O-Methylhydroxylamine as a New Trapping Reagent for Quantitative Studies of 4-Hydroxycyclophosphamide and Aldophosphamide

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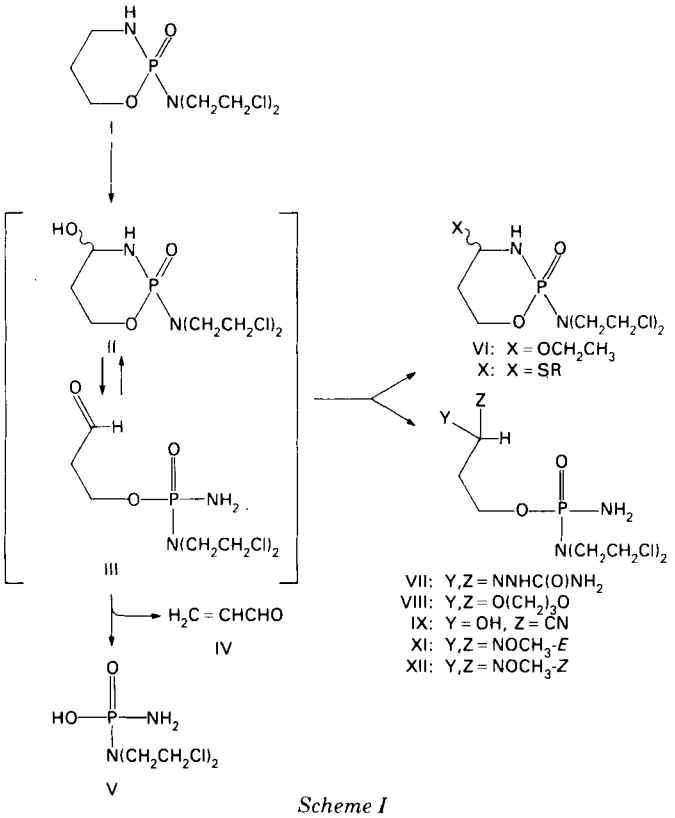
Abstract □ ^{31}P- and ^{1}H-NMR spectroscopy were used to demonstrate that the primary metabolites of the anticancer drug cyclophosphamide (4-hydroxycyclophosphamide and its acyclic tautomer, aldophosphamide) are quantitatively converted by *O*-methylhydroxylamine, at pH 7.4 and 37°, into the *E* and *Z* isomers of aldophosphamide *O*-methyl oxime. These trapping products are readily extracted from aqueous media with either chloroform or ethyl acetate, are stable at pH 6–8 toward oxime hydrolysis and elimination of phosphoramide mustard (a secondary metabolite of cyclophosphamide), and showed no evidence for transoximination with either ketone or aldehyde acceptors. All of these features support the use of aldophosphamide *O*-methyl oxime in quantitative studies related to cyclophosphamide metabolism.

Keyphrases □ Cyclophosphamide—metabolites, 4-hydroxycyclophosphamide, aldophosphamide, trapping with *O*-methylhydroxylamine □ Aldophosphamide—*O*-methyloxime derivative, *E* and *Z* isomers, ^{1}H-NMR identification □ NMR Spectroscopy—^{1}H analysis aldophosphamide *O*-methyloxime identification, ^{31}P analysis, decomposition kinetics

