Synthesis of Deuterium-Labeled Analogs of Cyclophosphamide and Its Metabolites

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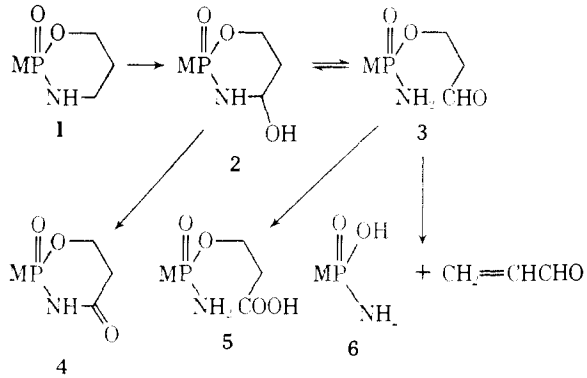
Convenient syntheses are described of d_4 analogs of cyclophosphamide and some of its metabolites, potential standards for the quantitative analysis of the drug and its metabolites in human body fluids by stable isotope dilution-mass spectrometry. Base-catalyzed H–D exchange on *N*-nitrosobis(2-hydroxyethyl)amine gave *N*-nitrosobis(1,1-dideuterio-2-hydroxyethyl)amine from which bis(2-chloro-1,1-dideuterioethyl)amine (nor-HN2-d_4) was readily obtained. Established synthetic routes were then used to convert nor-HN2-d_4 into d_4 analogs of cyclophosphamide [2-[bis(2-chloroethyl)amino]tetrahydro-2*H*-1,3,2-oxazaphosphorine 2-oxide], 4-ketocyclophosphamide [2-[bis(2-chloroethyl)amino]tetrahydro-2*H*-1,3,2-oxazaphosphorin-4-one 2-oxide], and carboxyphosphamide [2-carboxyethyl *N*,*N*-bis(2-chloroethyl)phosphorodiamidate], and these analogs were used in a preliminary investigation into the quantitation of the appropriate components in human plasma and urine. Also prepared were d_4 analogs of phosphoramide mustard [*N*,*N*-bis(2-chloroethyl)phosphorodiamidic acid (cyclohexylammonium salt)] and 3-(2-chloroethyl)oxazolidone and the methyl and trideuteriomethyl esters of phosphoramide mustard.

Studies[1] of the metabolism of cyclophosphamide, an anticancer agent in current widespread use, have shown that the drug **1a** is converted (Scheme I) by way of labile inter-

Scheme I. Metabolism of Cyclophosphamide (1a)



a series, M = $N(CH_2CH_2Cl)_2$
b series, M = $N(CD_2CH_2Cl)_2$

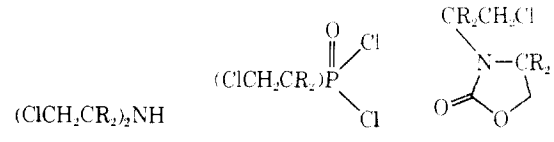
mediates, 4-hydroxycyclophosphamide (**2**) and its acyclic tautomer, aldophosphamide (**3**), into the relatively nontoxic 4-keto derivative **4a** and carboxyphosphamide (**5a**). Aldophosphamide (**3**) can also undergo a nonenzymic β-elimination reaction to yield acrolein and the markedly cytotoxic[1] phosphoramide mustard (**6a**). The antitumour activity of cyclophosphamide is probably due to the formation of **6a** so that the rates of the activating (**1a** → **2** ⇌ **3**) and deactivating (**2** → **4a**, **3** → **5a**) processes will determine the amount of **3** liberated and possibly influence the therapeutic effectiveness of the drug.

Thus, a knowledge of the metabolic profile shown in Scheme I could be important. Investigation of the variation of the total metabolic profile in Scheme I on an individual patient basis requires the determination of the time course of blood and urine levels of **1a**, **4a**, **5a**, and **6a** although routine chemotherapy might be based on a correlation between dose scheduling and the blood level of **6a**.

