

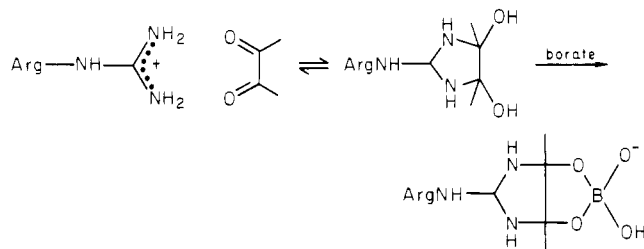
Synthesis of 9-(3,4-Dioxopentyl)hypoxanthine, the First Arginine-Directed Purine Derivative: An Irreversible Inactivator for Purine Nucleoside Phosphorylase[†]

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ABSTRACT: The synthesis of two potential arginine-directed purine-based analogues, 6-chloro-9-(3,4-dioxopentyl)purine (**6**) and 9-(3,4-dioxopentyl)hypoxanthine (**7**), is reported. Compound **7** was extensively tested as a potential affinity label of purine nucleoside phosphorylase (EC 2.4.2.1) from human erythrocytes. Evidence that **7** reacted with the catalytic center of purine nucleoside phosphorylase includes the following: (1) time-dependent inactivation of the enzyme by **7** was observed; (2) a plot of the pseudo-first-order rate constant for inactivation of the enzyme vs. concentration of **7** was hyperbolic, characteristic of saturation phenomenon; (3) substrates (P_i , arsenate, inosine) and a competitive inhibitor (formycin B) protected the enzyme from inactivation by **7**. Compound **7** was 25 times more effective in inhibiting purine nucleoside

phosphorylase than butanedione. Evidence that **7** modified arginine(s) includes the following: (1) when the inactivation was performed in borate, both the rate and the extent of inactivation were enhanced compared to those of the controls run in tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl) buffer; (2) dialysis of inactivator reversed the inactivation in Tris-HCl but not in borate buffer. All the above evidence combined with the previous demonstration [Jordan, F., & Wu, A. (1978) *Arch. Biochem. Biophys.* 190, 699-704] that butanedione modified only arginines in purine nucleoside phosphorylases and the results presented here demonstrating the similarities in the behavior of butanedione and **7** imply that compound **7** can be called an arginine-directed affinity label for purine nucleoside phosphorylase.

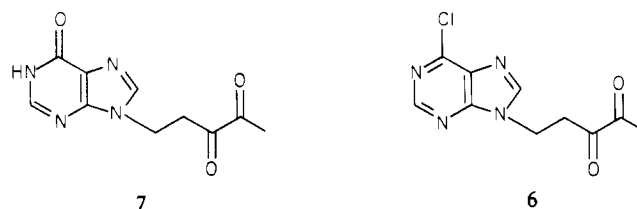
Arginine has been found to serve the function of an anion recognition site at the catalytic center of a large number of enzymes (Riordan, 1979). It has been amply demonstrated that an α -diketone functionality is capable of reacting with the guanidinium moiety of arginine in models (Yankeelov et al., 1966; Yankeelov, 1970) and in carboxypeptidase (Riordan, 1973). Such a reaction is made substantially more favorable (both faster and essentially irreversible) by the addition of borate. The presumed role of borate is the stabilization of the dicarbinolamine (dihydroxyimidazoline) adduct:



It was reported earlier from this laboratory that purine nucleoside phosphorylase (PNP) from human erythrocytes and calf spleen was inactivated by butanedione and phenylglyoxal (Jordan & Wu, 1978) and the inactivation was accompanied by specific reaction with arginine residues. On account of the widespread occurrence of functional arginine residues in enzymes that process nucleic base derivatives or employ nucleotide cofactors (Riordan, 1979), a program was undertaken to synthesize purine derivatives that also contain the α -diketone moiety. Such compounds may have the potential of being

arginine-directed affinity labels.

While there is a paper in the literature on the synthesis of pyrimidine derivatives containing the α -diketone moiety (Lee et al., 1978), this appears to be the first to give an account of the successful total synthesis of an α -diketone that is attached to purines: 6-chloro-9-(3,4-dioxopentyl)purine (**6**) and 9-(3,4-dioxopentyl)hypoxanthine (**7**). The synthetic route



outlined for both purine analogues is derived from 6-chloropurine; hence, the procedure would lend itself to the synthesis of the corresponding adenine analogue, as well as to several other potentially useful fluorescent and photoaffinity labels. The usefulness of **7** is demonstrated on PNP from human erythrocytes and calf spleen. According to accepted criteria, **7** is an affinity label for this enzyme, very likely by virtue of its interaction with arginine in the catalytic center.