There is considerable interest in the organic chemistry and quantitative analysis of 4-hydroxycyclophosphamide (II) and aldophosphamide (III) (Scheme I), as these primary metabolites of cyclophosphamide (I) are generally believed to play pivotal roles in the oncostatic selectivity of this widely used anticancer prodrug (1, 2). The presence of a hemiaminal moiety in II allows for facile tautomerization with III, which can fragment into acrolein (IV) and phosphoramide mustard (V). The combined alkylating activity of II, III, and V can be quantified using 4-(*p*-nitrobenzyl)pyridine (3), whereas the combined cytotoxicity of low levels of all lethal cyclophosphamide metabolites is measurable [*e.g.*, by an *in vitro* assay employing Walker 256 rat carcinosarcoma cells (4)]. One method for selectively measuring the total concentration of metabolites II and III is their conversion to a relatively stable derivative using reaction conditions which preempt loss of II and III by fragmentation. Reported examples of such trapping products (Scheme I) include 4-ethoxycyclophosphamide (VI) (5), aldophosphamide semicarbazone (VII) (6), aldophosphamide propanediol-1,3-acetal (VIII) (7), and aldophosphamide cyanohydrin (IX) (8). In contrast to these compounds, analytical utilization of 4-sulfidocyclophosphamides (X) must contend with their hydrolytic instability (9). Pulse Fourier-transform NMR spectroscopy is an alternative methodology which offers the unique advantages of providing direct quantitative measurements of II and III as individual solution components.



Scheme I

During the NMR kinetic studies of II and III, *O*-methylhydroxylamine, which serves as a derivatizing agent for GC–mass spectrometric analyses of aldoses (10), was conducive to this study because of (*a*) its super-nucleophilicity due to lone-pair repulsion; (*b*) the thermal (GC, GC–mass spectrometric) stability of *O*-methyl oximes; (*c*) the resistance of *O*-methyl oximes toward hydrolytic reversion to aldehydes; (*d*) the availability of *O*-methylhydroxylamine hydrochloride in various stable isotope-enriched (^{2}H, ^{13}C, ^{15}N) and radioactive (^{3}H, ^{14}C) forms; and (*e*) the possibility for chemically induced reformation of aldehyde III. Since all of these features are not applicable to VI–IX, this study reports the formation and characterization of the trapping products derived from the reaction of II/III with *O*-methylhydroxylamine, namely, the *E* (XI) and *Z* (XII) isomers of aldophosphamide *O*-methyl oxime.

EXPERIMENTAL

Hydroxyphosphamide (8), 4-ketocyclophosphamide (XIV) (11), and compound XV (12) were synthesized and compound XIII was prepared and purified by Colvin's[1] method. ^{31}P-Fourier-transform NMR spectra at 40.25 MHz[2] were obtained using 10-mm sample tubes, a 5-kHz spectral window, 8192 data points zero-filled to 16,384, a $\pi/2$ pulse of 18 μsec, low-power ^{1}H-decoupling, and a 2-sec pulse repetition time. Spectra were

[1] M. Colvin, Johns Hopkins University School of Medicine, personal communication.

[2] FX-100 spectrometer, JEOL USA, Inc.

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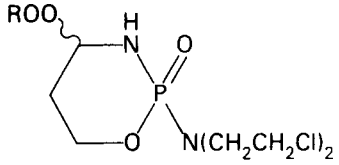
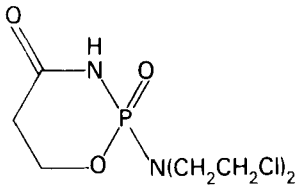


XIII: R = H
XV: R = I-4-yl
XVI: R = $C(CH_3)_3$



XIV

broadened by 1 Hz due to exponential multiplication prior to Fourier-transform. ^{31}P-Fourier-transform NMR spectra at 121.5 MHz[3] were recorded in a similar manner except for the use of a 20-μsec π/2 pulse, a 1.4-sec pulse repetition time, and 2.5 Hz of line-broadening. ^{1}H-Fourier-transform NMR spectra at 300 MHz[3] were recorded using 5-mm sample tubes, a 6-kHz spectral window, a 2-μsec π/2 pulse, a 5.4-sec pulse repetition time, and 0.2 Hz of line-broadening. ^{1}H-NMR spectra at 220 MHz[4] were recorded in the continuous-wave mode. Unless specified otherwise, ^{31}P-chemical shifts (δ, ppm) refer to deuterium oxide solvent and are relative to external 25% H_3PO_4. ^{1}H-Chemical shifts are relative to internal tetramethylsilane. NMR sample temperatures were measured by immersion of a precalibrated copper-constantan thermocouple. Values of pH measured[5] in deuterated water were not corrected for isotope effects (13). Electron-impact mass spectra[6] were scanned from samples introduced *via* a solids' inlet probe; the probe temperature was maintained at 20° for a 2-min period and was then elevated at 120°/min to a final temperature of 325°. Chemical-ionization mass spectra obtained with isobutane were scanned from samples similarly introduced. The ion-source temperature was 180°, the ionizing potential was 70 eV (150 eV for chemical-ionization), the ion-source pressure was 5×10^{-6} torr (10^{-3} torr for chemical ionization), and the ionizing current was 50 μamp.

