Oxime Derivatives of the Intermediary Oncostatic Metabolites of Cyclophosphamide and Ifosfamide: Synthesis and Deuterium Labeling for Applications to Metabolite Quantification

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Abstract □ There is ongoing interest in the selective, quantitative analysis of the cyclophosphamide metabolites 4-hydroxycyclophosphamide (**2a**) and aldophosphamide (**3a**) because these tautomers are generally believed to play a key role in oncostatic selectivity and metabolite transport. *O*-(2,3,4,5,6-Pentafluorobenzyl)hydroxylamine ($C_6F_5CH_2ONH_2$, 1 equiv) provided for the complete conversion (by ^{31}P NMR, 60% reaction within 15 min at 20 °C) of **2a/3a** (17 mM in H_2O/CH_3OH) to *E/Z*-aldophosphamide *O*-(2,3,4,5,6-pentafluorobenzyl)oxime [$C_6F_5CH_2ON{=}CHCH_2CH_2OP(O)(NH_2)N(CH_2CH_2Cl)_2$; *E*:*Z* = 54:46 (±3% average deviation)]. Under these conditions, the oxime exhibited little (6%) decomposition over 3 weeks. Parallel studies showed that 4-hydroxyifosfamide/aldoifosfamide reacted completely to give the analogous aldoifosfamide oxime [$C_6F_5CH_2ON{=}CHCH_2CH_2OP(O)(NHCH_2CH_2Cl)_2$; *E*:*Z* = 52:48 (±1% average deviation)] with 50% reaction within 15 min at 20 °C with no product decomposition over 3 weeks. In aqueous methanol and with 2 equiv $C_6F_5CH_2ONH_2$, clinically useful 4-hydroperoxycyclophosphamide (10 mM; $\tau_{1/2}$ = 10 min, 37 °C) and its isomer 4-hydroperoxyifosfamide (10 mM; $\tau_{1/2}$ = 25 min, 20 °C) underwent complete conversion to the corresponding aldehyde oximes. Each oxime was synthesized with deuterium in the chloroethyl moieties for use as internal standards in GC/MS applications.

Introduction

There is considerable, ongoing interest in the quantitative analysis of the intermediary metabolites of cyclophosphamide (**1a**), *cis/trans*-4-hydroxycyclophosphamide (**2a**) and aldophosphamide (**3a**) [Scheme 1 (in all schemes, structures denoting stereochemistry are shown only with the *R* configuration at phosphorus for the sake of simplicity)]. It is generally believed that **2a/3a** (which spontaneously interconvert) play a key role in oncostatic selectivity and that they act as unstable transport forms of the ultimate alkylating agent, phosphoramide mustard (**4a**).[1] Similar considerations apply to the metabolites of the clinically useful analog ifosfamide (**1b**), *i.e.*, *cis/trans*-4-hydroxyifosfamide (**2b**), aldoifosfamide (**3b**), and isophosphoramide mustard (**4b**) (Scheme 1).

The combined alkylating activity of **2/3/4** is frequently quantified using 4-(*p*-nitrobenzyl)pyridine;[2] however, the therapeutic value of extracellular **4a** and **4b** has not been clarified.[1,3-7] Thus, it is desirable to selectivity quantify **2a/3a** and **2b/3b** which are generally believed to be the circulating metabolites of greatest consequence in cytotoxicity, transport, and selectivity. One method which is currently used for clinical analyses of **2a/3a** requires that these metabolites be decomposed to acrolein; this product is then trapped and quantified by the use of fluorescence spectrophotometry.[8-10] Drawbacks to this procedure include the need for multiple sample manipulations, time constraints, and moderate sensitivity in the detection of the product.

One practical method for the quantification of **2a/3a** and **2b/3b** involves their conversion to stable derivatives in rapid and irreversible reactions which preclude metabolite loss by fragmentation to acrolein and **4a/4b**. Literature reports of such trapping reactions generally refer to analyses of **2a/3a** and include as trapping agents alcohols and thiols,[11-14] semicarbazides,[15] and cyanide.[5,16,17] To varying degrees, each of these examples suffers from product lability, complexity of product mixtures (mixtures of stereoisomeric, cyclic, and acyclic derivatives), difficulties in clinical applications, and/or problems in extending these methods to analyses of **2b/3b**. While the parent drugs **1a** and **1b** share many facets of a common metabolic pathway, there are differences among their metabolites in other chemical details involving reactivity.[18-20] Since many studies of cyclophosphamide and ifosfamide are comparative in nature, it is desirable that there be one method which is applicable to the quantification of **2a/3a** as well as **2b/3b**.

O-Methylhydroxylamine has been reported as a trapping agent for use in NMR-based, quantitative studies of **2a/3a**;[21] *O*-(2,3,4,5,6-pentafluorobenzyl)hydroxylamine has been used in analyses of various aldehydes and ketones in biological fluids.[22] The hydroxylamines offer advantages that, cummulatively, are not found in any of the other methods noted above. These include (a) predicted thermal and hydrolytic stabilities of the product oximes,[22,23] (b) formation of a single type of product (*albeit* as *E/Z* isomers), and (c) applicability to comparable reactions with **2b/3b**. Relative to the methylhydroxylamine, the fluorinated reagent introduces an additional number of attractive, analytical features into the product oximes, including increased volatility, improved sensitivity for halogen detectors, and applications to ^{19}F NMR studies. On the other hand, the kinetic advantage in the trapping reaction which results from the supernucleophilicity of hydroxylamines would predictably be muted by the presence of fluorine. The impact of this on the partitioning of **2a/3a** and **2b/3b** between pathways of oxime formation and fragmentation would have to be tested.