Materials and Methods

General Remarks. Melting points were determined on a Thomas-Hoover 6406-K melting point apparatus and are uncorrected. Refractive indices were determined on a Bausch & Lomb 33-45-58 refractometer. UV spectra were recorded in the thermostated ($\pm 0.1^\circ\text{C}$) cell compartment of a Cary 219 spectrophotometer. IR spectra were recorded on a Beckman IR 4240 spectrophotometer. ^1H NMR¹ spectra were

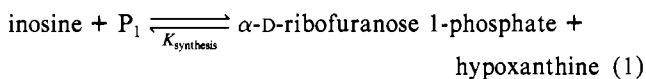
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¹ Abbreviations: NMR, nuclear magnetic resonance; Tris, tris(hydroxymethyl)aminomethane; HPLC, high-performance liquid chromatography; EDTA, ethylenediaminetetraacetic acid; TLC, thin-layer chromatography.

recorded on a JEOL JNM-PS FT 100 spectrometer operating in the Fourier transform mode. The ^1H chemical shifts are expressed as δ , parts per million, downfield relative to Me_4Si in organic solvents and $(\text{H}_3\text{C})_3\text{Si}(\text{CD}_2)_2\text{CO}_2\text{Na}$ (TSP) in D_2O . The mass spectra were recorded on a Finnigan Model 6000 spectrograph with methane as a reagent gas in the chemical ionization mode or on an AEI MS 30 fitted with a DEC PDP8 computer. pH readings were made on Radiometer Model 26 meter equipped with a combination electrode.

Sodium borate, sodium arsenate, sodium phosphate, potassium phosphate, Florosil (100–200 mesh), and thin-layer chromatography plates (Eastman Chromagram 13181, silica gel) were purchased from Fisher Scientific Co.; 5-hydroxy-2-pentanone, ethylene glycol, 6-chloropurine, and formic acid from Aldrich Chemical Co.; *n*-amyl nitrite and *n*-propyl nitrite from Pfaltz and Bauer, Inc.; inosine, hypoxanthine, formycin B, purine nucleoside phosphorylase (calf spleen, EC 2.4.2.1) as a crystalline suspension in 3.2 M $(\text{NH}_4)_2\text{SO}_4$ (26 units/mg), and xanthine oxidase as a crystalline suspension in 3.2 M $(\text{NH}_4)_2\text{SO}_4$ (1.68 units/mg) from Sigma Chemical Co. Purine nucleoside phosphorylase from human erythrocytes was isolated in this laboratory according to protocols devised by Zannis et al. (1978) and Wiginton et al. (1980). All deuterated solvents, Me_4Si , and TSP were purchased from Stohler Isotope Co.; silica gel and Tris-HCl from J. T. Baker. Dimethyl sulfoxide was freshly distilled at reduced pressure before use. Chloroform was distilled over molecular sieves, and all other solvents were purchased as HPLC or spectrophotometric grade.

Enzyme Assay. The reaction catalyzed by purine nucleoside phosphorylase has a $K_{\text{synthesis}}$ of 26 at 37 °C (Salamone et al., 1982). In the direction of nucleoside phosphorylase, the rate



can be assayed by employing xanthine oxidase and measuring the rate of appearance of urate (λ_{max} 293 nm, ϵ 1.2×10^4) from hypoxanthine (Kalckar, 1947). A typical reaction mixture contained 0.05 M borate or Tris, pH 7.6–8.0, 1 mM inosine, 1 mM phosphate, 0.14 unit of xanthine oxidase, and 0.03 unit of PNP in a 1 cm path cuvette containing 1 mL total solution. The absorbance was recorded at 293 nm as a function of time. All assays were performed at 37 °C.

Inhibition Studies. In studies examining inhibition of PNP, the inhibitors were dissolved in the same buffer used in the assay. Aliquots of the solution containing the inhibitor were equilibrated with the enzyme. The amount of protein used was 4.6 μg /0.5 mL of incubation mixture. The activity of the enzyme was determined periodically and compared to controls that did not contain inhibitor. In experiments testing for potential protection of the catalytic site from irreversible inactivation, substrates and competitive inhibitors of PNP were incubated with the enzyme for 30 min before addition of the inactivator. It is essential that the concentration of substrates in the assay mixtures be the same for all parallel studies, because phosphate and inosine not only protect the enzyme from inactivation by α -diketones but also activate the enzyme (Parks & Agarwal, 1972).

The test for the reversibility of the inactivation by α -diketones was performed by dialysis of the enzyme solution, subsequent to inactivation, for 18–20 h against three changes (200 volumes) of distilled water at 3 °C. The dialysis membranes were prepared by first boiling them in 0.5 M EDTA for 30 min and then washing them 8 times with boiling water (30 min each).

Syntheses. 5-Bromo-2-pentanone (**1**) (Grandberg & Zu-

yanova, 1968). 5-Hydroxy-2-pentanone (51.5 g, 500 mmol) was added to 200 mL of saturated aqueous HBr solution at 0 °C. The mixture was mechanically stirred at 0 °C for 0.5 h and then at room temperature for an additional 0.5 h. The dark reaction mixture was next poured into 1200 mL of chilled water. The aqueous solution was extracted twice with ether, and the combined organic layers were dried (MgSO_4). The ether was evaporated in vacuo at room temperature, and the residue was distilled at reduced pressure (35–45 °C, ~ 5 torr) to yield a colorless liquid (68 g, 410 mmol, 82%); n_D 1.4680; IR (film) 1715 cm^{-1} ($\text{C}=\text{O}$); ^1H NMR ($\text{CDCl}_3/\text{Me}_4\text{Si}$) δ 3.45 (t, 2 H, $J = 6$ Hz, 5- CH_2), 2.61 (t, 2 H, $J = 7$ Hz, 3- CH_2), 2.91 (s, 3 H, 1- CH_3), superimposed on 2.25–2.05 (2 H, complex multiplet).