Cyclophosphamide metabolites have been determined by a method[2] based on the blue color produced when alkylating species react with the Epstein reagent [4-(4-nitrobenzyl)pyridine]. A potentially more sensitive procedure employs radioactively labeled cyclophosphamide.[3] However, for these assay procedures to be specific and quantitative, the extraction efficiency for each metabolite must be determined and the homogeneity of each extracted product must be demonstrated. Moreover, the use of radioactively labeled drugs in man presents an ethical problem. These limitations can be overcome by using stable isotope dilution coupled with mass spectrometry.[4] Thus, to the fluid (blood, urine) containing the compound (drug, metabolite) to be assayed, a known weight of a deuterated analog is added. The concentration of the compound is then determined from the ratio of intensities of appropriate mass spectral signals for the protium and deuterium forms. This procedure is independent of extraction efficiency and may not require purification of the compound to be assayed. Stable isotope dilution–mass spectrometry can be used directly for routine monitoring of drug and metabolite concentrations in plasma and urine or to validate other less specific (e.g., colorimetric) methods.

We now report convenient syntheses of tetradeuterated analogs of cyclophosphamide (**1a**) and several metabolites for use as standards in analysis by stable isotope dilution. In addition to d_4 analogs (**4b–6b**) of the metabolites (**4a–6a**) shown in Scheme I, we have also synthesized d_4 analogs (**7b** and **9b**) of bis(2-chloroethyl)amine (nor-HN2, **7a**) and the oxazolidone (**9a**) which are urinary metabolites of **1a** in man.[5]

The use of d_4 analogs in analysis by stable isotope dilution–mass spectrometry simplifies spectra interpretation in relation to superposition of signals from protium and deuterium forms when signal intensities for ions containing a chlorine substituent are to be compared and we have thus used the ring-labeled compound, 4,4′,6,6′-tetradeuteriocyclophosphamide, in the determination of cyclophosphamide levels in the blood and urine of patients.[6] However, side-chain labeling would be more convenient since the $-N(CH_2CH_2Cl)_2$ function is present in cyclophosphamide and the metabolites **4a–6a** and **7a** and, in modified form (loss of one chlorine substituent), in **9a**. Moreover, $NH(CH_2CH_2Cl)_2$ (nor-HN2) is an intermediate in well-established syntheses of **1a**, **4a–6a**, and **9a** so that a deuterated analog could be easily utilized in the parallel syntheses shown in Scheme II. Such a tetradeuterated analog **7b** [$HN(CD_2CH_2Cl)_2$] of nor-HN2 is readily obtainable from *N*-nitrosodiethanolamine by taking advantage of the fact that base-catalyzed H–D exchange occurs readily in the



a series, R = H
b series, R = D

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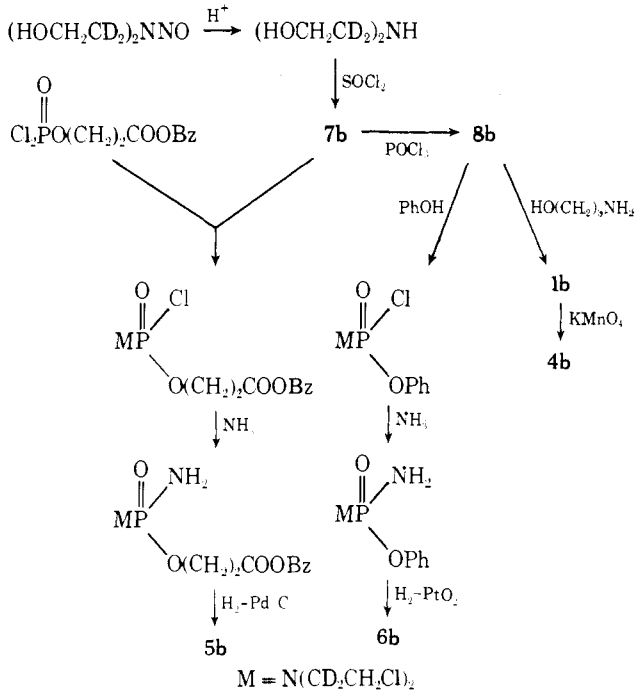
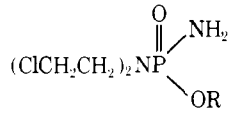
Scheme II. Synthetic Routes to d_4 Analogs of Cyclophosphamide and Its Metabolites



grouping CHNN=O (e.g., the conversion of dimethylnitrosamine into the d_6 analog[7]). Laboratory operations involving *N*-nitrosodiethanolamine are not unduly hazardous since the compound is only weakly carcinogenic.[8]