This investigation reports the formation and characterization of the oximes (**5a/5b**) derived from the reactions of *O*-(2,3,4,5,6-pentafluorobenzyl)hydroxylamine with **2a/3a** and **2b/3b** (Scheme 1). Each oxime was synthesized with deuterium in the chloroethyl moieties for use as internal standards in GC/MS applications (Schemes 2 and 3).

Experimental Section

In general, solvents and reagents were purchased through Aldrich Chemical Co. and Fisher Scientific Co. Tetrahydrofuran (THF), acetonitrile, benzene, pyridine, phosphorus oxychloride ($POCl_3$), and

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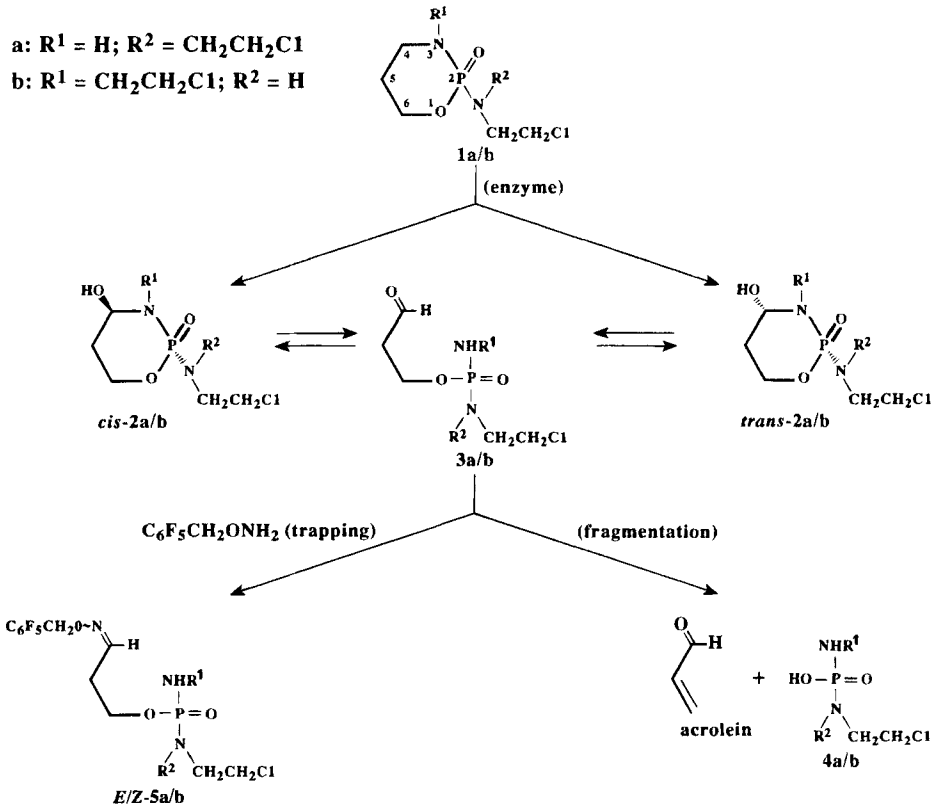
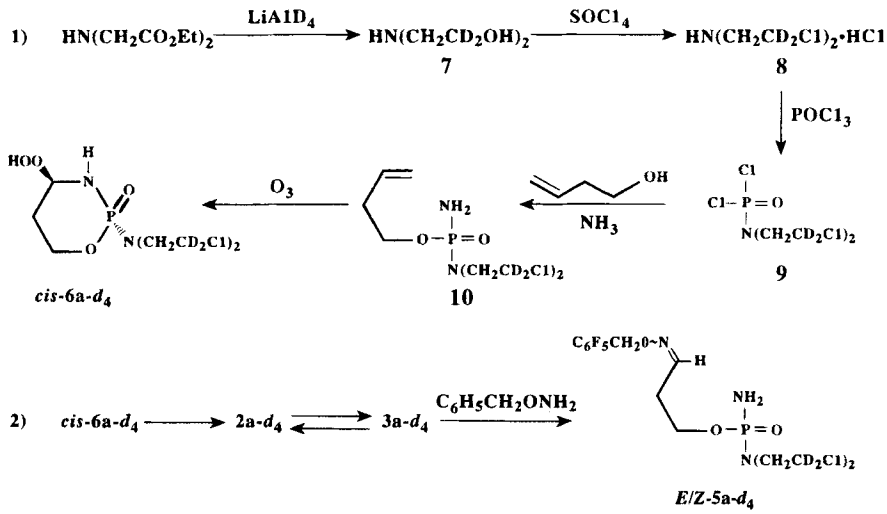