1-Bromo-4,4-ethylenedioxy-pentane (**2**) (Johnson et al., 1956). *p*-Toluenesulfonic acid (0.5 g), ethylene glycol (17 g, 274 mmol), and 5-bromo-2-pentanone (**5**) (8.3 g, 50 mmol) were added to 500 mL of dry benzene. The reaction mixture was refluxed for 18 h in a vessel that was also equipped with a Dean-Stark apparatus to remove the water being produced. The solution was then cooled and washed several times with saturated sodium bicarbonate and once with water. The combined aqueous layers were extracted with ether (5×150 mL), and the combined organic layers were dried (MgSO_4). The solvent was evaporated on a rotary evaporator, and the dark brown residue was distilled at reduced pressure (46–56 °C, ~ 5 torr) yielding a colorless liquid (9 g, 42 mmol, 84%). The IR spectrum indicated the absence or near absence of carbonyl stretching frequency; hence, the product was essentially free of starting material. ^1H NMR ($\text{CDCl}_3/\text{Me}_4\text{Si}$) gave the following: δ 4.19 (s, 4 H, ethylene ketal), 3.80 (t, 2 H, $J = 8$ Hz, 3- CH_2), 2.06–1.80 (m, 2 H, 2- CH_2), superimposed on 1.85 (t, 2 H, 1- CH_2), and 1.26 (s, 3 H, 2- CH_3). This compound could be stored below 0 °C without significant decomposition.

6-Chloro-9-(4,4-ethylenedioxy-pentyl)purine (**3**) (Montgomery & Temple, 1957). 6-Chloropurine (5 g, 32 mmol), 1-bromo-4,4-ethylenedioxy-pentane (**2**) (6.8 g, 32 mmol), and K_2CO_3 (4.86 g, 35 mmol) were added to 320 mL of freshly distilled Me_2SO , and the mixture was stirred for 18 h at room temperature under N_2 . The progress of the reaction was monitored by TLC (chloroform/ethanol, 97:3 v/v) on which **3** had an R_f of 0.54. The reaction mixture was filtered and the Me_2SO was evaporated at reduced pressure, yielding a tarry residue. The residue was triturated (4×60 mL) with ethyl acetate, dissolved in 5 mL of water, and extracted with ether (5×60 mL). The combined organic layers were dried (MgSO_4) and evaporated in vacuo. The resulting highly colored yellow solid was applied to a silica gel column (100 g) and eluted (chloroform/ethanol, 97:3 v/v) to yield a white crystalline solid **3** (5.8 g, 21 mmol, 65%). Recrystallization from ethyl acetate yielded the following: mp 75 °C; IR (thin film) 3100, 3070, 2980, 2950, 2880, 1590, 1505, 1495, 1440, 1400, 1375, 1300, 1250, 1210, 1140, 1060, 935, 850, 790, 630 cm^{-1} ; UV λ_{max} (ethanol) ($\epsilon \times 10^{-3}$) 265 nm (7.42); ^1H NMR ($\text{CDCl}_3/\text{Me}_4\text{Si}$) δ 8.74 (s, 1 H, 6-chloropurine C2 H), 8.18 (s, 1 H, 6-chloropurine C8 H), 4.36 (t, 2 H, $J = 6$ Hz, 1- CH_2), 3.95 (s, 4 H, ethylene ketal CH_2), 2.10 (t, 2 H, $J = 6$ Hz, 3- CH_2), 1.73 (m, 2 H, 2- CH_2), 1.31 (s, 3 H, 5- CH_3); MS ($M^+ + 1$) m/z (relative intensity) 283 (68), 247 (20), 177 (100), 129 (75), 117 (45). Anal. Calcd for $\text{C}_{12}\text{H}_{15}\text{N}_4\text{O}_2\text{Cl}$: C, 50.98; H, 5.35; N, 19.82. Found: C, 51.13; H, 5.10; N, 19.82.

6-Chloro-9-(4-oxopentyl)purine (**4**) (Lee et al., 1978). 6-Chloro-9-(4,4-ethylenedioxy-pentyl)purine (**3**) (3.18 g, 11.3 mmol) was added to 125 mL of refluxing 10% (v/v) formic

