Synthesis of 4-Hydroperoxy Derivatives of Ifosfamide and Trofosfamide by Direct Ozonation and Preliminary Antitumor Evaluation in Vivo

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SUMMARY

A one-step synthesis of 4-hydroperoxyifosfamide and 4-hydroperoxytrofosfamide is described. The method involves direct ozonation of ifosfamide and trofosfamide and offers improved yields in comparison with Fenton oxidation and greater convenience in comparison with ozonation of the appropriate 3-butenyl phosphorodiamidate. Evaluation of the 4-hydroperoxy derivatives of cyclophosphamide, ifosfamide, and trofosfamide against leukemia L1210 in vivo suggests a superior effect for the ifosfamide derivative.

INTRODUCTION

Cyclophosphamide and its congeners ifosfamide and trofosfamide (1, 3) (Chart 1) are effective antitumor agents in clinical and experimental use. Metabolic activation of cyclophosphamide is known to proceed by C4-hydroxylation by the mixed-function oxidase of liver microsomes (4, 7, 9, 13), the resulting 4-hydroxyphosphoramidate then spontaneously yielding the unstable aldophosphamide and subsequently the possible "ultimate" alkylating metabolite, phosphoramide mustard (5, 6, 14).

Synthesis of 4-hydroxycyclophosphamide (10, 17, 19, 20), as well as the "preactivated" derivatives, 4-hydroperoxycyclophosphamide (10, 17, 19, 20) and 4-peroxycyclophosphamide (15, 16, 20), has been accomplished. In addition, 4-hydroxy- and 4-hydroperoxyifosfamide have been prepared, and the latter is currently undergoing clinical trials in Japan (A. Takamizawa, personal communication). Experimental or clinical use of the peroxidized derivatives of cyclophosphamide and its congeners, rather than the 4-hydroxy derivatives, is preferred because the former are much more stable and spontaneously yield the hydroxy derivatives under physiological conditions (20, 21).

Because of the potential importance of the peroxidized derivatives of cyclophosphamide and its congeners, a direct synthesis of these types of proactivated derivatives was sought for the sake of convenience over the previously reported routes (15, 17 to 19, 20), as well as to improve the yields. Two of us (11) have recently described such a synthesis of 4-hydroperoxycyclophosphamide, and application of this method to ifosfamide and trofosfamide and evaluation of the products against leukemia L1210 in vivo are the subjects of this report. The method is advantageous because of its simplicity, its use of available starting materials, the ease of isolation of products, and respectable yields.

MATERIALS AND METHODS

Starting Materials. Cyclophosphamide, ifosfamide, and trofosfamide were obtained from Dr. Robert R. Engle, Drug Development Branch, National Cancer Institute, Silver Spring, Md., and from Asta Werke, 4800 Bielefeld, West Germany. [3H]Cyclophosphamide was obtained from Dr. E. Schaumlöffel, Radiologie-Zentrum der Phillipps-Universität, Marburg/Lahn, Germany.

Ozonation. A solution of cyclophosphamide or one of its congeners (1 g) in aqueous acetone and 30% hydrogen peroxide was treated with ozone (25 mmoles/hr) generated by a Welsbach Ozonator T-816 (Welsbach Corp., Philadelphia, Pa.) at a flow rate of 0.3 liter/min and 50 watts of power by bubbling into the ice bath cooled solution through a capillary tube.

TLC. TLC was performed on Analtech (Newark, Del.) precoated Silica Gel G plates (25 μm thick) in acetone:chloroform (1:3). The plates were activated at 120° for 1 hr and stored in a desiccated chamber.

Alkylating Activity. Thin-layer chromatograms were sprayed with a 1% solution of 4-(p-nitrobenzyl)pyridine (Aldrich Chemical Co., Milwaukee, Wis.) in acetone, heated in an oven for 15 min at 140°, and sprayed with a 3% solution of potassium hydroxide in methanol. Alkylating components yielded blue spots.

