Cyclophosphamide Potentiation and Aldehyde Oxidase Inhibition by Phosphorylated Aldehydes and Acetals

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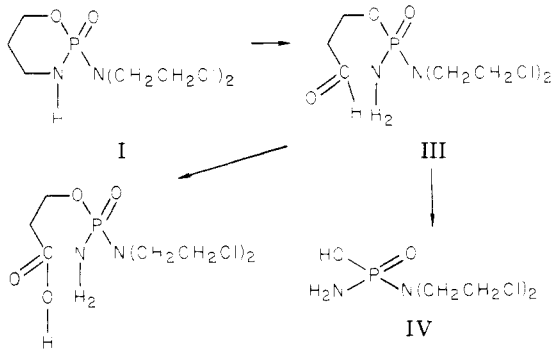
Fourteen phosphorylated acetals and aldehydes were synthesized for testing in vitro as inhibitors or substrates of aldehyde oxidase, an enzyme involved in the conversion of aldophosphamide to inactive carboxyphosphamide, and for concurrent in vivo administration with cyclophosphamide to mice bearing L1210 ascites tumor cells. Five phosphorus derivatives gave K_i values of 0.1–0.3 mM compared to 0.03 mM for pyridoxal, as determined in aldehyde oxidase assays using *N*-methylnicotinamide as the substrate. The most active phosphorus inhibitor, ethyl phenyl(2-formylethyl)phosphinate (**2b**), and pyridoxal were further shown to give competitive and mixed inhibition, respectively. Three aldehydes, administered concurrently with cyclophosphamide, produced greater increases in life span of L1210-implanted mice than did pyridoxal. All four agents gave an average increase in life span greater than 50% over that shown by cyclophosphamide alone.

Cyclophosphamide (I) is a widely used anticancer drug



I III II IV

whose metabolism has been extensively investigated.[1] The major urinary metabolite is inactive carboxyphosphamide (II), resulting from oxidation of aldophosphamide (III), the corresponding 2-formyl compound, which is a precursor to phosphoramide mustard (IV), a cytotoxic metabolite. Inhibition of this conversion, which is catalyzed by an aldehyde oxidase enzyme, would retard cyclophosphoramide catabolism and result in a potentiating cytotoxic effect. An earlier study of such inhibitors found pyridoxal to be the most active agent, when administered in combination with cyclophosphamide, for increasing the life span of mice implanted with L1210 cells.[2]

We here report the synthesis and in vitro and in vivo testing of a series of phosphorylated aldehydes and acetals, which are closely related structurally to aldophosphamide, as inhibitors or alternate substrates for aldehyde oxidase and potentiators of cyclophosphamide activity.

Chemistry. The phosphorylated aldehydes and acetals (Table I) were prepared by reaction of phosphites, phosphonites, or phosphinites with haloalkylaldehyde diethyl acetals, followed by hydrolysis (Scheme I), or by pyridinium chlorochromate oxidation of alcohols (Scheme II).

Certain products were observed to display physiochemical properties differing from closely related analogues. Compound **4** was initially isolated as a mixture of the aldehyde and what is believed to be its trimer or some other polymeric form, a situation not observed with its nitrogen isostere **5**. Prior to subjection to column chromatography, NMR examination of **4** showed peaks at both 5.20 and 9.57 ppm corresponding to methynyl and aldehydic protons, respectively. Only the latter chemical shift was present in chromatographed **4**.

Scheme I

$$R(R')(C_2H_5O)P + X(CH_2)_nCH(OC_2H_5)_2 \longrightarrow \underset{X = Cl;\ Br}{}$$

$$R(R')P(O)(CH_2)_nCH(OC_2H_5)_2 \xrightarrow[C_6H_4\text{-}1,4\text{-}(OH)_2]{H_3O^+}$$

1, R = R′ = C_6H_5; n = 1
1a, R = R′ = C_6H_5; n = 2
2a, R = C_6H_5; R′ = C_2H_5O; n = 1
2c, R = C_6H_5; R′ = C_2H_5O; n = 2
3a, R = R′ = C_2H_5O; n = 1
3c, R = R′ = C_2H_5O; n = 2
3e, R = R′ = C_6H_5O; n = 1

$$R(R')P(O)(CH_2)_nCHO$$

2, R = C_6H_5; R′ = C_2H_5O; n = 1
2b, R = C_6H_5; R′ = C_2H_5O; n = 2
3, R = R′ = C_2H_5O; n = 1
3b, R = R′ = C_2H_5O; n = 2
3d, R = R′ = C_6H_5O; n = 1