acid. After a 25-min reflux, the solution was cooled, and the solvent was removed at reduced pressure. The residue was dissolved in 20 mL of water, and the pH was adjusted (5 N NaOH) to 8.0. The solution was next extracted with chloroform (5 × 25 mL), the combined chloroform layers were dried (MgSO₄), and the solvent was evaporated on a rotary evaporator to yield a yellow oil. The oil was applied to a short silica gel column and eluted (chloroform/ethanol, 97:3 v/v) to yield white crystals (2 g, 8.3 mmol, 75%) of compound 4. An analytical sample was prepared by recrystallization from ethanol: TLC (Chloroform/ethanol, 97:3 v/v) *R_f* 0.44; mp 63–64 °C; IR (thin film) 3110, 3080, 2950, 1710, 1590, 1560, 1500, 1435, 1400, 1370, 1330, 1215, 1180, 1150, 940, 855, 790, 680 cm⁻¹; UV λ_{max} (ethanol) (ε × 10⁻³) 265 nm (11.45); ¹H NMR (CDCl₃/Me₄Si) δ 8.74 (s, 1 H, 6-chloropurine C2 H), 8.16 (s, 1 H, 6-chloropurine C8 H), 4.36 (t, 2 H, *J* = 6 Hz, 1-CH₂), 2.51 (t, 2 H, *J* = 6 Hz, 3-CH₂), 2.21 (m, 2 H, 2-CH₂), 2.15 (s, 3 H, 5-CH₃) (in D₂O in the presence of base or acid the resonances corresponding to the 5-CH₃ and 3-CH₂ disappeared due to exchange with solvent deuterons and the multiplet corresponding to 2-CH₂ became a triplet); MS (*M*⁺ + 1) *m/z* (relative intensity) 239 (100) 203 (10). Anal. Calcd for C₁₀H₁₁N₄O₄Cl₁: C, 50.32; H, 4.65; N, 23.47. Found: C, 50.52; H, 4.70; N, 23.47.

The procedure that was employed to remove the ketal group also converted some of the 6-chloropurine to hypoxanthine to yield 9-(4-oxopentyl)hypoxanthine. This compound precipitated (0.4 g, 1.6 mmol) during the chloroform extraction: TLC (acetonitrile/ethanol, 50:50 v/v) *R_f* 0.40; mp 202–205 °C decomposition (taken from 50 °C); IR (KBr pellet), 3400 (br), 3040, 2950, 2860, 1700, 1680, 1590, 1410, 1365, 1200, 1120, 920, 895 cm⁻¹; UV λ_{max} (ethanol) (ε × 10⁻³) 250 nm (6.17); ¹H NMR (Me₂SO-*d*₆/Me₄Si) δ 12.4 (br s, 1 H, hypoxanthine 1-NH), 8.06 (s, 1 H, hypoxanthine 8-CH), 8.02 (s, 1 H, hypoxanthine 2-CH), 4.11 (t, 2 H, *J* = 7 Hz, 1-CH₂), 2.48 (s, 2 H, *J* = 7 Hz, 3-CH₂), 2.05 (s, 3 H, 5-CH₃), superimposed on 2.10–1.97 (m, 2 H, 2-CH₂) (in D₂O in the presence of base or acid the resonances corresponding to the 5-CH₃ and 3-CH₂ disappeared due to exchange with solvent deuterons); MS (*M*⁺) *m/z* (relative intensity) 220 (15), 177 (9.3), 163 (43.4), 150 (16.5), 149 (17.4), 137 (13.5), 136 (12.5), 85 (27.9).

Alternate Synthesis for 6-Chloro-9-(4-oxopentyl)purine (4). 6-Chloropurine (2.5 g, 16 mmol), 5-bromo-2-pentanone (2.66 g, 16 mmol), and K₂CO₃ (2.43 g, 17.6 mmol) were added to 160 mL of freshly distilled Me₂SO, and the solution was stirred for 16 h at room temperature. The progress of the reaction was monitored by TLC (chloroform/ethanol, 97:3 v/v) on which the N9 substituted product had an *R_f* of 0.44. The same workup and purification that was employed in the synthesis of 6-chloro-9-(4,4-ethylenedioxypentyl)purine yielded 2.1 g of 4 (8.8 mol, 55%).

6-Chloro-9-(3-oximido-4-oxopentyl)purine (5) (Lee et al., 1978). 6-Chloro-9-(4-oxopentyl)purine (4) (2.68 g, 10 mmol), *n*-propyl nitrite (2.9 mL, 35 mmol), and 0.5 mL of concentrated HCl were added to 200 mL of 2-propanol. After the solution was stirred for 18 h at room temperature, the solvents were evaporated under reduced pressure. A light yellow powdery product resulted (2.6 g, 9.7 mmol, 97%). This powder was applied to a silica gel column (60 g). Elution (chloroform/ethanol, 98:2 v/v) yielded a white powder that could be recrystallized from chloroform/acetonitrile: TLC (chloroform/ethanol, 97:3 v/v) *R_f* 0.29; mp 165 °C decomposition (start reading from 100 °C); IR (thin film) 3340 (br), 2990, 2900, 1685, 1590, 1560, 1500, 1440, 1400, 1335, 1255, 1215, 1175, 1140, 1095, 1025, 990, 940, 900, 825, 790, 730, 675,

625 cm⁻¹; UV λ_{max} (ethanol) (ε × 10⁻³) 264 nm (7.41); ¹H NMR (Me₂SO-*d*₆/Me₄Si) δ 12.26 (br s, 1 H, oxime OH), 8.75 (s, 1 H, 6-chloropurine 2-CH), 8.64 (s, 1 H, 6-chloropurine 8-CH), 4.43 (t, 2 H, *J* = 7 Hz, 1-CH₂), 3.00 (t, 2 H, *J* = 7 Hz, 2-CH₂), 2.20 (s, 3 H, 5-CH₃); MS (*M*⁺ + 1) *m/z* (relative intensity) 268 (10), 208 (90), 155 (50), 114 (100). Anal. Calcd for C₁₀H₁₀N₅O₂Cl₁: C, 44.87; H, 3.77; N, 26.16. Found: C, 44.79; H, 3.57; N, 26.23.