Scheme 1



Scheme 2

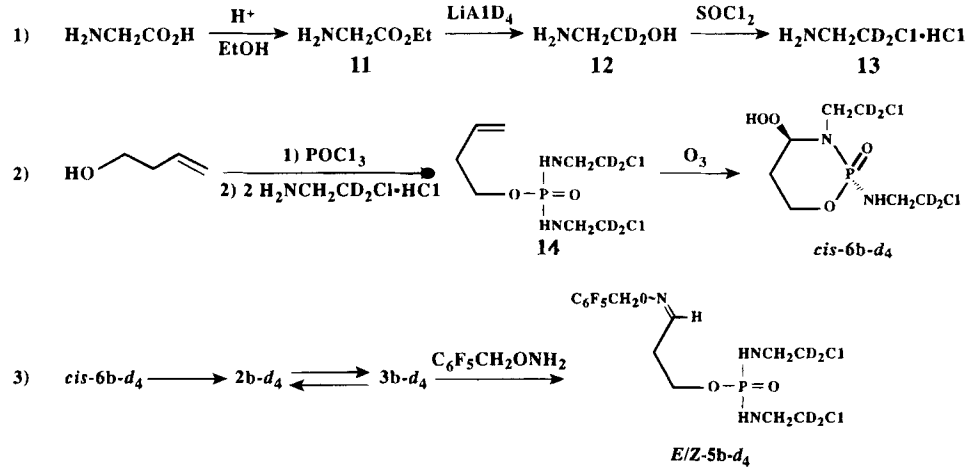
thionyl chloride ($SOCl_2$) were dried and/or distilled prior to use. Reactions that did not include water were carried out under nitrogen. Melting points were obtained with a Fisher-Johns melting point apparatus and are uncorrected. Ozone was produced by a Welsbach T-408 ozone generator with a rated output of 4–8 g of ozone/h (Welsbach Ozone Systems Corp.). Elemental analyses were performed by Atlantic Microlab, Inc. Gravity chromatography columns utilized silica gel (60–200 mesh) from J. T. Baker, Inc. Flash chromatography used silica gel (finer than 230 mesh) from EM Science; the weight of silica gel used in each column was 10 times the weight of the crude material being chromatographed. Analytical TLC employed 2.5 × 10 cm plates coated with a 250 μm layer of silica gel GF (Analtech); a 250-nm UV lamp and I_2 were used for component visualization. Reactions done at 5 °C were performed in an ice bath.

^{1}H spectra at 500 MHz and ^{31}P spectra at 202.5 MHz were obtained on a Bruker MSL500 spectrometer. A Bruker AMX300 was used for ^{1}H spectra at 300 MHz and ^{31}P spectra at 121.5 MHz. Unless specified otherwise, ^{1}H NMR chemical shifts were referenced to tetramethylsilane (TMS) as an internal reference and ^{31}P δ values to external 1% H_3PO_4 in H_2O.

Values of pH were measured with a precalibrated standard glass microelectrode; solution pH values correspond to observed readings and are uncorrected for D_2O or methanol.

Phosphoramide mustard (**4a**, as the cyclohexylammonium salt) was a gift from the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, The National Cancer Institute. The synthesis of isophosphoramide mustard (**4b**) has been reported.[4,17]

^{31}P NMR Trapping Studies—^{31}P NMR acquisitions at 202.5 MHz used a 10.2 kHz spectral window, 16 000 data points, a 45° pulse of 10 μs, gated low-power ^{1}H decoupling, and a pulse recycle time of 6.13 s. On the basis of our previous work with cyclophosphamide metabolites and related species in aqueous and organic solutions,[17,24] phosphorus peak heights were judged to be reliable measures of component concentration because (1) possible differential nuclear



Scheme 3

Overhauser effects were supressed with gated decoupling and (2) the pulse delay time was sufficient to compensate for differences in relaxation times among the components of interest. For relative measurements of *E* and *Z* isomers, this judgement was supported by the proton NMR data where relative integrated signal intensities were in accord with the relative peak height measurements obtained from the ^{31}P NMR spectra.

*1. Using Prereduced cis-4-Hydroperoxycyclophosphamide (cis-**6a**)*—Hydroxperoxide *cis*-**6a**[14] (0.034 mmol, 10 mg) was dissolved (sonication for 5–10 min) in a mixture of H_2O (0.6 mL) and D_2O (0.4 mL) and to this was added sequentially (1) sodium thiosulfate pentahydrate (0.136 mmol, 22 mg), (2) a solution of *O*-(2,3,4,5,6-pentafluorobenzyl)hydroxylamine hydrochloride (0.034 mmol, 9 mg) in CH_3OH (0.2 mL), and (3) methylphosphonic acid as an internal standard [0.016 mmol; 0.1 mL of a solution containing 12 mg (0.125 mmol) of $CH_3P(O)(OH)_2$ per 0.78 mL of H_2O]. More CH_3OH (0.6 mL) was then added until the resultant solution was clear. The ambient "pH" of this solution was 3.98. An aliquot of this sample was added to a 5 mm NMR tube, the tube was inserted into the NMR probe at ambient temperature (20 °C) and ^{31}P (202.5 MHz) spectral acquisition was initiated.

In the experiment where the addition of *O*-(2,3,4,5,6-pentafluorobenzyl)hydroxylamine hydrochloride was delayed, the same amounts of reagents were used but the following modifications were employed. Sodium thiosulfate pentahydrate was dissolved in H_2O/D_2O/methanol (0.6 mL/0.4 mL/0.9 mL) and this solution was added to a solid sample of *cis*-**6a**. After spectral observations of this sample, the hydroxylamine was added, and then additional NMR spectra were taken.

*2. Using cis-4-Hydroperoxycyclophosphamide (cis-**6a**)*—A solution of *O*-(2,3,4,5,6-pentafluorobenzyl)hydroxylamine hydrochloride (0.060 mmol, 15 mg) in CH_3OH (1.5 mL) was added to a solution of *cis*-**6a**[14] (0.031 mmol, 9 mg) in 0.1 M triethanolamine (1.35 mL of a 0.1 M solution which was adjusted to pH 7.4 by the addition of 1 M HNO_3) with added D_2O (0.15 mL). Methylphosphonic acid (0.021 mmol, 2 mg) was added as an internal standard and the resultant solution (ambient pH 3.01) was transfered to a 10 mm NMR tube for spectral acquisitions (^{31}P NMR, 202.5 MHz) at a probe temperature of 37 °C.

