70°: wt of syrup 29 g, $n^{25}D$, 1.4991. Add 4 g of KF to syrup and repeat: wt of syrup 30 g, $n^{25}D$, 1.4985. The final syrup from either procedure was distilled through a falling film molecular still at BuOH refluxing temp: wt 23 g, $n^{25}D$, 1.4992. The colorless distillate was distilled through a short-path distillation head at 129° (0.005 mm), yield 22 g (67%) $n^{25}D$, 1.4993.

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(7) Heating medium for falling film molecular still. Distillation carried out at a pressure of *ca.* 0.005 mm.