4-(*tert*-Butylperoxy)cyclophosphamide (XVI)[7]—A solution of cyclophosphamide monohydrate[8] (2.048 g, 7.34 mmole) and *tert*-butyl hydroperoxide (1.0 ml, 70% aqueous solution) in 34 ml of acetone–water 1:2 was cooled with an ice bath, and ozone[9] (25–50 mg/min) was then bubbled into this solution through a glass frit. More acetone (5 ml) was added at 30-min intervals, and more *tert*-butyl hydroperoxide (1 ml) was added at 1.0-hr intervals during the course of the reaction (3 hr). Acetone was removed *in vacuo* on a rotary evaporator at room temperature and the remaining aqueous solution was extracted with methylene chloride (5 × 25 ml). The combined extracts were dried over anhydrous magnesium sulfate and solvent was then removed *in vacuo* without heating. The residual oil was chromatographed at 6° on a column (2.2 × 26-cm) of silica gel[10] using acetone–chloroform (2:1) and a flow rate of 8 ml/hr. Product [R_f 0.80, 250-μm silica gel, acetone–chloroform (2:1), iodine visualization] contaminated with *tert*-butyl hydroperoxide (R_f 0.95) and XIV (R_f 0.60) eluted in fractions 7–9 (8 ml/fraction), which were combined and concentrated *in vacuo* to give material (380 mg) for rechromatography in hexane–acetone–chloroform (3:2:1). The two diastereomers of XVI were detected in fractions 12–16 as overlapping TLC components [R_f 0.58 and 0.52, 250-μm silica gel, hexane–acetone–chloroform (3:2:1)]. Removal of solvent from these fractions afforded a pure sample (144 mg) of the faster eluting diastereomer and a mixture (113 mg) of the faster and slower eluting diastereomers; total yield 10% (257 mg). ^{31}P-NMR (40.25 MHz, deuterochloroform) of faster eluting XVI: δ 6.04; for slower eluting XVI, δ 6.44. ^{1}H-NMR (220 MHz, deuterochloroform) of faster eluting XVI: 5.09 (d, J_{HP} = 25 Hz of q, J_{HH} = 3 Hz, 1H, C_4—H), 4.64 (m, 1H, 1-C_6—H), 4.09 (m, 1H, 1-C_6—H), 3.82 (broad s, 1H, NH), 3.59 (t, J_{HH} = 7 Hz, 4H, 2-NCH_2CH_2Cl), 3.41 (m, 4H, 2-NCH_2CH_2Cl), 2.02 (m, 2H, 2-C_5—H), and 1.23 [s, 9H, $C(CH_3)_3$] ppm; electron-impact mass spectrum: *m/z* 57 (55%) $[(CH_3)_3C]^+$, 73 (2%) $[(CH_3)_3CO]^+$, 92 (70%) $(^{35}ClCH_2CH_2NHCH_2)^+$, and 142 (4%) $[(^{35}ClCH_2CH_2)_2NH_2]^+$; chemical-ionization—mass spectrum: *m/z* 93 (38%) $(^{35}ClCH_2CH_2NHCH_2 + 1)^+$, and 143 (100%) $[(^{35}ClCH_2CH_2)_2N + 3]^+$; 349 $(M + 1)^+$ not observed.