*3. Using Prereduced cis-4-Hydroperoxyifosfamide (cis-**6b**)*—The experiments using hydroperoxide *cis*-**6b** were performed exactly as those detailed above for *cis*-**6a** (NMR expt 1). The final solutions had an ambient pH value of 3.15.

*4. Using cis-4-Hydroperoxyifosfamide (cis-**6b**)*—The experiment using *cis*-**6b** was carried out as described above for *cis*-**6a** (NMR expt 2) except that an ambient probe temperature of 25 °C was used during NMR spectral acqusitions. The NMR solution had an ambient pH value of 2.51.

Bis(2,2-dideuterio-2-hydroxyethyl)amine (7). Using modifications to published procedures,[25] a solution of diethyl iminodiacetate (25 mmol, 4.73 g, Kodak) in THF (25 mL) was added dropwise to a suspension of $LiAlD_4$ (119 mmol, 5.00 g) in THF (100 mL) at 5 °C. Upon complete addition, the mixture was stirred briefly (5 min) at 5 °C followed by 20 min at room temperature. The reaction flask was fit with a drying tube (Drierite) and then the mixture was heated at reflux overnight. After cooling to room temperature, the reaction was quenched by the slow, sequential addition of water (2.0 mL), 15% NaOH (2.0 mL), and water (6.1 mL). The resultant mixture was stirred for 2 h and then vacuum filtered. The filtrate was concentrated on a rotary evaporator and the residual material was taken up in $CHCl_3$, dried ($MgSO_4$), filtered, and concentrated to give a yellow oil (0.44 g, 4 mmol, 16%) which was used without further purification.

The solids obtained from the vacuum filtration of the reaction mixture were subjected to a Soxhlet extraction with THF (250 mL) for 2 days. The THF phase was concentrated and the residual material was taken up in $CHCl_3$, dried ($MgSO_4$), filtered, and concentrated to give a yellow oil (1.78 g, 16 mmol, 64%) which was used without further purification. The net yield of crude **7** was 20 mmol (80%).

Bis(2-chloro-2,2-dideuterioethyl)amine Hydrochloride (8)—By analogy to a literature procedure for ^{15}N-labeled material,[26] a solution of $SOCl_2$ (65 mmol, 4.75 mL) in CH_3CN (32 mL) was added dropwise to a solution of **7** (16 mmol, 1.78 g) in CH_3CN (33 mL). Upon complete addition, the reaction mixture was refluxed (1.5 h). After cooling to room temperature, ether (10 mL) was added and the reaction mixture was stored at −20 °C overnight. The resultant suspension of flocculent powder was transferred to centrifuge tubes and centrifuged, and the mother liquor was removed by pipet. The solids were washed with ether and then dried under vacuum to give the product as an off-white powder (0.07 g, 2%). The mother liquor and ether washings were combined and stored at −20 °C to afford more product. This procedure, along with a periodic concentration of the mother liquor, was repeated 7 times to give the product in a total yield of 1.29 g, 7 mmol, 44%.

Bis(2-chloro-2,2-dideuterioethyl)aminophosphoramidic Dichloride (9)—By analogy to a literature procedure for ^{15}N-labeled material,[26] a suspension of **8** (13 mmol, 2.38 g) in benzene (12 mL) was cooled at 5 °C and to this was added pyridine (13 mmol, 1.06 mL). After stirring at low temperature for 20 min, more pyridine (13 mmol, 1.06 mL) was added followed by the dropwise addition of a solution of $POCl_3$ (13 mmol, 1.21 mL) in benzene (5 mL). Upon complete addition, the ice bath was allowed to melt and the reaction mixture was stirred overnight at room temperature. The mixture was then vacuum filtered and the filtrate was concentrated on a rotary evaporator. The residual oil was chromatographed on silica gel (3 cm × 25 cm column, dry-packed, $CHCl_3$ eluent) to give the product (R_f 0.6) as a pale yellow oil (0.98 g, 3.7 mmol, 28%). Product identification was based on TLC comparisons with unlabeled material.

***cis*-4-Hydroperoxycyclophosphamide-β,β,β′,β′-d_4 [*cis*-2-[Bis-(2-chloro-2,2-dideuterioethyl)amino]-4-hydroperoxytetrahydro-2*H*-1,3,2-oxazaphosphorin 2-Oxide] (*cis*-6a-β,β,β′,β′-d_4)**—This compound was made according to literature procedures for unlabeled material.[14] Using **9** (3.7 mmol, 0.98 g), the intermediate 3-butenyl *N,N*-bis(2-chloro-2,2-dideuterioethyl)phosphorodiamidate (**10**) was obtained as a pale yellow oil [0.45 g, 1.6 mmol, 43%, R_f 0.33 (ether) and 0.56 ($CHCl_3$–CH_3OH, 9:1)]. Ozone was bubbled (15 min) through a solution of **10** (1.6 mmol, 0.45 g) in acetone-water (2:1, 13.5 mL). The usual workup, including the addition of H_2O_2 (0.69 mL of a 30% solution in water), afforded the product as a white microcrystalline solid (recrystallized from ether, 0.11 g, 0.37 mmol, 23%).