Scheme II

$$(C_6H_5X)_2P(O)Cl + HO\sim\sim OH \longrightarrow$$

$$(C_6H_5X)_2P(O)O\sim\sim OH \xrightarrow{C_5H_5NH\cdot ClCrO_3}$$

6, X = O
7, X = NH

$$(C_6H_5X)_2P(O)O\sim\sim CHO$$

4, X = O
5, X = NH

Contrary to an earlier report,[3] compound **3** was found to display aldo–enol tautomerism, existing in an equilibrium of forms V and VI.

$$(EtO)_2P(O)CH_2C(=O)H \rightleftharpoons (EtO)_2P(O)CH{=}C(OH)H$$

V VI

The neat IR spectrum of freshly distilled **3** showed not only strong absorption at 1720 cm^{-1}, characteristic of the carbonyl group, but also an equally intense absorption in the C=C region (1610 cm^{-1}). The ^1H NMR spectrum of **3** in deuterated chloroform also supports this phenomenon.

(1) P. J. Cox, B. J. Phillips, and P. Thomas, *Cancer Treat. Rep.*, **60**, 321 (1976), and pertinent references cited therein.
(2) D. L. Hill, W. R. Laster, and R. F. Struck, *Cancer* Res., **32**, 658 (1972).
(3) C. E. Griffin and S. K. Kundu, *J. Org. Chem.*, **34**, 1532 (1969).

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Table I. Phosphorylated Aldehydes and Acetals

$(R)_2P(O)(CH_2)_nCHX$ 1, 1a; $(R)(R'O)P(O)(CH_2)_nCHX$ 2, 2a-c; $(RO)_2P(O)(CH_2)_nCHX$ 3, 3a-e; $(RO)_2P(O)O(CH_2)_nCHO$ 4; $(RNH)_2P(O)O(CH_2)_nCHO$ 5

no.	R	R'	n	X	mp or bp (mm), °C	n_D^{20}	formula
1[a]	C_6H_5		1	$(OC_2H_5)_2$	96.5-97.5		$C_{18}H_{23}O_3P$
1a	C_6H_5		2	$(OC_2H_5)_2$	78-80[b]		$C_{19}H_{25}O_3P$
2	C_6H_5	C_2H_5	1	O	140-141 (0.1)[c,d]	1.5180[e]	$C_{10}H_{13}O_3P$
2a	C_6H_5	C_2H_5	1	$(OC_2H_5)_2$	139-141 (0.15)[f]	1.4949[g]	$C_{14}H_{23}O_4P$
2b[a]	C_6H_5	C_2H_5	2	O	133 (0.01)		$C_{11}H_{15}O_4P \cdot 0.5H_2O$
2c	C_6H_5	C_2H_5	2	$(OC_2H_5)_2$	170-172 (0.4)[h]	1.4950[i]	$C_{15}H_{25}O_4P$
3	C_2H_5		1	O	90-92 (0.2)[j,k]	1.4369	$C_6H_{13}O_4P$
3a	C_2H_5		1	$(OC_2H_5)_2$	88-90 (0.15)[m]	1.4300[n]	$C_{10}H_{23}O_5P$
3b	C_2H_5		2	O	91-97 (0.05)[o,p]	1.4400[q]	$C_7H_{15}O_4P$
3c	C_2H_5		2	$(OC_2H_5)_2$	94 (0.05)[r]	1.4300[s]	$C_{11}H_{25}O_5P$
3d	C_6H_5		1	O	*t,u*		$C_{14}H_{13}O_4P$
3e[a]	C_6H_5		1	$(OC_2H_5)_2$	197-199 (0.15)	1.5275[v]	$C_{18}H_{23}O_5P$
4[a]	C_6H_5		3		84-86		$C_{16}H_{17}O_5P$
5[w]	C_6H_5		3		69-74[x]		$C_{22}H_{23}N_6O_6P$