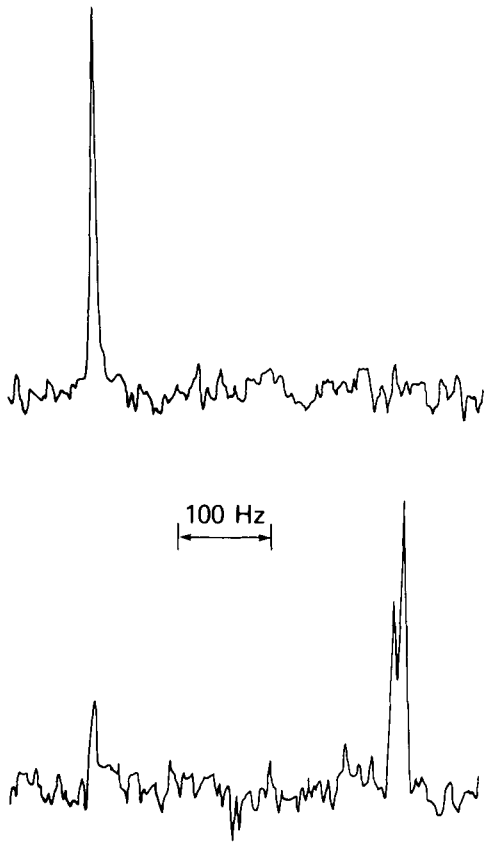


Figure 1—*^{31}P-NMR (40.25 MHz) spectra recorded in 0.05 M lutidine buffer at 37°; bottom:* cis- *and* trans-*II (δ 12.62, 12.93) and III (δ 20.83); top: ~10 min after reaction with O-methylhydroxylamine to form O-methyl oximes XI/XII (δ 20.83).*

Reaction of II/III with *O*-Methylhydroxylamine—After recording the ^{31}P-NMR (40.25 MHz) spectrum of a solution of *cis*-XIII (10 mg, 0.034 mmole) in deuterochloroform (2 ml) at 25°, triphenylphosphine (12.5 mg, 0.048 mmole, 1.4 equivalents) was added and the spectrum was recorded after 5 min. The original signal for *cis*-XIII (δ 11.07) was replaced by that of *cis*-II (δ 10.89), and residual triphenylphosphine (δ −6.70) was detected in addition to the triphenylphosphine oxide (δ 27.42) by-product. Solvent was removed *in vacuo* on a rotary evaporator without heating, and the residue was then sonicated for 10 min with lutidine buffer (2 ml of 0.05 *M*, pH 7.4, 5 v/v% deuterium oxide). The filtered solution was clarified by centrifugation and was then analyzed by ^{31}P-NMR before and after (10 min) addition of *O*-methylhydroxylamine hydrochloride (14 mg, 0.170 mmole, 5 equivalents; sample pH adjusted to 7.4 with 1 *N* sodium hydroxide); spectra are shown in Fig. 1. The solution was saturated with sodium chloride and then extracted with deuterochloroform (2 × 1 ml) for ^{1}H- and ^{31}P-NMR analyses, which showed the presence of XI/XII as a single phosphorus resonance (δ 14.13); XI/XII were not detectable (^{31}P-NMR) in the remaining aqueous layer. Similar results were obtained by ethyl acetate extraction of a duplicate reaction mixture.