***E/Z*-Aldophosphamide-β,β,β′,β′-d_4 *O*-(2,3,4,5,6-Pentafluorobenzyl)oxime (*E/Z*-5a-β,β,β′,β′-d_4)**—To a stirring solution of *cis*-**6a**-

$\beta,\beta,\beta',\beta'$-$d_4$ (0.37 mmol, 110 mg) in water (7 mL) was added sodium thiosulfate pentahydrate ($Na_2S_2O_3{\cdot}5H_2O$, 0.37 mmol, 92 mg). Upon dissolution of the thiosulfate, a solution of *O*-(2,3,4,5,6-pentafluorobenzyl)hydroxylamine hydrochloride (0.37 mmol, 92 mg) in CH_3OH (7 mL) was added dropwise to the reaction mixture. After stirring overnight, the CH_3OH was removed on a rotary evaporator and the resultant aqueous mixture was extracted with CH_2Cl_2 (5 × 20 mL). The organic layers were combined, dried ($MgSO_4$), filtered, and concentrated at reduced pressure. The resultant oil solidified upon storage at 5 °C (150 mg, 0.31 mmol, 84%, mp 48–52 °C). Elemental analysis [found (theory)]: C, 35.15 (35.31); H + D as H (for this analysis, the molecular weight was calculated with 4 D atoms but the instrumentation analyzed each deuterium as though it were hydrogen), 3.69 (3.61); N, 8.72 (8.83). For the isolated mixture of *E*/*Z* isomers: ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.36 and 6.75 (2 t, $^3J_{HH}$ = 5 Hz each, 1H, CH=N, *E* and *Z* isomers, respectively), 5.13 and 5.08 (2 s, 2H, benzylic CH_2, *Z* and *E* isomers, respectively), 4.18–3.95 (m, 2H, CH_2OP), 3.35 (d, $^3J_{HP}$ = 12 Hz, 4H, 2 $PNCH_2$), 3.17–3.02 (br s, 2H, NH_2), and 2.59 and 2.48 (2 apparent q, J = 6 Hz each, 2H, CH_2C=N, *Z* and *E* isomers, respectively); ^{31}P NMR (121.5 MHz, $CDCl_3$) δ 15.6. Based on ^{1}H NMR data, the average ratio of *E* to *Z* isomers was 61:39 (±6% average deviation).

Synthesis of Unlabeled *E*/*Z*-Aldophosphamide *O*-(2,3,4,5,6-Pentafluorobenzyl)oxime (*E*/*Z*-5a)—As for the synthesis of the deuterium-labeled oxime (*E*/*Z*-5a-$\beta,\beta,\beta',\beta'$-$d_4$), a solution of unlabeled *cis*-**6a**[14] (0.24 mmol, 70 mg) in water (5 mL) was treated first with $Na_2S_2O_3{\cdot}5H_2O$ (0.24 mmol, 60 mg) and second with a solution of *O*-(2,3,4,5,6-pentafluorobenzyl)hydroxylamine hydrochloride (0.24 mmol, 60 mg) in CH_3OH (5 mL). After it was stirred overnight, the reaction mixture was worked up as described for labeled material and this gave the product as an oil (70 mg, 0.15 mmol, 63%). For the isolated mixture of *E*/*Z* isomers: ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.43 and 6.83 (2 t, $^3J_{HH}$ = 6 and 5 Hz, respectively, 1H, CH=N, *E* and *Z* isomers, respectively), 5.19 and 5.13 (2 s, 2H, benzylic CH_2, *Z* and *E* isomers, respectively), 4.20–4.04 (m, 2H, CH_2OP), 3.69–3.59 (m, 4H, 2 CH_2-Cl), 3.49–3.37 (m, 4H, 2 $PNCH_2$), 3.17–3.08 (br s, 2H, NH_2), and 2.67 and 2.50 (2 apparent q, J = 6 Hz each, 2H, CH_2C=N, *Z* and *E* isomers, respectively); ^{31}P NMR (121.5 MHz, $CDCl_3$) δ 15.6. Based on ^{1}H NMR data, the average ratio of *E* to *Z* isomers was 56:44 (±2% average deviation).

Ethyl 2-Aminoacetate (Glycine Ethyl Ester; 11)—This compound was synthesized from glycine (71.6 mmol scale) according to a literature procedure for ^{15}N-labeled material.[27] The desired product was obtained as a pale yellow oil (5.75 g, 55.7 mmol, 77.8%): ^{1}H NMR ($CDCl_3$) δ 4.19 (q, J = 7.2 Hz, 2H, OCH_2), 3.43 (s, 2H, NCH_2), 1.60–1.35 (br s, 2H, NH_2) and 1.28 (t, J = 7.2 Hz, 3H, CH_3).

2,2-Dideuterioethanolamine (12)—On the basis of a literature procedure for the synthesis of ^{15}N-labeled material,[27] a solution of **11** (4.21 g, 39.7 mmol) in THF (25 mL) was added dropwise to a cold (5 °C) suspension of $LiAlH_4$ (5.00 g, 119 mmol) in THF (80 mL). The reaction mixture was stirred at 5 °C for 5 min, at room temperature for 20 min, and then was refluxed overnight. After cooling to room temperature, the reaction was quenched by the slow, dropwise, sequential addition of water (1.75 mL), aqueous NaOH (15%, 1.75 mL), and water (5.35 mL). The reaction mixture was stirred for 2 h and was then filtered and concentrated at reduced pressure to afford the product as a colorless oil (1.23 g, 19.5 mmol, 49.1%). The solids were extracted with a Soxhlet extractor (THF, 2 days) which afforded additional (more impure) material (1.27 g, 20.1 mmol, 50.6%): total crude yield, 2.50 g, 39.6 mmol, 99.7%; ^{1}H NMR ($CDCl_3$) δ 2.83 (s, 2H, CH_2N) and 2.58–2.20 (br s, 3H, NH_2, OH).