[a] Analyzed for C and H. [b] 76-77 °C. [c] 137-140 (0.02 mm) °C. [d] 2,4-Dinitrophenylhydrazone (DPH), mp 132-135 °C; 133-134 °C (ref 8). [e] 1.5163 (ref 8). [f] 142-143 (0.2 mm) °C (ref 8). [g] 1.4943 (ref 8). [h] 151-153 (0.25 mm) °C (ref 8). [i] n_D^{22} 1.4930 (ref 8). [j] 78-80 (0.17 mm) °C (ref 6). [k] DPH, mp 110-112 °C; 111-112 °C (ref 6). [l] 1.4355 (ref 6). [m] 97-98 (0.12 mm) °C (ref 6). [n] 1.4280 (ref 6). [o] 92-93 (0.23 mm) °C (ref 6). [p] DPH, mp 148-149 °C; 148-149 °C (ref 6). [q] 1.4465 (ref 6). [r] 98-100 (0.07 mm) °C (ref 6). [s] n_D^{24}; 1.4290 (ref 6). [t] Undistillable oil. [u] DPH, mp 147-149 °C. [v] n_D^{24}. [w] Analyzed for C, H, and N. [x] DPH, mp 152-154 °C.

In the spectrum, methylene protons (P – CH_2) appear as a doublet of doublets (splitting due to phosphorus) at δ 3.24 and 2.90. In addition, there are poorly defined multiplets at δ 4.65 and 7.13 which may be attributed to the α- and β-ethylenic protons of the enol tautomer, respectively.[4] Upon addition of D_2O to the spectral sample, the following observations were made: (a) complete exchange of the upfield doublets and (b) an increase in intensity of the signal at δ 7.13, with a corresponding decrease in intensity of the signal due to the aldehydic proton (δ 9.59). Thus, compound **3** exists in tautomeric equilibrium with enol form VI, and from the spectral data the presence of water appears to drive the equilibrium toward this form. These observations are compatible with those made on a group of analogous compounds which possessed an α-alkyl substituent.[5]

Phosphorylated aldehydes isolated by vacuum distillation are likely hydrated, whereby a water molecule is hydrogen bonded between two aldehyde groups. Two different workers synthesized **2b**, and separate elemental analyses showed identical results which agree exactly with a 0.5 mol of water of hydration being associated with this compound. Also, a 100-MHz NMR analysis of the agent showed an integration value between 4.5 and 6 ppm corresponding to almost one proton. A survey of 17 phosphorylated aldehydes synthesized and analyzed for phosphorus only by Russian chemists[5-9] found that 15 of these had low average experimental phosphorus values and five were off by 0.4% or more when compared to the lower of two determinations. Also, German workers[9] reported **3** bearing an α-methyl group as analyzing 0.52% higher for hydrogen, whereas a 0.37% higher value is calculated when a 0.5 mol of water of hydration is included.

Biology and Discussion of Results

Prior to this study, the only aldehydic compounds investigated for aldehyde oxidase inhibition or cyclophosphamide potentiation were acetaldehyde, benzaldehyde, and pyridoxal.[2] The former two agents inhibited enzymatic conversion of III to II to the extent of 17 (80 mM) and 22% (7 mM), respectively, while pyridoxal increased the life span of L1210 tumor bearing mice up to 154% with an average increase of 53%.

The purpose of this study was to design, synthesize, and biologically evaluate aldehydes bearing a P(O) group as inhibitors or alternative substrates of aldehyde oxidase in vitro and potentiators of cyclophosphamide in vivo antitumor activity. The two criteria established for these agents were that they possess no antitumor activity per se and that they resemble aldophosphamide in structure with the expectation that the P(O) moiety may play a role in increasing enzyme affinity or otherwise conferring a potentiating effect. A previous study concerning the inhibition of pyridoxal phosphate binding to apoaspartate aminotransferase by aldehyde phosphonic acids indicated the importance of the presence of both aldehydo and phosphoro portions.[10]