Column Chromatography. Column chromatography was performed on Silica Gel 40 (70 to 230 mesh; EM Laboratories, Elmsford, N. Y.) in acetone:chloroform (3:1) for ozonized product of cyclophosphamide and in acetone:chloroform (1:3) for the ifosfamide and trofosfamide products.

Instrumentation. Mass spectral analysis was performed...
with a Varian MAT Model 311A mass spectrometer and PMR measurements with a Varian XL-100-15 spectrometer (Varian, Inc., Palo Alto, Calif.). Infrared analysis was performed with Perkin-Elmer Model 521 and 621 infrared spectrophotometers (Perkin-Elmer Corp., Norwalk, Conn.). Radiochromatograms were scanned with a Berthold-Radiochromatogram-scanner II, LB 2722 (Laboratory Professor Dr. Bert- hold, 7547 Wildbad/Schwarzwald, West Germany).

**Evaluation against L1210 Leukemia.** Compounds were administered i.p. on the 1st day of inoculation of 10⁵ or 10⁶ leukemia cells in C57BL × DBA/2 mice. Control and test animals were observed for days of survival.

**RESULTS AND DISCUSSION**

Two synthetic routes to 4-hydroperoxycyclophosphamide and 4-hydroperoxyifosfamide were heretofore available. Fenton oxidation was used successfully for synthesis of 4-hydroperoxycyclophosphamide (C. Benckhuysen, personal communication), as well as 4-peroxycyclophosphamide, although characterization of products generated by this route was not completed until after Takamizawa et al. (17, 19) reported their synthesis by ozonation of 3-butenyl N,N-bis(2-chloroethyl)phosphorodiamidate, a route used unsuccessfully by Struck and Hill (12) for the synthesis of aldo- phosphamide. Van der Steen et al. (20) and Montgomery and Struck (10) were able to produce 4-hydroperoxycyclophosphamide in yields of 4% and 9%, respectively, by the Fenton method. Takamizawa et al. (17) synthesized this hydroperoxide in 44% overall yield by their procedure. Preparation of 4-hydroperoxyifosfamide was also reported by both methods (10, 18).

Because of the availability of cyclophosphamide and its congeners, synthesis of the corresponding hydroperoxides by use of the drugs themselves appeared to be a potential, convenient route, and such a procedure was developed by Peter and Hohorst (11) for 4-hydroperoxycyclophosphamide. The general applicability of the method is confirmed by this report on the synthesis of 4-hydroperoxyifosfamide and -trofosfamide.

**4-Hydroperoxyifosfamide (NSC 207117 and NSC 227114).** Ifosfamide (1 g) in 18 ml acetone and 9 ml water containing 1.5 ml 30% H₂O₂ was treated with ozone at 0 °C. After removal of acetone by evaporation under reduced pressure at 15 °C, leaving a colorless oil. While the flask was shaken and scratched with a glass rod, 20 ml diethyl ether were added portionwise until cloudiness appeared. Using too much diethyl ether precipitated an oily bottom layer, which disappeared with several drops of CH₂Cl₂. After this procedure, the flask was allowed to stand at room temperature. About 15 min later, 4-hydroperoxyifosfamide began to separate as white crystals. After the compound stood for some hours at room temperature, crystallization was com-

**4-Hydroperoxytrofosfamide (NSC 260608).** Ozonation of trofosfamide under identical conditions yielded 25% of 4-hydroperoxytrofosfamide, as indicated by PMR analysis. Column chromatographic separation of the chloroform extract of the aqueous solution gave a fraction containing only the hydroperoxide. Concentration of the column eluate and refrigeration resulted in the isolation of 18% crystalline material (3 crops) that was homogeneous upon TLC analysis: m.p. 145 °C with explosive decomposition (Heizbank); PMR, infrared (cm⁻¹): 3150, 3015, 2965, 2950, 2820, 1485, 1445, 1370, 1345, 1310, 1260, 1235, 1215, 1140, 1105, 1085, 1060, 1030, 1010, 980, 955, 930, 905, 875, 805, 765, 740, 655, 540, 515: field desorption mass spectrum (m/e): 355 (3 Cl, M + 1)⁺, 336 (3 Cl, M – H₂O)⁺, 320 (3 Cl, M – H₂O)⁺: TLC: Rf 0.48 in acetone:chloroform (1:3) (RF of trofosfamide, 0.59). Elemental analysis:

\[
\text{C}_8\text{H}_6\text{Cl}_2\text{N}_2\text{O}_4\text{P}
\]