6-Chloro-9-(3,4-dioxopentyl)purine (6) (Lee et al., 1978). 6-Chloro-9-(3-oximido-4-oxopentyl)purine (5) (0.54 g, 1.78 mmol) was added to 25 mL of refluxing *n*-amyl nitrite. After a 4-min reflux, the reaction flask was allowed to cool to room temperature, and the solvent was removed at reduced pressure. The yellow residue that resulted was dissolved in 1 mL of chloroform, and the solution was applied to a silica gel column (2.5 cm × 25 cm) or a Lobar type B silica gel column and eluted (chloroform/ethanol, 98:2 v/v). The band corresponding to *R_f* 0.49 on TLC (chloroform/ethanol, 97:3 v/v) proved to be the desired compound and was isolated as a light yellow crystalline compound (0.089 g, 0.36 mmol, 20%): mp 106 °C; IR (thin film) 3110, 3070, 2920, 2960, 1710, 1590, 1555, 1495, 1430, 1400, 1330, 1255, 1205, 1175, 1060, 930, 850, 790, 625 cm⁻¹; UV λ_{max} (ethanol) (ε × 10⁻³) 264 nm (4.93); ¹H NMR (CDCl₃/Me₄Si) δ 8.64 (s, 1 H, 6-chloropurine 2-CH), 8.17 (s, 1 H, 6-chloropurine 8-CH), 4.55 (t, 2 H, *J* = 7 Hz, 1-CH₂), 3.38 (t, 2 H, *J* = 6 Hz, 3-CH₂), 2.27 (s, 3 H, 5-CH₃); MS (*M*⁺ + 1) *m/z* (relative intensity) 253 (100), 209 (10), 189 (15), 155 (60). Anal. Calcd for C₁₀H₉N₄O₂Cl₁: C, 47.54; H, 3.59; N, 22.18. Found: C, 47.42; H, 3.59; N, 22.03.

9-(3,4-Dioxopentyl)hypoxanthine (7). 6-Chloro-9-(3,4-dioxopentyl)purine (6) (0.05 g, 0.2 mmol) was refluxed in 8 mL of 0.1 N HCl for 1 h. Next, the solution was cooled and lyophilized. The dark yellow residue was triturated (ethyl acetate), and the solvent was evaporated under reduced pressure, yielding 9-(3,4-dioxopentyl)hypoxanthine hydrochloride (36 mg, 0.13 mmol, 65%). A sample was prepared for analysis by dissolving 20 mg of 7 in alcohol, applying this solution to a silica gel column (1 × 12 cm), and eluting (ethanol/acetonitrile, 50:50 v/v): TLC (ethanol/acetonitrile, 50:50 v/v) *R_f* 0.30; mp 105–106 °C decomposition (taken from 50 °C); IR (thin film) 3420 (br), 3080, 2960, 2910, 1710, 1680, 1590, 1560, 1500, 1435, 1400, 1335, 1210, 1185, 1180, 1065, 935, 910, 850, 790, 725 cm⁻¹; UV λ_{max} (ethanol) (ε × 10⁻³) 249 nm (7.56); ¹H NMR (Me₂SO-*d*₆/Me₄Si) δ 12.60 (br s, 1 H, hypoxanthine 1-NH), 8.05 (s, 1 H, hypoxanthine 8-CH), 8.02 (s, 1 H, hypoxanthine 2-CH), 4.37 (t, 2 H, *J* = 7 Hz, 1-CH₂), 3.32 (t, 2 H, *J* = 6 Hz, 2-CH₂), 2.22 (s, 3 H, 5-CH₃); MS (*M*⁺ + 1) *m/z* (relative intensity) 235 (20), 234 (20), 209 (80), 188 (80), 156 (30), 155 (35), 154 (100), 153 (75), 152 (30), 136 (35), 125 (40), 119 (50), 111 (30). Anal. Calcd for C₁₀H₁₁N₄O₃Cl₁: C, 44.36; H, 4.09. Found: C, 44.37; H, 4.37.

In D₂O (TSP) the α diketone was found to exist in three different forms (Greenzaid et al., 1967): the unhydrated form [δ 2.32 (s, 5-CH₃), 3.46 (t, 2-CH₂)], the 3-C=O monohydrate [δ 2.22 (s, 5-CH₃), 2.48 (t, 2-CH₂)], and the 4-C=O monohydrate [δ 1.40 (s, 5-CH₃), 3.46 (t, 2-CH₂)].