The yield of 4-ketocyclophosphamide (**4a**) obtained[3] via the phosphoramidic dichloride **8a** and β-hydroxypropionamide is poor (<1%) and the alternative synthesis[9] via cyclization of carboxyphosphamide (**5a**) is lengthy. The direct oxidation[10] by $KMnO_4$ of cyclophosphamide (**1a**) to **4a** was therefore applied for the synthesis of **4b** from the cyclophosphamide-d_4 (**1b**). The synthesis of the trideuteriomethyl ester **10b** of the phosphoramide mustard **6a** is also described by the successive reaction of methanol-d_4 and ammonia with the phosphoramidic dichloride **8a**. Compound **10b** would be a more readily accessible internal standard than **6b**, provided that methylation of **6a** present in biological fluids can be either quantitatively or at least reproducibly achieved. Although the mass spectrum of the methyl ester **10a** has been reported,[1,11] no synthesis of **10a** appears to have been described.



10a, R = CH_3
b, R = CD_3

The simultaneous quantitation of cyclophosphamide and some of its metabolites in human blood and urine using the previously reported direct insertion technique[6] and deuterated internal standards described here is currently under investigation in our laboratories and some preliminary findings are now reported using plasma and urine samples obtained from a man undergoing treatment for oat cell carcinoma of the lung. These samples were treated with the appropriate internal standards (in this investigation, **1b**, **4b**, and **5b**) prior to chromatography on Amberlite XAD-2 resin (see **Experimental Section**). The concentrated methanol eluates were allowed to react with diazomethane, which converts 4-ketocyclophosphamide into an N^3-methyl derivative and carboxyphosphamide into a methyl ester. The

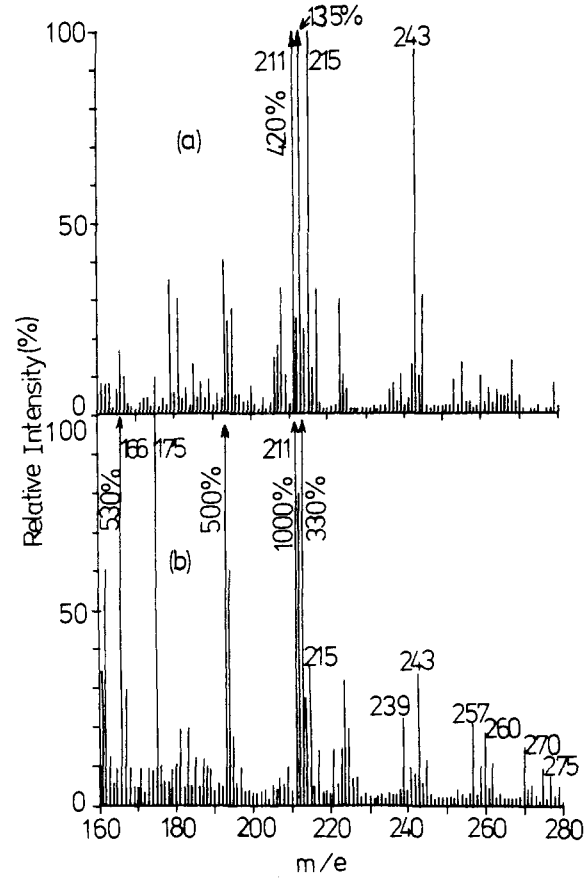


Figure 1. Mass spectra showing cyclophosphamide and methylated metabolites (4-ketocyclophosphamide, carboxyphosphamide) after isolation from (a) 1 hr plasma; (b) 4 hr urine. Patient given 1 g iv; 10 μg/ml of tetradeuterated standards was added.

mass spectrum of the concentrate was obtained using the direct insertion method. Blank samples of plasma and urine, obtained from the patient before treatment, were also subjected to the same procedure, and it was verified that the level of background signals which are obscured, in the test samples, by the signals from the drug, its metabolites, and the internal standards was not significantly greater than the background levels in the immediate vicinity of such peaks, as depicted in Figure 1.