The effect on life span of mice bearing 1×10^5 L1210 ascites cells produced by five phosphorylated aldehydes (**2, 2b, 3, 4,** and **5**), six diethyl acetals (**1, 2a, 2c, 3a, 3c,** and **3e**), and pyridoxal, administered alone and concurrently with cyclophosphamide, was studied. No increase in life span over the cell control or groups given cyclophosphamide only was observed in any singly administered test compound or with **1, 2, 2a, 3a, 4,** and **5** when injected concurrently with cyclophosphamide. The average life span of the six control animals administered vehicle once on day 1 was 9.2 ± 0.3 days. Those agents concurrently administered with cyclophosphamide which gave increases in life span greater than cyclophosphamide alone are shown in Table II. All acetals were heated under acidic conditions just prior to administration in vivo for con-

(4) V. V. Moskva, V. M. Ismailov, T. V. Zykova, and A. I. Razumov, *Zh. Obshch. Khim.*, 41, 1676 (1971).
(5) V. V. Moska, G. F. Nazvanova, T. V. Zykova, A. I. Razumov, and L. A. Chemodanova, *Zh. Obshch. Khim.*, 41, 1680 (1971).
(6) A. I. Razumov and V. V. Moskva, *Zh. Obshch. Khim.*, 34, 2589 (1964).
(7) G. Kamai and V. A. Kuhtin, *Zh. Obshch. Khim.*, 27, 2436 (1957).
(8) A. I. Razumov and G. A. Savicheva, *Zh. Obshch. Khim.*, 34, 2595 (1964).
(9) L. Reichel and H. J. Jahns, *Justus Liebigs Ann. Chem.*, 751, 69 (1971).
(10) A. J. Rudinskas and T. L. Hullar, *J. Med. Chem.*, 19, 1367 (1976).

Table II. Effect of Compounds Potentiating the Activity of Cyclophosphamide on Mice Bearing L1210 Ascites Cells

compd[a]	ILC, %[b]	cures, %[c]
2b	175	83
2c	166	50
3	180	67
3c	204	83
pyridoxal	164	33
cyclophosphamide[d]	114	17

[a] Compounds corresponding to the numbers and pyridoxal were administered once on day 1 at a dose of 200 mg/kg. [b] The increase in life span of mice compared to the control. Mice surviving for at least 30 days were calculated as surviving for only 30 days when calculating the % ILS. Percent increase in life span (ILS, %) was calculated as follows: (average life span of drug treated − average life span of controls/average life span of controls) × 100. [c] Those animals in a group of six surviving 50 days beyond day 1, expressed as percent. [d] Administered alone and concurrently with the preceding compounds on day 1 at a dose of 100 mg/kg.

version to the corresponding aldehydes. Thus, **2,2a**, **2b,2c**, and **3,3a** represent only three different phosphorylated aldehyde preparations. The completeness of such conversion was monitored using **3c** in a separate NMR study by following the change of chemical shift of the acetal proton to the aldehydic proton. Such quantitative in situ conversion, as shown by NMR analysis, has also been used for aldehydophosphonic acetals.[10] Three phosphorylated derivatives gave life-span increases greater than pyridoxal, with **3c** showing the maximum effect. The close results between **2b** and its corresponding converted acetal **2c** provide additional evidence that the administered compounds were of a similar nature, and the aforementioned hydrolysis proceeded to completion. However, a comparison of activity of aldehyde **3** and its acetal **3a** in combination inexplicably showed only the former to be active. Also, with **2** and **2a**, similarly administered, the former produced four toxic deaths (those animals in a group of six dying on or before day 5) and none was observed in the latter. The only other toxic deaths, one each, were seen with **2b**, **3c** and **4** when given in combination with cyclophosphamide. The results of this portion of the investigation indicate that the greatest potentiation is achieved in compounds with a two-carbon separation between the aldehydic group and the phosphorus atom and with at least one *P*-ethoxy group. This latter relationship may involve solubility and/or steric factors.