Results and Discussion

Synthesis of 6-Chloro-9-(3,4-dioxopentyl)purine (6) and 9-(3,4-Dioxopentyl)hypoxanthine (7). Various attempts to synthesize 7 by direct alkylation of hypoxanthine failed (Salamone, 1982). On the basis of reports by several groups that indicated the much enhanced solubility in organic solvents of *N*-alkyl-6-chloropurines compared to the virtual insolubility

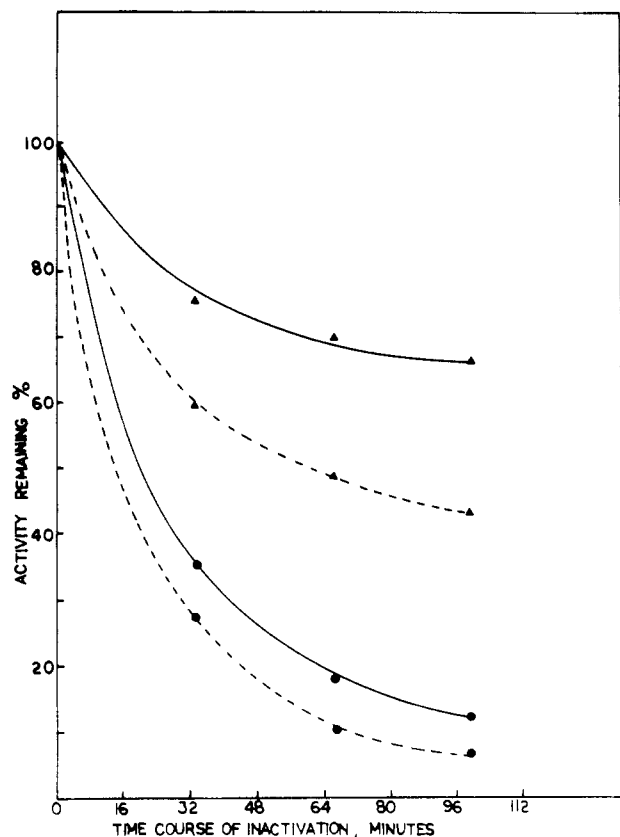


FIGURE 1: Time course of inactivation of purine nucleoside phosphorylase from calf spleen (—) and human erythrocytes (---) by butanedione [(●) 57 mM] and 9-(3,4-dioxopentyl)hypoxanthine [(▲) 0.8 mM] in the presence of 0.05 M borate (pH 8.0) at 37 °C.

of *N*-alkylhypoxanthines, Scheme I was finally adopted (see details under Materials and Methods).

Alkylation of 6-chloropurine with **1** or **2** gave the N9-alkylated compound as the major product and the N7-alkylated compound as a minor product. These isomers were easily separated and the N9-substituted compound could be differentiated from the N7-substituted 6-chloropurine as follows: (1) In all reported alkylations of 6-chloropurine employing the experimental conditions used here, the N9-alkylated 6-chloropurine is the major component and the N7-substituted isomer in many cases is formed only in trace amounts (Montgomery & Temple, 1957, 1961; Schaeffer & Odin, 1966; Lister, 1971). (2) The N9 isomer is less polar and more soluble in organic solvents than the N7-substituted 6-chloropurine. (3) The proton resonances corresponding to the methylene or methyl protons attached to N9 in 6-chloropurine or hypoxanthine are shifted upfield (~0.2 ppm) relative to those in the N7-substituted isomer (Montgomery et al., 1966; Bergmann et al., 1969). (4) The 6-chloro-9-(4,4-ethylene-dioxopentyl)purine produced by an unequivocal scheme (Salamone, 1982) had ¹H NMR, UV, mp, and TLC characteristics identical with those of compound **3**.

Studies on Inactivation of PNP by α -Diketones. Time Course of Inactivation. Figure 1 demonstrates that **7** inhibits PNP from human erythrocytes and from calf spleen more effectively than does butanedione and that PNP from human erythrocytes is more susceptible to modification by both α -diketones than is the enzyme from calf spleen. Figure 2 illustrates the early time course of inactivation of PNP (human erythrocyte) by varying concentrations of **7**. At later times all curves indicated distinct biphasic behavior, where the first phase appeared to depend on the initial concentration of α -diketone present and the second, slower one did not. A plot