Preparative-Scale Isolation of XI/XII, Decomposition of XIII in the Presence of *O*-Methylhydroxylamine—A sample of crude XIII (175 mg, ~0.5 mmole), which contained ~20 moles % XIV by ^{31}P-NMR, was dissolved in water (10 ml) and was then treated with an alkaline solution of *O*-methylhydroxylamine that had been prepared from its hydrochloride salt (100 mg, 1.2 mmoles in 6.2 ml of 0.2 *M* sodium hydroxide). After 24 hr at room temperature, the sodium chloride-saturated

[3] WM-300 spectrometer, Bruker Instruments, Inc.
[4] HR-220 spectrometer, Varian Associates, Inc.
[5] Model PHM 64 pH meter, Radiometer A/S.
[6] Model 2091 GC-MS and 2130 Data System, LKB Instruments, Inc.
[7] Compound XVI has been independently prepared by R. B. Brundrett, Johns Hopkins University School of Medicine.
[8] Aldrich Chemical Co.
[9] Model 03V5-0 ozone generator, Ozone Research & Equipment Corp.
[10] Merck 60, <230 mesh, EM Laboratories.

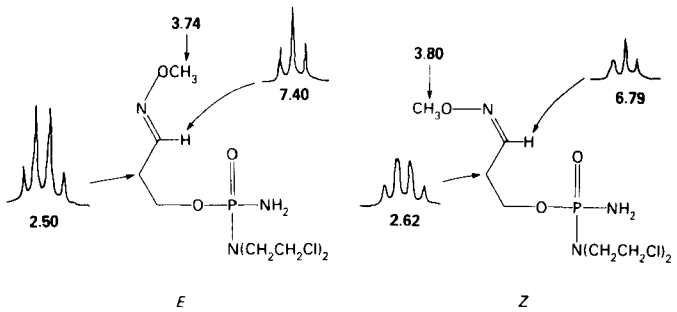


Figure 2—*Structures of* E *and* Z *aldophosphamide* O*-methyl oximes (XI and XII) with* ^{1}*H-NMR (300 MHz) spectral inserts and chemical shifts for the indicated protons.*

reaction mixture was extracted with chloroform (3 × 15 ml) and the combined extract was then dried with anhydrous magnesium sulfate. Removal of chloroform *in vacuo* gave a residue (54 mg) which was subjected to TLC (1000-μm silica gel, acetone–chloroform 1:1) and afforded two overlapping components (R_f 0.1–0.2) identified as a 62:38 mixture of *E* (XI) and *Z* (XII) *O*-methyl oximes of III (17 mg of oil, 12% corrected yield); ^{1}H-NMR (300 MHz, acetone-d_6) for XI and XII, respectively (Fig. 2): δ 7.40 and 6.79 (2-t, J_{HH} = 5.8 and 5.1 Hz, 61:39, 1H total, nonequivalent CH_2CH=N), 4.07 (m, 2H, CH_2O), 3.74 and 3.80 (2-s, 63:37, 3H total, nonequivalent OCH_3), 3.90 (broad s, 2H, NH_2), 3.69 (t, *J* = 7.2 Hz, 4H, 2-NCH_2CH_2Cl), 3.31 (m, 4H, 2-NCH_2CH_2Cl), 2.50 and 2.62 (2-q, J_{HH} = 6.2 and 5.9 Hz, respectively, 61:39, 2H total, nonequivalent CH_2CH=N) ppm; electron-impact–mass spectrum: *m/z* 274 (0.8%) and 276 (0.4%) $(M-CH_3O)^+$, 165 (70%) $[M-(ClCH_2CH_2)_2N]^+$, 142 (3%) $[(^{35}ClCH_2CH_2)_2NH_2]^+$, 92 (87%) $(^{35}ClCH_2CH_2NHCH_2)^+$, and 86 (69%) $(CH_3ON{=}CHCH_2CH_2)^+$; chemical-ionization–mass spectrum: 87 (100%) $(CH_3ON{=}CHCH_2CH_2+1)^+$, 93 (26%) $(^{35}ClCH_2CH_2NHCH_2+1)^+$, 143 (58%) $[(^{35}ClCH_2CH_2)_2N+3]^+$, 308 (39%) and 310 (25%) $(M+3)^+$. Upon standing in a freezer, the initial oily sample of XI/XII crystallized, mp 72–75°.