2-Chloro-2,2-dideuterioethylamine Hydrochloride (13)—The title compound was synthesized based on the literature procedure for the synthesis of the ^{15}N-labeled compound.[27] Thionyl chloride (14.3 mL, 195 mmol) was added dropwise to a solution of 2,2-dideuterioethanolamine (1.25 g, 19.5 mmol) in CH_3CN (120 mL). The resulting solution was stirred for 20 h. Ether (200 mL) was then added and the mixture was allowed to sit for 10 min. This resulted in the appearance of precipitating product, which was collected by centrifugation. The product was isolated as a light beige solid (960 mg, 8.1 mmol, 41.5%): ^{1}H NMR (D_2O/TSP reference) δ 3.40 (s, CH_2N).

3-Butenyl *N*,*N*′-Bis(2-chloro-2,2-dideuterioethyl)phosphorodiamidate (14)—With minor modifications to reported methods,[28] a solution of $POCl_3$ (0.22 mL, 2.22 mmol) in CH_2Cl_2 (6.5 mL) was cooled to 5 °C. To this was added dropwise 3-buten-1-ol (0.20 mL, 2.32 mmol) followed by triethylamine (0.32 mL, 2.22 mmol). The reaction mixture was stirred at 5 °C for 3 h. Compound **13** (526 mg, 4.46 mmol) was then added slowly, as a solid, via an additional funnel. Triethylamine (1.27 mL, 8.92 mmol) was then added dropwise, resulting in fuming and bubbling. The mixture was stirred at room temperature overnight. Filtration and concentration at reduced pressure afforded a crude reaction mixture which was taken up in ethyl acetate, filtered, and concentrated again. The residue was flash chromatographed ($CHCl_3$ eluent) to afford the product [R_f 0.5 (CH_3OH–$CHCl_3$, 5:95)] as a pale yellow oil (508 mg, 1.8 mmol, 81.1%): ^{1}H NMR (500 MHz, $CDCl_3$) δ 5.87–5.73 (m, 1H, vinylic), 5.18–5.10 (m, 2H, vinylic), 4.15–4.01 (m, 2H, CH_2O), 3.30–3.24 (m, 4H, 2 CH_2N), 2.99 (br, 2H, 2 NH), 2.46–2.39 (m, 2H, allylic). ^{31}P NMR (202.5 MHz, $CDCl_3$) δ 14.2.

cis*-4-Hydroperoxyifosfamide-$\beta,\beta,\beta',\beta'$-$d_4$ [*cis*-2-[(2-Chloro-2,2-dideuterioethyl)amino]-3-(2-chloro-2,2-dideuterioethyl)-4-hydroperoxytetrahydro-2*H*-1,3,2-oxazaphosphorin 2-Oxide] (*cis*-6b-$\beta,\beta,\beta',\beta'$-$d_4$)**—As previously described for the synthesis of 4-hydroperoxycyclophosphamide (**6a**),[14] ozone was bubbled (15 min) through a solution of 14 (235 mg, 0.84 mmol) in acetone-water (2:1, 10.5 mL). The usual workup including the addition of H_2O_2 (0.56 mL of a 30% solution in water) afforded the product as a white microcrystalline solid (recrystallized from CH_2Cl_2/ether, 29 mg, 0.098 mmol, 11.7%, mp 93–95 °C): ^{1}H NMR ($CDCl_3$) δ 5.11–5.01 (m, 1H, C_4-H), 4.68–4.13 (m, 2H, CH_2OP), 3.65–3.13 (m, 5H, NH and 2 NCH_2), 2.33–2.08 (m, 2H, CH***$_2CH_2O$). ^{31}P NMR (202.5 MHz, $CDCl_3$) δ 10.5.

***E*/*Z*-Aldoifosfamide-$\beta,\beta,\beta',\beta'$-$d_4$ *O*-(2,3,4,5,6-Pentafluorbenzyl)-oxime (*E*/*Z*-5b-$\beta,\beta,\beta',\beta'$-$d_4$)**—Hydroperoxide *cis*-**6b**-$\beta,\beta,\beta',\beta'$-$d_4$ (60.0 mg, 0.20 mmol) was dissolved in CH_3OH (3 mL) and the solution was then diluted with water (3 mL). Reduction ($Na_2S_2O_3{\cdot}5H_2O$, 50.1 mg, 0.20 mmol) and trapping [*O*-(2,3,4,5,6-pentafluorbenzyl)hydroxylamine hydrochloride, 50.4 mg, 0.20 mmol in CH_3OH (4 mL)] agents were added, and the reaction was worked up as described above for the synthesis of the aldophosphamide oxime (*E*/*Z*-**5a**-$\beta,\beta,\beta',\beta'$-$d_4$). The crude material was flash chromatographed (CH_3OH–$CHCl_3$, 1:99) yielding a colorless oil [R_f 0.19 (CH_3OH–$CHCl_3$, 2:98); 35 mg, 0.074 mmol, 37%]. For the isolated mixture of *E*/*Z* isomers: ^{1}H NMR ($CDCl_3$) δ 7.42 and 6.81 (2 t, $^3J_{HH}$ = 5.6 an 5.2 Hz, respectively, 1H, CH=N, *E* and *Z* isomers, respectively), 5.19 and 5.13 (2 s, 2H, benzylic CH_2, *Z* and *E* isomers, respectively), 4.18–4.08 (m, 2H, CH_2OP), 3.33–3.19 (m, 4H, 2 $PNCH_2$), 3.17–3.03 (m, 2H, 2 NH), and 2.67 and 2.55 (2 apparent q, J = 5.8 and 6.0 Hz, respectively, 2H, CH_2C=N, *Z* and *E* isomers, respectively); ^{31}P NMR (202.5 MHz, $CDCl_3$) δ 14.3. Based on ^{1}H NMR data, the average ratio of *E* to *Z* isomers was 54:46 (±1% average deviation).