Scheme I

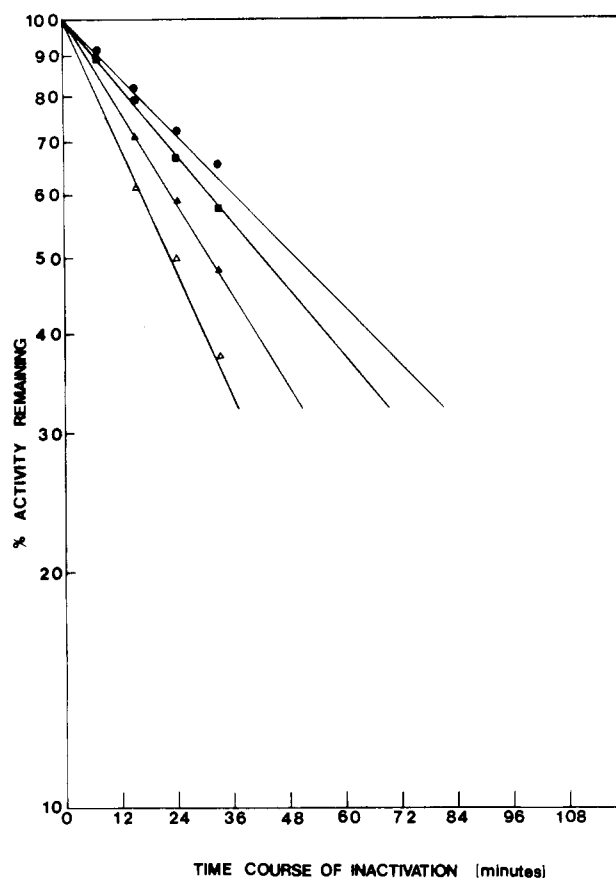
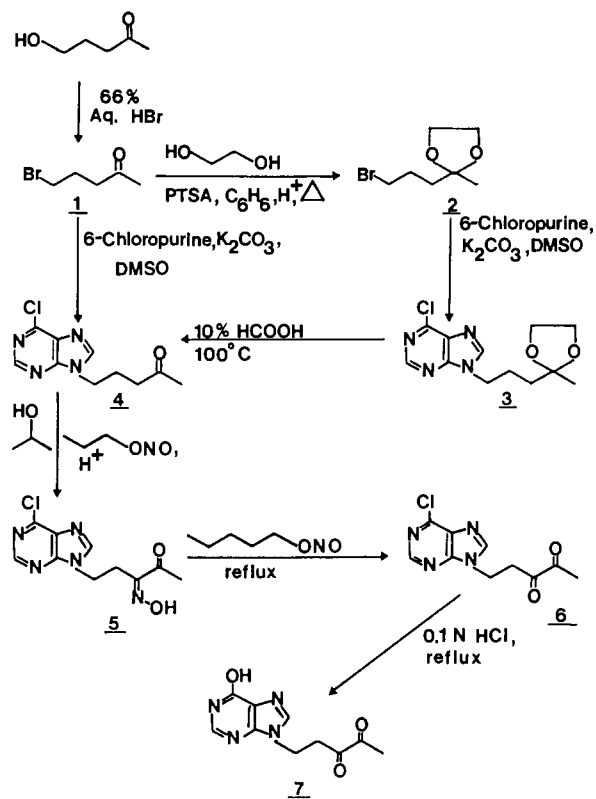


FIGURE 2: Time course of inactivation of purine nucleoside phosphorylase from human erythrocytes in 0.05 M borate (pH 8.0) at 37 °C by 1.1 (●), 1.33 (■), 2.0 (▲), and 4.0 mM (△) 9-(3,4-dioxopentyl)hypoxanthine.

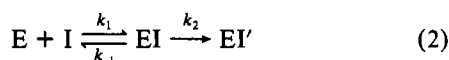
of k_{obsd} for inactivation vs. concentration of **7** was distinctly hyperbolic, a behavior characteristic of saturation kinetics

Table I: Effect of Substrates and a Competitive Inhibitor on Inactivation of Purine Nucleoside Phosphorylase from Human Erythrocytes by 9-(3,4-Dioxopentyl)hypoxanthine^a

chemical modifier	substrate added in preliminary treatment (concn)	protection (%)	k_{obsd}^b (min^{-1})
9-(3,4-dioxopentyl)-hypoxanthine (1.1 mM)	phosphate (30 mM)	100	0
	phosphate (10 mM)	75	2.6×10^{-3}
	arsenate (10 mM)	57	4.4×10^{-3}
	inosine (15 mM)	62	4.1×10^{-3}
	formycin B (2 mM)	33	7.9×10^{-3}
	none	0	13.3×10^{-3}

^a The enzyme was incubated with inhibitor for 1 h at 37 °C in 0.05 M borate buffer (pH 8) as described under Materials and Methods before the standard assay was performed. ^b The first-order rate constant for inactivation by 7 in the presence of the indicated concentration of inhibitor.

indicating that the following kinetic scheme was being followed (Kitz and Wilson, 1962):



where EI and EI' are reversibly and irreversibly inhibited PNP, respectively. A steady-state treatment of this mechanism yields

$$1/k_{\text{obsd}} = K_I/(k_2[I]) + 1/k_2 \quad (3)$$

and

$$K_I = [E][I]/[EI] \quad (4)$$

Employing k_{obsd} obtained from Figure 2 and eq 3 results in Figure 3, from which k_2 (0.057 min^{-1}) and K_I (3.3 mM) may be obtained for inactivation of PNP from human erythrocytes by compound 7. Thus the data are consistent with rapid, reversible binding of the inhibitor followed by a slower conversion to an irreversibly inhibited form. The K_I obtained is larger than the K_M s for inosine ($K_M = 0.058\text{--}1.5 \text{ mM}$), hypoxanthine ($K_M = 0.019 \text{ mM}$), arsenate ($K_M = 1.8 \text{ mM}$), and phosphate ($K_M = 0.32\text{--}0.74 \text{ mM}$), as well as for formycin B ($K_I = 0.1 \text{ mM}$) [see Table VI in Parks & Agarwal (1972)] and 1- α -D-ribofuranosyl-1,2,4-triazole-3-carboxamide ($K_I = 5 \mu\text{M}$, Willis et al., 1980). The latter two compounds are the most effective reversible inhibitors known to date.

Compound 7 is ca. 25 times more effective as an irreversible inactivator than is butanedione.