Cyclophosphamide (**1a** $[M - CH_2Cl]^+$ at *m/e* 211) and its d_4 analog (**1b**, *m/e* 215) and the d_4 analog of 4-ketocyclophosphamide (*N*-methyl derivative of **4b** $[M - CH_2Cl]^+$ at *m/e* 243) were recovered from plasma. In urine, 4-ketocyclophosphamide itself (**4a**, signal for the *N*-methyl derivative at *m/e* 239) and carboxyphosphamide (**5a**, signals for the methyl ester at *m/e* 275 $[M - OCH_3]^+$, 270 $[M - HCl]^{\cdot+}$, 257 $[M - CH_2Cl]^+$, and 166 $[M - N(CH_2CH_2Cl)_2]^+$) were additionally present. Prior acidification of the urine was necessary for the detection of carboxyphosphamide as its methyl ester. Presumably, **5a** (or **5b**) must be present as the free acid, either for successful recovery or for conversion into the methyl ester, and the low abundance of the appropriate base peak (*m/e* 166) in the mass spectrum of the plasma-derived sample [Figure 1 (a)] may be due to buffering of the added acid by plasma constituents.

Cyclophosphamide in plasma and urine and 4-ketocyclophosphamide in urine were quantitated by measurement of the peak height ratios, 211:215 and 239:243, respectively. Using diazomethane-treated standard mixtures (cf. ref 6) of the appropriate compounds (**1a**, **1b**; **4a**, **4b**) it was found, as expected, that conversion of these ratios into absolute concentrations of **1a** and **4a** required the appropriate cor-

rection (see Experimental Section) for the presence of the d_3 analog in the standards. From the signal slightly exceeding background intensity at *m/e* 239 in the mass spectrum [Figure 1 (a)] of the plasma-derived products is derived a maximum level of ~1 μg/ml for 4-ketocyclophosphamide in the 1-hr plasma sample, and for none of the blood samples taken was the ratio 239:243 indicative of a plasma level of **4a** exceeding 1 μg/ml.

The carboxyphosphamide analog **5b** is not suitable for the accurate quantitation of carboxyphosphamide (**5a**) as its methyl ester by the present procedure since, after allowing for the isotopic contribution from *m/e* 260 ($M^{\cdot+}$ for cyclophosphamide), the most abundant deuterium-containing ion given by **5b** methyl ester, *m/e* 261 ($[M - CH_2Cl]^+$, 6% of base peak at *m/e* 166), was not detected above background level. From the ratio 261:257 (corresponding ion for **5a** methyl ester) a minimum level for the carboxyphosphamide content in the 4-hr urine sample of 50 μg/ml could be calculated, but the true value is probably much higher. In a previous study,[3] in which ^{14}C-labeled cyclophosphamide was used, a urinary level of **5a** comparable with that of cyclophosphamide was found, but the level of 4-ketocyclophosphamide was, as in the present study, relatively much lower.

Recently, direct insertion chemical ionization–mass spectrometry coupled with stable isotope dilution has been used to quantitate drugs (quinidine, lidocaine) in human plasma[12] and it is likely that this minimal fragmentation procedure could prove more sensitive than the corresponding electron-impact procedure as well as permitting the effective use of the carboxyphosphamide analog **5b** as an internal standard by giving abundant deuterium-containing ions in the mass spectrum of the methyl ester. A sensitive gas chromatography–mass spectrometry (GC–MS) method based on the formation of volatile N^3-trifluoroacetyl derivatives has been developed[13] for cyclophosphamide and 4-ketocyclophosphamide (**4a**) and would permit the quantitation of nanogram quantities of the latter component in human plasma, using **4b** as internal standard. Field desorption mass spectrometry allows structural information to be obtained directly (i.e., without derivative formation) on compounds which are thermally unstable [e.g., carboxyphosphamide (**5a**)] or involatile [e.g., phosphoramide mustard (**6a**)] and has been used[14] to obtain abundant molecular ions of all the components in a mixture of cyclophosphamide **1a** and the metabolites **4a–6a**.