Results and Discussion

Trapping Studies of Intermediary Cyclophosphamide Metabolites—As in our previous studies,[7,14,18,21] *cis*-4-hydroperoxycyclophosphamide (*cis*-**6a**, Scheme 2) was utilized as a convenient, synthetic precursor to **2a/3a**. The solution chemistry of the subsequent trapping reaction was monitored by ^{31}P NMR.[14,21] Thus, an aqueous solution of *cis*-**6a** was reduced with sodium thiosulfate and then treated with *O*-(2,3,4,5,6-pentafluorobenzyl)hydroxylamine (1 equiv) in methanol. Methylphosphonic acid (δ 24.0) was added as an internal standard. At an ambient pH of 4 and a probe temperature of 20 °C, the initial spectrum (accumulated about 50 min after mixing) revealed the presence of two major resonances in nearly equal intensities at δ 19.4 and 19.3. Some smaller upfield signals were also detected (δ *ca.* 11 and 10.5). Spectra taken over the next hour revealed no significant changes. The sample was stored at room temperature and monitored periodically by NMR; at 24 h, the upfield resonances were no longer visible. Spectra taken at 24 h and 10 and 21 days were essentially identical, showing signals at δ 19.4 and 19.3 in an average ratio of 54:46 (±3% average deviation), respectively. Over this extended period, the ratio of the sum of the two product signals to that of the internal standard remained constant within experimental error (±2% average deviation). With time, however, a new component (δ 18) was observed and it did appear to increase slowly in intensity (4% of the

phosphorus intensity at 10 days and 6% at 21 days). No resonances indicative of phosphoramide mustard (**4a**) were observed during the course of the experiment, as verified by the addition of authentic material to the NMR sample.[29]

The chemical shifts and stabilities of the downfield signals (δ 19.4 and 19.3) were consistent with those expected for the *E* and *Z* isomers of the expected acyclic product, aldophosphamide *O*-(2,3,4,5,6-pentafluorobenzyl)oxime (*E/Z*-**5a**, Scheme 1).[21] Subsequently, the isomers were isolated as a mixture and identified by ^{1}H NMR. In the proton spectrum, triplets attributable to the *E/Z* imino proton (CH=N) were observed at 7.43 and 6.83 ppm in a ratio of 3:2, respectively. In proton NMR studies of other *O*-methyl oximes, the imino proton in the *E* isomer generally displayed a shift *ca.* 0.6–0.9 ppm downfield relative to the resonance for the same proton in the *Z* diastereomer.[30,31] On the basis of this shift data, the triplets at 7.43 and 6.83 ppm ($\Delta\delta$ 0.6) were assigned, respectively, as the *E* and *Z* isomers of **5a**. Resonances attributable to *E* and *Z* isomers were also clearly observed for the benzylic protons (CH_2ON, singlets) and the CH_2C=N moiety (apparent quartets). The relative integrated intensities of the signals in each pair were used to determine an average *E*:*Z* ratio of 56:44 (±2% average deviation) in the isolated product.

In the above experiment, the identities of the components giving rise to upfield resonances (δ 11 and 10.5) were uncertain. The chemical shifts were in a region expected for the isomers of 4-hydroxycyclophosphamide (*cis/trans*-**2a**), but the persistence of the signals did not correlate with the kinetics of oxime formation. To clarify these assignments, the NMR experiment was repeated but with a delay in the addition of *O*-(2,3,4,5,6-pentafluorobenzyl)hydroxylamine. Based on our previous experience with this chemistry,[3,14,18,21,32] the following conclusions were drawn. Following the reduction of hydroperoxide **6a** (17 mM) with thiosulfate in aqueous methanol, ^{31}P resonances for *cis/trans*-**2a** and aldehyde **3a** (or their equivalents[33]) were observed at δ 11.5, 11.9, and 19.4, respectively. As expected under the acidic conditions,[14,19,20] **3a** was the favored species: after *ca.* 30 min of reaction time at 20 °C, *cis*-**2a**:*trans*-**2a**:**3a** = 26:19:55. The fluorobenzylhydroxylamine was added to this sample and the spectrum accumulated 15 min later revealed a 60% loss of intensity in the signals attributed to **2a/3a**; this loss was accompanied by a corresponding increase in resonances for *E/Z*-**5a**. Extraneous signals which were present *before* the addition of the hydroxylamine and which were not significantly affected by the subsequent addition of the trapping reagent were attributed to **4a** and relatively stable products of side reactions beween **2a** and thiosulfate (multiple resonances between δ 3 and 12).[14,29,34] The intensities (up to *ca.* 30% of the phosphorus intensity in these experiments) and number of signals resulting from the thiosulfate reduction reaction were apparently dependent on whether or not **6a** had fully dissolved prior to the addition of thiosulfate (less dissolved, more side reactions).

In a third experiment, a methanol solution of *O*-(2,3,4,5,6-pentafluorobenzyl)hydroxylamine (2 equiv) was added to a buffered (0.1 M triethanolamine, pH 7.4) solution of hydroperoxide *cis*-**6a** *without added reducing agent*. As determined by ^{31}P NMR, the signal for the hydroperoxide (δ 11.5) rapidly decreased ($\tau_{1/2}$ *ca.* 10 min, 37 °C, ambient pH of 3, 10 mM **6a**) while those for trapping products *E/Z*-**5a** (δ 20.0 and 19.9) rapidly increased in intensity. Within 1 h, the signal for the hydroperoxide had disappeared; only the resonances for the oximes were detectable. A transient signal of relatively low intensity at δ 11.2 was also observed but was unidentified. This experiment demonstrated that hydroperoxide **6a**, which is used clinically as a spontaneous generator of **2a/3a**, was not subject to any extraneous reactions in the absence of thiosulfate and, as a result, the trapping reaction with the hydroxylamine was very clean.