Evidence for Chemical Modification at or near the Catalytic Center. There are reported 10–11 arginines per subunit of PNP isolated from calf spleen (Edwards et al., 1973). It is of importance therefore to demonstrate whether or not chemical modification is occurring at the catalytic center. It was demonstrated earlier (Jordan & Wu, 1978) that the

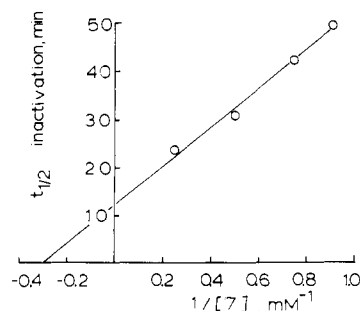


FIGURE 3: Plot of half-life for inactivation of purine nucleoside phosphorylase from human erythrocytes by 9-(3,4-dioxopentyl)-hypoxanthine (from Figure 2) against reciprocal concentration of inhibitor (according to eq 3).

presence of 66.7 mM phosphate or arsenate or inosine provided approximately 50% protection from modification by 25 mM butanedione.

Table I summarizes the experiments testing protection by substrates and a competitive inhibitor against inactivation by 7. The data indicate that 30 mM P_i effectively protects the catalytic center from inactivation from 1.1 mM 7. Arsenate is less effective than phosphate in protection from inactivation by 7 when employed at equimolar concentrations (10 mM) during the preincubation. Finally, formycin B and inosine also offer protection from inactivation. The fact that P_i is more effective than inosine in protecting the catalytic center is consistent with earlier data employing butanedione (Jordan & Wu, 1978). The essentially quantitative protection by 30 mM P_i from inactivation by 7 very strongly suggests that the chemical modification is occurring at the catalytic center.

Evidence for Interaction of 7 with Arginine. It was already demonstrated earlier employing amino acid analysis that butanedione reacted with arginines of PNP (Jordan & Wu, 1978). Several experiments were performed to confirm that 7 reacted with PNP in an analogous fashion. These experiments were based on Riordan's (1979) protocol for confirming that the α -diketones reacted with arginines specifically: the presence of borate enhances the rate of inactivation by α -diketone and stabilizes the product. All of the experiments were performed by employing both 7 and butanedione in parallel reactions.

Table II indicates that the rate of inactivation of PNP by 7 or butanedione is enhanced by the presence of borate buffer. Furthermore, equilibrating 7 or butanedione in Tris buffer and adding borate prior to assaying brings about an instantaneous increase in inactivation. This indicates that the ternary arginine-inhibitor-borate complex is formed subsequent to the rate-determining step, which is probably the formation of the arginine- α -diketone complex. This mechanism had been previously suggested by Riordan (1973).

Table II: Change of Purine Nucleoside Phosphorylase (Human Erythrocyte) Activity upon Incubation with 9-(3,4-Dioxopentyl)hypoxanthine and Butanedione: Instantaneous Augmentation of Inactivation by Borate

	incubation and assay with Tris-HCl ^a	incubation and assay with borate ^a	incubation with Tris-HCl and assay with borate ^a
PNP	100	100	100
↓ 9-(3,4-dioxopentyl)hypoxanthine (2 mM)			
PNP-modifier ^b	81	50	45
PNP	100	100	100
↓ butanedione (50 mM)			
PNP-modifier ^b	66	50	45

^a The concentrations of borate and Tris were 50 mM. ^b The incubation with inhibitor lasted for 1 h (pH 7.8) at 37 °C prior to the standard assay.

In addition, the activity of the enzyme inactivated by either **7** or butanedione in borate buffer is not reversed upon dialysis whereas the activity of the enzyme inhibited by **7** or butanedione in Tris buffer is completely regenerated upon dialysis. These observations are characteristic of the irreversible nature of the reaction of α -diketone with arginine in the presence of borate (Riordan, 1973).

On account of the sum of the above evidence and the similarity of behavior of **7** and butanedione (in conjunction with earlier results demonstrating that butanedione modified arginines specifically in PNP), it is apparent that **7** reacts with arginine(s). Furthermore, the effective protection of the catalytic center by P_i or inosine from inactivation by **7** implies that an Arg at the catalytic center is being modified. Therefore, **7** can be appropriately labeled an arginine-directed affinity label.

Very recently it was demonstrated (Makinen et al., 1982) that under certain conditions butanedione provides photochemically induced inactivation of proteins. That the α -diketones employed in the present study did not inactivate PNP by a photochemical pathway was demonstrated as follows. When the reaction with **7** or butanedione was performed in the dark or under ordinary laboratory conditions, the same time course of inactivation was observed. The inactivation was totally reversible when Tris buffer was employed. This would not have been the case had **7** and butanedione led to photochemical processes.

Conclusions. It was demonstrated that an α -diketone moiety attached to a purine derivative can serve as an arginine-directed affinity label. N9-Substituted 6-chloropurines can be readily transformed chemically to yield both hypoxanthine and adenine analogues. Therefore, the synthetic route (Scheme I) outlined should be a useful starting point for targeting other enzymes that bind a purine base and possess arginine at their catalytic centers [see Riordan (1979) for numerous examples]. Elaboration to fluorescent analogues (e.g., ϵ -ethenoadenine derivatives) and photoaffinity labels (purine \rightarrow 8-bromopurine \rightarrow 8-azidopurine) would be a further desirable extension. Efforts along these lines are underway.

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