Anal.—Calc. for $C_8H_{18}N_3O_3PCl_2$: C, 31.38; H, 5.94; N, 13.73. Found: C, 31.34; H, 5.89; N, 13.64.

Decomposition of XV in the Presence of *O*-Methylhydroxylamine—A solution of lutidine buffer (1.75 ml of 0.05 *M*, 5 v/v% deuterium oxide) and *O*-methylhydroxylamine hydrochloride (20 mg, 0.24 mmole), adjusted to pH 7.4 with 1 *N* sodium hydroxide, was sonicated with crystalline XV (10 mg, 0.018 mmole) to achieve rapid dissolution. The sample was placed in the ^{31}P-NMR (121.5 MHz) probe at 37°, and after 10 min of temperature equilibration, the free-induction decay signal for each spectrum (320 pulses, 7.56 min) was automatically accumulated and stored. The average concentration of XV in each frequency-domain spectrum was measured as a relative percentage, using the peak height of XV (δ 12.60) and all other reaction components: XI/XII (δ 20.83), XIII (δ 13.43), and XIV (δ 9.93). A plot of $\ln([XV]_0/[XV]_t)$ *versus* time (*t*) using eight data points was linear over the monitored decomposition period (75%) and gave $\tau_{1/2}$ = 0.5 hr.

Decomposition of XVI in the Presence of *O*-Methylhydroxylamine—The procedure with the faster eluting diastereomer of XVI (15 mg, 0.043 mmole) was the same as that described above for compound XV, except for the use of pyridine buffer (1.30 ml of 0.1 *M*, 5 v/v% deuterium oxide). From the ^{31}P-NMR peak heights of XVI (δ 11.57), XIII (δ 12.84), and XI/XII (δ 20.80) in eight spectra (300 pulses, 10.00 min/spectrum) obtained over 9 hr of monitoring (67% decomposition), a linear plot of $\ln([XVI]_0/[XVI]_t)$ *versus t* gave $\tau_{1/2}$ = 5.2 hr.

Stability Studies with XI/XII—All buffers refer to 0.05 *M* aqueous solutions. An aliquot (0.10 ml) of a stock solution of XI/XII (0.073 *M*) in methanol was added to potassium dihydrogen phosphate (1 ml) at pH 6.00, 7.03, and 8.03, and lutidine (1 ml) at pH 7.01. A standard solution of the oximes was similarly prepared in chloroform (1 ml). Each solution was magnetically stirred at room temperature and was analyzed as a function of time using TLC [250-μm silica gel, acetone–chloroform (3:1)], XI/XII R_f = 0.62. There was no detectable oxime decomposition after 6 days.

An aliquot (25 μl) of a stock solution of XI/XII (0.146 *M*) in methanol was added to both the phosphate buffer at pH 7.03 and the lutidine buffer at pH 7.01, giving an oxime concentration of 7.0 m*M*. The former solution was combined with acetone (15 μl, 0.2 mmole, 55-fold molar excess), while the latter solution was treated with acetaldehyde (10 μl, 0.18 mmole, 48-fold molar excess). Daily TLC monitoring as described showed no evidence of reaction after 6 days at room temperature.

The initial oxime solutions in phosphate buffer at pH 6.00, 7.03, and 8.03, and lutidine buffer at pH 7.01, were each combined after 6 days with aqueous formaldehyde (6 μl of 37 w/w %, 0.073 mmole, 10-fold molar excess) and were stirred at room temperature. TLC analysis showed no detectable oxime decomposition after 4 days.