Calculated: C 30.40, H 5.10, N 7.88

Found: C 30.37, H 4.94, N 7.81

For a larger scale preparation, ifosfamide (10 g) was dissolved in a mixture of 70 ml acetone, 30 ml water, and 10 ml 30% hydrogen peroxide. Ozone (1 mmole/min) was bubbled into the solution through a sintered glass disc at 0 °C over a 4.5-hr period. After evaporation of acetone under reduced pressure, the reaction mixture, which showed an oily bottom layer, was extracted with CH₂Cl₂ (3 x 100 ml), and the combined organic layers were dried over Na₂SO₄. After filtration, CH₂Cl₂ was evaporated under reduced pressure at 15 °C, leaving a colorless oil. While the flask was shaken and scratched with a glass rod, 20 ml diethyl ether were added portionwise until cloudiness appeared. Using too much diethyl ether precipitated an oily bottom layer, which disappeared with several drops of CH₂Cl₂. After this procedure, the flask was allowed to stand at room temperature. About 15 min later, 4-hydroperoxyifosfamide began to separate as white crystals. After the compound stood for some hours at room temperature, crystallization was com-

**Direct Ozonation of Ifosfamide and Trofosfamide**

[Chart 1. Cyclophosphamide and its congeners.]
complete. The crystals were collected by suction and washed with diethyl ether to yield 1.6 g of product (14%).

In a similar way 4-hydroxytrofosfamide was obtained in a larger amount. Trofosfamide (3 g) was dissolved in 6 ml acetone, 12 ml water, and 2 ml 30% hydrogen peroxide. Ozone (1 mmole/min) was bubbled through a sintered glass disc into the solution at 0°C over a 4-hr period. From the reaction emulsion from which an oily bottom layer separated on standing, acetone was evaporated under reduced pressure. The residue was extracted with CH$_2$Cl$_2$ (3 x 40 ml) and dried over Na$_2$SO$_4$. The CH$_2$Cl$_2$ extract was filtered and evaporated under vacuum, leaving a colorless oil, which was dissolved in 1 to 2 ml CH$_2$Cl$_2$. While the flask was scratched with a glass rod, 4-hydroperoxytrofosfamide separated at room temperature as white crystals. After some hours at room temperature, the crystals were collected by suction and washed with 1 ml cold CH$_2$Cl$_2$ (−25°C) and subsequently with diethyl ether to yield 510 mg (15.5%) of the chromatographically homogeneous product.

4-Hydroperoxytrofosfamide (NSC 181815). For comparison purposes, the preparation of 4-hydroperoxytrofosfamide was performed under conditions identical to those used for the ifosfamide and trofosfamide derivatives; a 10% yield was obtained and 20% of cyclophosphamide was recovered. When ozonation was continued for longer periods, more complete utilization of cyclophosphamide was realized. For example, ozonation for 7 hr gave a 9% yield of 4-hydroperoxytrofosfamide and a 17% yield of 4-hydroperoxycyclophosphamide; no starting material was recovered, but a 50% yield of 4-ketocyclophosphamide was isolated.

The relative yield of 4-hydroperoxytrofosfamide could be enhanced by increasing the polarity of the reaction solvent using acetone:water (1:2). By this way, the yield of 4-hydroperoxytrofosfamide was increased to 20% and that of 4-hydroperoxycyclophosphamide was reduced to 3%. The relative amount of 4-ketocyclophosphamide was 50%. The yields were determined by radioscanning the chromatogram of the final reaction mixture with side-chain-labeled [1H]cyclophosphamide as starting material. The yield of crystalline 4-hydroperoxytrofosfamide, obtained by fractional crystallization and recrystallization from CHCl$_3$, was 12%.