Thus, while the present report on the quantitation of cyclophosphamide and its metabolites in human body fluids by the previously reported direct insertion method[6] is only preliminary, the existence of the several alternative approaches mentioned above has prompted a more comprehensive report of the synthesis of the deuterated reference standards.

Experimental Section

Melting points were determined on a Kofler block and are corrected. ^1H NMR spectra were determined on 10% w/v solutions (internal Me_4Si) using a Perkin-Elmer R10 spectrometer operating at 60 MHz. Mass spectra were determined using an AEI MS-12 spectrometer at an ionizing voltage of 70 eV and an ion-source temperature of ~100°. TLC was performed on 0.25-mm layers of Merck Kieselgel G supported on standard glass plates (20 × 5 cm or 20 × 20 cm) or, in the preparation of **4b**, on grooved plates obtained from May and Baker Ltd., Dagenham, Essex, England. Rotary evaporation was performed at 30° (10 mm).

Deuterated reagents were obtained from Koch-Light Laboratories Ltd., Colnbrook, Buckinghamshire, England. Isotopic purity was ≥99.7 atom % D for D_2O, ≥99% atom % for CD_3OD, and ≥98 atom % D for DCl in D_2O. The solution of NaOD in D_2O was prepared by dissolution of sodium in D_2O.

In reporting mass spectral data, only molecular ions and fragment ions of value in determining deuterium content are given. Although only those Cl-containing ions containing exclusively ^{35}Cl isotope are reported, the corresponding ^{37}Cl-containing ions of the appropriate intensity[15] were also always present. The ions used for determining the ratio between isotopic species were the most intense ions known to retain all deuterium atoms. Of these, $[M - CH_2OH]^+$ ions (deuterated analogs of nitrosodiethanolamine and diethanolamine) were of low relative abundance, and the quoted d_4:d_3 ratios are less accurate than those determined from $[M - CH_2Cl]^+$ ions (100% relative intensity except for **5b**) and from which the small contribution due to dideuterated species (1% of the total deuterated species present) could also be determined. The small contribution (<2%) to the signal for each major deuterated component by the ^{13}C-containing peak given by the minor deuterated species was neglected.

Bis(2-chloro-1,1-dideuterioethyl)amine Hydrochloride (Nor-HN2-d_4, 7b). (a) ***N*-Nitrosobis(1,1-dideuterio-2-hydroxyethyl)amine.** A solution of *N*-nitrosobis(2-hydroxyethyl)amine[16] (2.2 g) in D_2O (10 ml) was concentrated and the process was repeated. A solution of the residue in D_2O (20 ml) containing 4% w/v NaOD was then heated under reflux for 1 hr. The cooled solution was neutralized with 20% DCl in D_2O (~1.5 ml) and then concentrated, and the residue was triturated with EtOH (20 ml). To the filtered solution silicic acid (5 g) was added, the mixture was concentrated, and the residue was applied to a column (2 cm diameter) of dry silicic acid (30 g). Elution with $CHCl_3$–EtOH (9:1) afforded the partially deuterated nitrosamine (1.5 g) in fractions 15–45 (10-ml fractions). At this stage the ratio of d_4- to d_3-labeled product (see below) was 100:70. Repetition of the entire exchange procedure afforded 1.1 g of the product as a yellow oil: mass spectrum *m/e* 138 (1%, $M^{\cdot+}$ for d_4 analog), 107 (9, $[M - CH_2OH]^+$, d_4 analog), 106 (0.9, d_3 analog), 93 (60, d_4 analog), 92 (4, d_3 analog) 78 (33), 77 (35), 75 (47), 60 (40, $[CD_2{=}NCD_2CH_2]^+$), 59 (4, d_3 analog), 47 (60), 46 (100, partly $[CD_2{=}N{=}CD_2]^+$), 45 (11), 42 (6). Starting material gave *m/e* 134 (0.5%, $M^{\cdot+}$), 103 (9), 102 (0.2), 91 (55), 90 (2), 74 (30), 73 (36), 72 (53), 56 (40), 55 (3), 46 (68), 42 (100). The ratio *m/e* 107/106 indicated that the product contained *N*-nitrosodiethanolamine-d_4 and the d_3 analog in the ratio ca. 10:1.