RESULTS AND DISCUSSION

Triphenylphosphine deoxygenation of 4-hydroperoxycyclophosphamide (XIII) is commonly employed for synthetic entry to *cis*-II, *trans*-II, and III, which give rise to ^{1}H-decoupled, ^{31}P-NMR singlet absorptions at δ 12.63, 12.93, and 20.83, respectively, in 0.05 *M* lutidine buffer at 37° (Fig. 1)[11]. Hydroxyphosphamide (δ 21.01), which is the 3-hydroxypropyl analog of aldophosphamide (III), is a good chemical shift model for ^{31}P-NMR identification of III; however, it should be noted that the proportion of free aldehyde in III *versus* its hydrate (15) has not been established. The absolute and relative signal intensities for *cis*-II, *trans*-II, and III vary (not shown) during the course of their gradual tautomerization and fragmentation of III to give IV and V, which is relatively short-lived and was previously studied by ^{31}P-NMR (16). In contrast to these spectral changes, addition of a 5-fold molar excess of *O*-methylhydroxylamine hydrochloride leads to rapid (<10 min) consumption of II/III with formation of a single resonance (δ 20.83) due to the *E* and *Z* aldophosphamide *O*-methyl oxime (XI and XII) trapping products (Fig. 1)[12]. ^{1}H-NMR analysis of an analytically pure oxime sample, which was obtained from spontaneous decomposition of XIII in the presence of *O*-methylhydroxylamine followed by extraction and TLC, showed resonance doubling (Fig. 2) for three types of protons: CH_3O (singlets), CH_2CH_2CH=N (quartets), and CH_2CH_2CH=N (triplets). Other investigators (17, 18) have compared the ^{1}H-NMR spectra of nine *E/Z* pairs of *O*-methyl oximes, and have concluded that the imino proton (CH=N) in the *E* isomer generally exhibits an ~0.6–0.9 ppm downfield shift relative to the corresponding resonance from the *Z* form. On this basis, the triplets at δ 7.40 and 6.79 (Δδ = 0.61 ppm) are respectively assigned to the *E* (XI) and *Z* (XII) *O*-methyl oximes of III, while averaged signal integrations gave a 62:38 (±1%) *E/Z* product ratio. Selective irradiation of each imino proton triplet led to the observation of a triplet for the corresponding CH_2CH_2CH=N nuclei, while irradiation of the overlapping absorptions for the CH_2O protons led to simultaneous collapse of both CH_2CH_2CH=N quartets into doublets, thus confirming the internal spectral assignments.

The utility of *O*-methylhydroxylamine in simplifying kinetic studies of 4-peroxy derivatives of I and its analogs (2) was exemplified by the spontaneous decomposition of 4-peroxycyclophosphamide (XV) in lutidine buffer (0.05 *M*) containing a 13-fold molar excess of this trapping reagent. ^{31}P-NMR spectra recorded as a function of time at 37° show the gradual disappearance of XV (δ 12.60) with the transient intermediacy of XIII (δ 13.43) and the steady accumulation of XI/XII (δ 20.83), together with the formation of 4-ketocyclophosphamide (XIV, δ 9.93) as a minor by-product (18%). The disappearance of XV obeys a first-order rate law, with $\tau_{1/2}$ = 0.5 hr, whereas other investigators (19), who monitored the time-course for release of acrolein from XV, reported an initial lag phase and postulated a coupled set of homolytic reactions. While the mechanistic origins for these kinetic differences require further investigation, the competing production of XIV is tentatively ascribed to free-radical cage reactions prior to formation of II and then III, which is finally intercepted as XI/XII. Analogous kinetic measurements with diastereomerically pure 4-(*tert*-butylperoxy)cyclophosphamide (XVI) revealed an ~10-fold increase in stability ($\tau_{1/2}$ = 5.2 hr), relative to XV ($\tau_{1/2}$ = 0.5 hr), and the absence of XIV (<1%) during formation of XI/XII. The contrasting results obtained for XV and XVI clearly reveal that the details for spontaneous decomposition of 4-peroxy derivatives of I are more complex than anticipated, *a priori*.