Under these conditions crystallization of the product was much easier than using acetone:water (2:1), because in the latter case the presence of 4-peroxycyclophosphamide seemed to prevent crystallization of 4-hydroperoxytrofosfamide.

As illustrated by the experiments described herein, the direct synthesis of the hydroperoxy derivatives of cyclophosphamide, ifosfamide, and trofosfamide can be accomplished conveniently by ozonation of the appropriate, commercially available drug. Although the yield does not equal that reported by Takamizawa et al. (18-20) for these preactivated derivatives of cyclophosphamide and ifosfamide, the necessity of preparation of the required acyclic intermediates from phosphorus oxychloride is eliminated.

This method is superior to the Fenton method for 4-hydroperoxytrofosfamide and ifosfamide because of comparable yield and easier isolation. Synthesis of the trofosfamide derivative by the Fenton method gave only a bicyclic peroxide in 2 attempts. Synthesis of 4-hydroperoxytrofosfamide is also particularly convenient by this direct ozonation method. Ozonation can be continued until little cyclophosphamide remains; evaporation of the acetone from the reaction solution and filtration removes the major part of the 4-keto derivative. Washing a chloroform extract of the resulting filtrate with aqueous base removes any remaining 4-ketocyclophosphamide and simultaneously converts the 4-hydroperoxide to the peroxy derivative. Consequently, the chloroform extract then contains 4-peroxycyclophosphamide slightly contaminated with unreacted starting material. Column chromatography on silica gel in acetone:chloroform (3:1) gives a purified product which crystallizes upon trituration with ether containing a small amount of acetone.

Although the reaction mechanism of this synthesis of the various hydroperoxides is unknown, several routes appear to be possible. Reaction of C$_4$ of the oxazaphosphorine ring with ozone to give a transition state with radical character followed by elimination of molecular oxygen would yield the corresponding 4-hydroxy derivative as an intermediate. An alkane oxidation route discussed by Hamilton et al. (8). Conversion of the hydroxy derivative to the hydroperoxide would be expected in the presence of hydrogen peroxide. Such a conversion has been observed (17). The intermedi-

### Table 1

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<thead>
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<th>Compound</th>
<th>10^5 cells</th>
<th>10^6 cells</th>
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<tr>
<td></td>
<td>Dose (mg/kg)</td>
<td>% 30-day survivors</td>
</tr>
<tr>
<td>4-Hydroperoxytrofosfamide</td>
<td>100</td>
<td>30</td>
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</tr>
<tr>
<td>4-Hydroperoxytrofosfamide</td>
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<td>70</td>
</tr>
<tr>
<td>Cyclophosphamide</td>
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<td>70</td>
</tr>
<tr>
<td>Ifosfamide</td>
<td>40</td>
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* Dose of drug required to kill 10% of test animals.
acy of the hydroxy derivatives has not been established or disproved, although the high yield of the 4-keto derivatives is consistent with their presence in the reaction solution. Attack of hydrogen peroxide on the transition state to give the hydroperoxides directly is also a possibility. Since the P(O)—N₃—C₄ group is a somewhat deactivated alkyl amine, electrophilic attack of ozone on N₃ to give an adduct followed by proton abstraction from C₄ and subsequent hydroxylation of C₄ and elimination of molecular oxygen, a route discussed by Bailey et al. (2), would also yield the 4-hydroxy derivative. However, solvent effects reported for side-chain oxidation of amines by ozone and the deactivated state of N₃ make this latter mechanism appear less likely.

Experimental antitumor evaluation has been reported (10, 17—19, 20) for the hydroperoxy and hydroxy derivatives of cyclophosphamide and ifosfamide and for peroxycyclophosphamide (15). Comparative data versus L1210 leukemia in vivo are given in Table 1 along with data for 4-hydroperoxytrofosfamide, cyclophosphamide, ifosfamide, and phosphoramide mustard, the possible ultimate alkylating metabolite of cyclophosphamide. The results indicate a superior effect for 4-hydroperoxyifosfamide against L1210 leukemia.

REFERENCES

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