Three aldehydes, four acetals, and pyridoxal were also investigated for ability to inhibit rabbit liver aldehyde oxidase catalyzed oxidation of *N*-methylnicotinamide (NMN) (Table III). This part of the study was restricted to pyridoxal and those compounds (**2a–c**, **3**, and **3a–c**) possessing one or two *P*-ethoxy groups and, thus, sufficient water solubility for in vitro use. Comparing aldehydes and acetals, **3b** and **3c** gave identical inhibitory effects, whereas there were dissimilar activities between **2b** and **3** and their corresponding acetals **2c** and **3a**, respectively. Except in the case of pyridoxal, there is a fairly good correlation between in vitro inhibitor effect and in vivo potentiation. Four of the most active inhibitors (**2b**, **3b**, **3c**, and **3**) were agents that showed the highest potentiation, and three of these possess a two-carbon separation between the phosphorus atom and the aldehydic or acetal group. In this portion, the acetals were used without prior treatment, and it is not known whether these were hydrolyzed to aldehydes during the test procedure or were active as inhibitors per se. A previously referenced study of 2-formylethynyl-

Table III. Aldehyde Oxidase Inhibition

compd	K_i, mM[a]	substrate act.[b]
pyridoxal	0.03	−
2b	0.1	+
3b	0.2	+
3c	0.2	+
3	0.3	+
2c	0.3	−
2a	0.4	−
3a	1.5	−

[a] Determined by means of the NMN assay (ref 15) and the Dixon plot methods (ref 10). [b] Determined by use of the ferricyanide assay methods (ref 10).

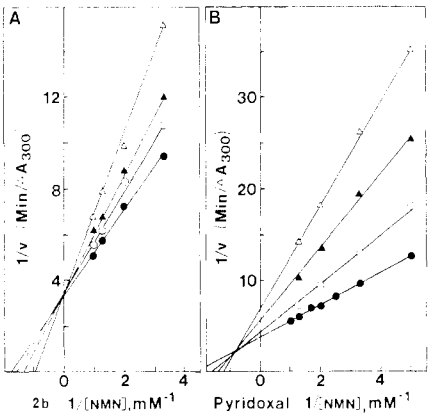

Figure 1. Lineweaver–Burk double-reciprocal plot of aldehyde oxidase activity vs. substrate (NMN) concentration in the presence (A) of **2b** as the inhibitor at a concentration of 0 (●), 43.20 (○), 75.6 (▲), and 130 (△) μM and (B) of pyridoxal at 0 (●), 15 (○), 40 (▲), and 65 (△) μM.

and 2-formylethylphosphonic acids and their corresponding acetals as competitive inhibitors for apoaspartate aminotransferase showed the aldehydic forms to be about tenfold more potent and the acetals not hydrolyzed during the enzymatic evaluation.[10]

The phosphorylated aldehydes are structurally closely similar to aldophosphamide. Therefore, the potentiating activities probably can be attributed to ability to inhibit the aldehyde oxidase catalyzed oxidation of aldophosphamide by competing with the substrate aldophosphamide in binding to the enzyme site. To test this hypothesis, the nature of aldehyde oxidase inhibition by **2b** was examined by two types of steady-state kinetic analysis, the Lineweaver-Burk and the Dixon plots. In the former analysis, the enzyme activity was determined as a function of substrate concentration, both in the absence and presence of several constant levels of **2b** and pyridoxal as inhibitor (Figure 1). The results were analyzed by the double-reciprocal plots of the initial velocity vs. substrate concentration. In the Dixon analysis, the enzyme activity was determined as a function of inhibitor concentration at several constant levels of substrate concentration. The reciprocal of the initial velocity was then plotted vs. inhibitor concentration (Figure 2). Although the observed pattern of the Lineweaver–Burk plot alone cannot rule out partially competitive inhibition, and that of the Dixon plot alone cannot exclude mixed inhibition, these results together unambiguously identify **2b** to be a competitive inhibitor for aldehyde oxidase.[11] The inhibition patterns of the other phosphorylated aldehydes were also deter-

(11) J. L. Webb, "Enzyme and Metabolic Inhibitors", Vol. 1, Academic Press, New York, 1963, pp 149–191.