The stability of XI/XII toward either hydrolytic reversion to II/III or fragmentation into IV and V was demonstrated by TLC monitoring of the *O*-methyl oximes in buffered solutions at pH 6, 7, and 8, which showed no evidence of either oxime disappearance or formation of acrolein (IV) after 6 days at room temperature. Treatment of these solutions at pH 7 with excess acetone (55 equivalents), acetaldehyde (48 equivalents), or formaldehyde (10 equivalents) gave no TLC evidence for transoximination.

[11] The ^{31}P-NMR chemical shifts of I and its metabolites in aqueous solution are dependent upon pH, temperature, and the type of buffer (14).

[12] TLC and ^{1}H-NMR analyses of a deuterochloroform extract confirmed the presence of oximes XI and XII, thus ruling out the possibility of catalyzed conversion of II into III, which happens to have the same chemical shift as XI/XII under the stated reaction conditions.

CONCLUSIONS

O-Methylhydroxylamine rapidly reacts with tautomers II and III at neutral pH to quantitatively produce an ~60:40 mixture of the *E* and *Z* isomers of aldophosphamide *O*-methyl oxime, XI and XII. Assuming that oxime formation occurs by condensation of the amine with aldehyde III, it follows that the rate of ring-opening of *cis*- and *trans*-II to give III must be relatively fast, since ^{31}P-NMR analysis of the reaction mixture showed that these hemiaminals are no longer detectable after ~10 min at 37°. The *O*-methyl oximes of III are resistant toward hydrolysis of the oxime functionality, fragmentation into IV and V, and transoximination with either acetone, acetaldehyde, or formaldehyde. In concert, these features lend themselves to the use of *O*-methylhydroxylamine as an effective trapping agent for studies of cyclophosphamide metabolites II and III. Investigations of enzymatic and chemical regeneration of III from XI/XII in the design of new anticancer prodrugs will be reported in another study.

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High-Performance Liquid Chromatographic Analysis of Hydrocortisone Drug Substance, Tablets, and Enema

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Abstract □ Methods for the analysis of hydrocortisone drug substance, tablets, and enema were developed using adsorption high-performance liquid chromatography (HPLC). This HPLC system was shown to be capable of isolating hydrocortisone from its degradation products, synthesis precursor, and related corticosteroids. The accuracy, precision, and linearity of the HPLC assay methods and their applicability to commercial products has been demonstrated.

Keyphrases □ High-performance liquid chromatography—analysis of hydrocortisone drug substance, tablets, and enema □ Hydrocortisone—analysis of drug substance, tablets, and enema using high-performance liquid chromatography □ Degradation products—separation from hydrocortisone, high-performance liquid chromatography

The majority of reported analytical methods for corticosteroids utilize blue tetrazolium (1–10), isoniazid (11), or phenylhydrazine (12, 13) reactions, UV spectrophotometry (14), or high-performance liquid chromatography (HPLC)[1] (15–22).

The methods for determining hydrocortisone products in the last four revisions of the United States Pharmacopeia (USP) (2, 23–25) have employed the blue tetrazolium reaction as the final determinative step. This is preceded by extraction or thin layer chromatographic (TLC) isolation of the active ingredient. Interferences and critical parameters of the reaction have been reported (6–9, 26).

HPLC is rapidly becoming the method of choice for the analysis of many drugs, and numerous applications of this technique to hydrocortisone are reported in the literature. The majority of published methods utilize reversed-phase systems (15–20). Recent reports, however, demonstrate that normal-phase adsorption chromatography offers greater selectivity for closely related corticosteroid structures[2] (21–22).

This study was undertaken to develop an HPLC system suitable for the analysis of hydrocortisone products and to compare the relative advantages of the USP and HPLC methods. Accuracy, precision, specificity, indication of

[1] E. Bunch, Food and Drug Administration, Seattle, Wash., unpublished work (1975).

[2] M. J. Walters, Food and Drug Administration, Detroit, Mich., unpublished work, presented at the 6th Annual Meeting of the Federation of Analytical Chemistry and Spectroscopy Societies (1979).

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