Comparable ^{31}P NMR experiments were carried out with **2a/3a** and the aldehyde trapping agents carboxyphenyl semicarbazide, dansyl hydrazine, and azidobenzoyl hydrazide. None gave products with the stability of **5a**.

Trapping Studies of Intermediary Ifosfamide Metabolites—In parallel with the above experiments using 4-hydroperoxycyclophosphamide, an aqueous solution of *cis*-4-hydroperoxyifosfamide (*cis*-**6b**, Scheme 3) was reduced with thiosulfate and to this was added 1 equiv of *O*-(2,3,4,5,6-pentafluorobenzyl)hydroxylamine in methanol. The uncorrected pH value of the resultant solution was 3.15. ^{31}P NMR spectra of this sample exhibited two major signals at δ 17.32 and 17.29 which were indicative of the *E/Z* isomers of aldoifosfamide *O*-(2,3,4,5,6-pentafluorobenzyl)oxime (*E/Z*-**5b**, Scheme 1). These resonances were invariant in spectra taken of this mixture after 9, 14, and 22 days at room temperature [respective signal ratio of 52:48 (±1% average deviation)]. During this time period, no signals indicative of **4b** (verified by addition of authentic material) or other decomposition products were detected. On the other hand, the thiosulfate reduction of hydroperoxide **6b** sometimes gave a number of undesired side products (δ 10–13; up to *ca.* 35% of the phosphorus intensity in these experiments) similar to those seen in the reduction of **6a** (*vide supra*). Control of these thiosulfate-induced side reactions was not straightforward.

That the ^{31}P NMR signals at δ 17.32 and 17.29 were attributable to *E/Z*-**6b** was confirmed during a large-scale repeat of the NMR experiment which happened to use deuterium-labeled precursor (*vide infra*). The isolated and purified reaction product gave a proton NMR spectrum characteristic of the expected oxime as a mixture of *E* and *Z* isomers (see Experimental Section for details).

In a second experiment wherein the addition of the hydroxylamine was delayed, the ^{31}P spectrum of an aqueous methanol solution of thiosulfate-reduced **6b** exhibited signals indicative of *cis/trans*-**2b** (δ 12.66/12.56) and **3b** (or its equivalent; δ 17.45).[18,19,33] After *ca.* 15 min of reaction time at 20 °C, the relative ratio of *cis*-**2b**:*trans*-**2b**:**3b** was 22:26:52. Upon addition of the pentafluorobenzylhydroxylamine, the signals assigned to *cis/trans*-**2b** and **3b** rapidly disappeared, giving rise to those for **5b**. The reaction was approximately 50% complete after 15 min (20 °C, pH 3).

In a third experiment, *O*-(2,3,4,5,6-pentafluorobenzyl)hydroxylamine (2 equiv in methanol) was added to a buffered solution of *cis*-**6b** *without added reducing agent*. The ^{31}P NMR signal for the hydroperoxide at δ 12.3 decreased ($\tau_{1/2}$ = 25 min, 20 °C, ambient pH 2.5) while those for trapping products *E/Z*-**5b** increased in intensity [δ 18.10 and 18.07 with a respective peak height ratio of 57:43 (±1% average deviation)]. Relatively small signals for species which were apparently intermediates between **6b** and **5b** were observed at δ 13.5, 13.0, and 12.5.

Deuterium-Labeled Oximes—For applications of this derivatizing method to the quantitative analysis of **2a/3a** and **2b/3b**, internal standards are required. An internal standard is only as reliable as its ability to chemically mimic the material being quantified. Quantifications by MS are particularly attractive not only because of the sensitivity levels which can be achieved but also because internal standards can be used which differ from the analyte only by isotopic enrichment. Thus, according to Schemes II and III, deuterium labeled analogs of **5a** and **5b** (*E/Z*-**5a**-$\beta,\beta,\beta',\beta'$-$d_4$ and *E/Z*-**5b**-$\beta,\beta,\beta',\beta'$-$d_4$) were synthesized for use as internal standards in MS quantifications of derivatized **2a/3a** and **2b/3b**.

Conclusion

These experiments demonstrated that the presence of just 1 equiv of *O*-(2,3,4,5,6-pentafluorobenzyl)hyroxylamine re-

sulted in the rapid and irreversible conversion of the cyclophosphamide metabolites **2a/3a** (and, indirectly, any species interconverting with **2a/3a**)[33] to the *E/Z* diastereomers of **5a**. These product oximes were then relatively stable under the reaction conditions. Extraneous components were determined to be the result of thiosulfate-induced side reactions produced during the reduction of hydroperoxide **6a**. These reactions were unrelated to the trapping reaction and would be absent under clinical conditions. Similar observations were made for the conversion of the ifosfamide metabolites **2b/3b** to *E/Z*-**5b**. These chemical characteristics indicated that this derivatizing reaction could be used for the selective quantifications of the intermediary, oncostatic metabolites of cyclophosphamide and ifosfamide. Using the deuterated oxime analogs as internal standards, clinical applications of this method to GC/MS analyses of **2a**/3a have been conducted and are reported elsewhere.[35] In brief, metabolite detection limits have been lowered approximately 10-fold relative to any non-GC/MS method previously used in clinical settings. In addition, levels of reproducibility and practicality have been achieved which have not been demonstrated previously by other techniques. Similar GC/MS quantitation studies of clinical samples of **2b/3b** are in progress.

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