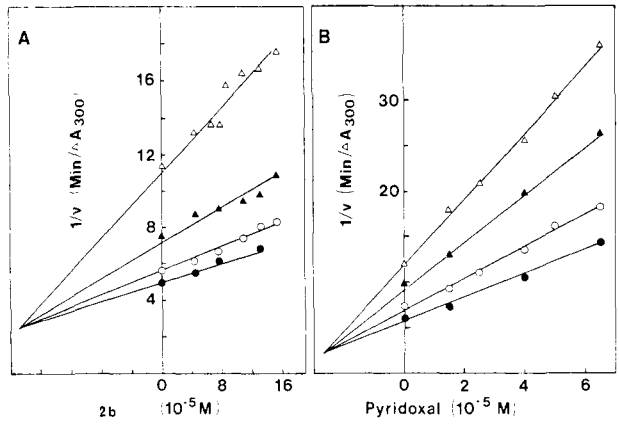

Figure 2. Dixon plot in which the enzyme activity was assayed, similar to that described in Figure 1A, over a range of 4 to 15 × 10^{-5} M of **2b** as the inhibitor in the presence of 1.0 (●), 0.8 (○), 0.5 (▲), and 0.2 (△) mM NMN as the substrate and (B) over a range of 1.5 to 6.5 × 10^{-5} M of pyridoxal as the inhibitor in the presence of 0.8 (●), 0.5 (○), 0.3 (▲), and 0.2 (△) mM as the substrate.

mined by the method of the Dioxn plot. The results indicate that **2b** is a competitive inhibitor for aldehyde oxidase.

The nature of aldehyde oxidase inhibition by pyridoxal was also determined as described above. Both Lineweaver–Burk (Figure 1) and Dixon (Figure 2) plots similarly and unambiguously identified mixed inhibition of the enzyme by pyridoxal. Such a finding suggests that the enzyme–pyridoxal complex is inactive, but the binding of substrate and of pyridoxal are not completely exclusive. It should be noted that, although pyridoxal is the most effective inhibitor tested, its activity as a potentiator of cyclophosphamide is surpassed by several phosphorylated aldehydes. This is perhaps not surprising, since after administration pyridoxal can be phosphorylated in vivo to the corresponding cofactor form, pyridoxal phosphate, which is essential to the activity of, and forms tight complexes with, numerous enzymes. This would be expected to reduce the amount of pyridoxal effective in the potentiation of the aldophosphamide anticancer activity.

A low degree of acute toxicity was observed in mice treated with the phosphorylated aldehydes and with these compounds administered concurrently with cyclophosphamide, except in the case of **2**. Although the toxicology of the agents has not, as yet, been extensively pursued, this lack of adverse effects at the relatively high dose of 200 mg/kg coupled with the potentiating activity of certain of these compounds is encouraging as concerns their potential application in cancer chemotherapy.

Experimental Section

Melting points were determined on a Thomas-Hoover unimelt capillary melting point apparatus and are corrected to reference standards; boiling points are uncorrected. Infrared spectra obtained using a Perkin-Elmer 283 spectrophotometer and ^{1}H NMR spectra, with Me_4Si as the internal standard, recorded on a Varian EM 360 or Varian XL-100 (for **4**) spectrophotometer were consistent with assigned structures. Visible–UV spectral determinations were made on a Perkin-Elmer Hitachi 200 spectrophotometer, and the mass spectrum was recorded by means of a Finnigan Model 3300 GC/mass spectrometer equipped with a 6100 data process system. TLC's were performed on products using fluorescent silica gel plates, and spots were detected by UV followed by exposure to I_2 vapor. Elemental analyses were performed by Atlantic Microlabs, Atlanta, GA, and were within ±0.4% of theoretical values.

(Diphenylphosphinyl)acetaldehyde (1) and 3-Propionaldehyde (1a) Diethyl Acetal. According to the procedure of Razumov and Savicheva,[8] a mixture of diphenylethylphosphinite[12] (50 mmol) and bromoacetaldehyde or 3-chloropropionaldehyde diethyl acetal (75 mmol) was stirred under a N_2 atomosphere for 2.5 h at 150 °C (oil bath). Distillation of the reaction mixtures yielded colorless oils.

Ethyl Phenyl(formylmethyl)phosphinate Diethyl Acetal (2a) and Ethyl Phenyl(2-formylethyl)phosphinate Diethyl Acetal (2c). Prepared according to the previous section using diethylphenylphosphinite[13] in lieu of diphenylethylphosphinite.

Diethyl and Diphenyl (Formylmethyl)phosphonate Diethyl Acetal (3a and 3e) and Diethyl (2-Formylethyl)-phosphonate Diethyl Acetal (3c). Following the method of Razumov and Muskva,[6] a mixture of triethyl or diphenyl ethylphosphite[5] and bromoacetaldehyde or 3-chloropropionaldehyde diethyl acetal was heated for 3 h at 185–190 °C (oil bath) under a N_2 atmosphere. Distillation of the reaction mixtures gave the products as colorless oils.

Phosphorylated Aldehydes 2, 2b, 3, and 3b. Equal molar amounts of the appropriate diethyl acetal **2a**, **2c**, **3a**, or **3c** were stirred under a N_2 atmosphere with 1 drop of concentrated HCl and a few crystals of hydroquinone for 2–5 h.[6,8] The products were obtained as colorless oils by distillation. Undistillable **3d** was characterized by means of its 2,4-dinitrophenylhydrazone and NMR spectroscopy of the aldehyde and this derivative.

Diphenyl 4-Hydroxybutyl Phosphate (6) and 4-Hydroxybutyl *N,N'*-Diphenylphosphorodiamidate (7). Diphenyl phosphorochloridate or *N,N*-diphenylphosphorodiamidochloridic acid[14] in THF (250 mL) was added dropwise to a stirred solution of 1,4-butanediol (200 mmol) and Et_3N (150 mmol) in THF (250 mL) at 10 ± 5 °C. After stirring to room temperature and refluxing for 2 h, the reaction mixture was filtered and the filtrate evaporated to remove the solvent. The residue was mixed with H_2O and extracted with Et_2O, the extract was dried over $CaCl_2$ and filtered, and the filtrate was evaporated to yield **6**. To obtain **7**, the residue was washed with H_2O and recrystallized from EtOH–H_2O. **6**: mp 88–89 °C. Anal. ($C_{16}H_{19}O_5P$) C, H. **7**: mp 132–134 °C. Anal. ($C_{16}H_{21}N_2O_3P$) C, H, N.

Diphenyl 3-Formylpropyl Phosphate (4) and 3-Formylpropyl *N,N'*-Diphenylphosphorodiamidate (5). Esters **6** and **7** were oxidized to the corresponding aldehydes using the method of Corey and Suggs.[15] To a stirred suspension of pyridinium chlorochromate (150 mmol) and NaOAc (30 mmol) in CH_2Cl_2 (200 mL) was added **6** and **7** (90 mmol) in CH_2Cl_2 (20 mL). After 3 h, Et_2O (200 mL) was added and the supernatant decanted from the resulting black gum. The residue was washed with Et_2O and the washings were filtered through a pad of Florisil. The filtrate was evaporated and the residue chromatographed on a silica gel column (70–230 mesh ASTM) using 3 or 5% MeOH in $CHCl_3$ as the eluant to yield pure **4** and **5**, respectively.

Biological. Enzyme Inhibition Studies. The enzyme activity was assayed spectrophotometrically by both the *N*-methylnicotinamide (NMN) and ferricyanide methods at 23 °C and in 1 mL of 0.05 M phosphate buffer, pH 7.8, containing 0.005% EDTA and 0.3 unit of enzyme. In the former method, the reaction was monitored by observing the increase in absorbance at 300 nm accompanying the oxidation of NMN to 6-pyridinone. With the second method, ferricyanide served as the electron acceptor, and the substrate oxidation was followed by a decrease in absorbance at 420 nm resulting from the ferricyanide reduction. One unit of activity is defined as that amount of enzyme which produced 1 ΔA_{300}/min at 23 °C using the substrate NMN at a saturating level. The aldehyde oxidase was purified from rabbit liver to a specific activity of 2 units/mg[16] and is free

(12) A. M. Shakivoa and M. G. Imaev, *Zh. Obshch. Khim.*, **37**, 468 (1967).
(13) G. Kamai, F. M. Kharrasova, R. B. Sultanova, and S. Y. Tukhvatullina, *Zh. Obshch. Khim.*, **31**, 3550 (1961).
(14) H. G. Cook, J. D. Ilett, B. C. Saunders, G. C. Stacey, H. G. Watson, I. G. E. Wilding, and S. J. Woodcock, *J. Chem. Soc.*, 2921 (1949).
(15) E. J. Corey and J. W. Suggs, *Tetrahedron Lett.*, **31**, 2647 (1975).
(16) V. V. Rajagopalan and P. Handler, *Methods Enzymol.*, **9**, 164 (1966).

of NAD-linked aldehyde dehydrogenase activity.

Cyclophosphamide Potentiation Studies. In a manner similar to that described by Hill et al.,[2] groups of six BDF_1 female mice weighing 18–20 g were implanted ip with 10^5 L1210 ascites cells. After 24 h (day 1), one group was administered an equal volume of vehicle (5% Tween 80 in normal saline, pH 4) ip to serve as a control; other groups were similarly injected with solutions or suspensions once only with drug or drug and cyclophosphamide on day 1. The acetals **2a**, **2c**, **3a**, **3c**, and **3e** were heated at 80 °C for 2 h just prior to injection. The groups were observed daily and any deaths recorded. Any animals surviving 30 days from day 1 were considered to be "cures" and increase in life span over controls as a percent (% ILS) were calculated.

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Mechanism of Decomposition of *N*-Hydroxyacetaminophen, a Postulated Toxic Metabolite of Acetaminophen

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The decomposition of *N*-hydroxyacetaminophen (*N*-acetyl-*N*-hydroxy-*p*-aminophenol, **2**), a postulated toxic metabolite of acetaminophen (*N*-acetyl-*p*-aminophenol, **1**) in aqueous solution is quantitatively accounted for by the appearance of equimolar amounts of *p*-nitrosophenol and acetaminophen. The rate of decomposition depends on initial concentration and varies with pH. Antioxidants decrease the rate of decomposition and change the products. In the presence of cysteine, *N*-acetyl-3-(*S*-cysteine)-*p*-aminophenol, an in vivo metabolite of acetaminophen, is a product of decomposition.

The toxicity of acetaminophen (**1**, *N*-acetyl-*p*-aminophenol), a widely employed mild analgesic, has been attributed to the formation of the reactive metabolite *N*-hydroxyacetaminophen (**2**, *N*-acetyl-*N*-hydroxy-*p*-aminophenol).[1] Studies on its toxicity have been prompted by the severe liver damage associated with excessively large acute doses, as well as by the possibility that a similar mechanism may be involved in the analgesic-related nephropathy which occurs in man with chronic abuse.[2,3] Neither the hepatic nor renal toxicity can be attributed to the parent compound (**1**) or to its major urinary metabolites, namely, the glucuronide and sulfate conjugates.

The mechanism of toxicity which has been proposed is that a small portion of the administered dose of **1** is oxidized to **2** by the mixed function oxidase system, principally in the liver but also in the kidney. As the result of the formation of **2**, intracellular stores of glutathione become depleted and **2** then becomes covalently bound to tissue proteins, presumably by the formation of thiol adducts. This is then followed by tissue necrosis.[4] This mechanism is supported by the urinary excretion of the cysteine and *N*-acetylcysteine 3 adducts of acetaminophen, which are presumed to have been derived from the prior formation of the glutathione 3 adduct.[5] It has been postulated that **2** decomposes to *N*-acetyl-*p*-benzoquinone imine (**4**), which is the proximate reactant with tissue thiols.[6]

Table I. Product Distribution as a Function of pH[a]

pH	*n*	concn, mM 1	concn, mM 9	unidentified peak ht, mm
6.8	2	0.69	0.50	8.3
7.2	3	0.72	0.59	6.7
7.6	3	0.70	0.66	4.4
8.2	1	0.72	0.72	1.3
9.6	1	0.74	0.74	0.0
10.0	1	0.74	0.73	0.0
10.5	1	0.75	0.73	0.0

[a] Initial conditions: 1.5 mM **2**, 200 mM PO_4, 37 °C. Incubation for 3 h or until the decomposition of **2** was complete. Unidentified peak had a retention time of 7.4 min. Standard deviation of replicate analyses was ±0.02 mM.

The above mechanism is based primarily on correlations observed in experimental animals in vivo. To date, **2** has not been detected in any biological fluid. When **2** became available through a synthetic route,[7,8] it was of pharmacological interest to determine its mechanism of decomposition in vitro with particular emphasis on the factors which might be operative in vivo.

Kinetic Studies. In measuring the disappearance of **2**, we obtained the same results with the ferric chloride assay as with high-performance liquid chromatography.

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