(b) Bis(1,1-dideuterio-2-hydroxyethyl)amine. The product from (a) was heated under reflux in 1 *N* HCl (10 ml) for 16 hr. The cooled solution was neutralized by passage through a column (13 × 2 cm) of Amberlite IR-45 (HO^-) resin and washing with H_2O (50 ml). Concentration of the eluate afforded deuterated diethanolamine as a colorless oil (0.9 g): mass spectrum *m/e* 109 (3%, $M^{\cdot+}$ for d_4 analog), 78 (100, $[M - CH_2OH]^+$).

(c) **Nor-HN2-d_4 (7b).** To a stirred mixture of the product from (b) and $CHCl_3$ (EtOH-free, 3 ml) at 0°, $SOCl_2$ (1.5 ml) was added during 2 min. After 16 hr at room temperature, the mixture was concentrated with petroleum ether (bp 40–60°). Recrystallization of the brown residue from Me_2CO gave the hydrochloride of **7b** as colorless plates (softening, mp 165–190°): mass spectrum *m/e* 145 (4%, $M^{\cdot+}$ for d_4 analog), 96 (100, $[M - CH_2Cl]^+$, d_4 analog), 95 (14, d_3 analog), 94 (2). Nor-HN2 (**7a**)[17] gave *m/e* 141 (5% $M^{\cdot+}$), 92 (100). The ratio *m/e* 96/95 indicated that **7b** contained the d_4 and the d_3 analogs in the ratio 88:12.

2-Carboxyethyl *N,N*-Bis(2-chloro-1,1-dideuterioethyl)phosphorodiamidate (Carboxyphosphamide-d_4, 5b). (a) **2-Benzyloxycarbonylethyl *N,N*-Bis(2-chloro-1,1-dideuterioethyl)phosphoroamidochloridate.** To a stirred mixture of 2-benzyloxycarbonylethyl phosphorodichloridate[9] (0.9 g, 3.03 mmol) and bis(2-chloro-1,1-dideuterioethyl)amine (**7b** hydrochloride, 0.48 g, 2.63 mmol) in dry CH_2Cl_2 (5 ml) at 0° was added Et_3N (0.82 ml) in CH_2Cl_2 (2.5 ml). After 35 min at 0° and 1.5 hr at room temperature the filtered mixture was applied to a column (42 × 2.2 cm) of silicic acid (70 g) and eluted with Et_2O (10-ml fractions). The product (0.13 g, 12%, homogeneous on TLC in Et_2O, R_f 0.5) appeared in fractions 5–10, was an oil, and was used for stage (b) without further purification.

(b) 2-Benzyloxycarbonylethyl *N,N*-Bis(2-chloro-1,1-dideuterioethyl)phosphorodiamidate. Ammonia was passed through a solution in PhH (8 ml) of the product from (a). After 40 min, NH_4Cl was removed by centrifugation. Chromatography (10-ml fractions) of the supernatant on a column (23 × 1.4 cm) of silicic acid (10 g) using Et_2O (35 ml) followed by EtOH–Et_2O (1:9) afforded the required product in fractions 29–40 which was isolated as a colorless oil (0.116 g, 93%): R_f 0.43 (TLC, EtOH–Et_2O, 1:9); mass spectrum *m/e* 386 (2% $M^{\cdot+}$, d_4 analog), 242 (20, $[M - N(CD_2CH_2Cl)_2]^+$), 91 (100, $C_7H_7^+$).

(c) Carboxyphosphamide-d_4 (5b). A solution of the product from (b) in MeOH (4 ml) was stirred with 5% Pd/C (0.1 g) under

H_2 for 1 hr at room temperature. The mixture was then filtered and concentrated to yield **5b** as a white crystalline solid (0.077 g, 86%) which was recrystallized from Et_2O–MeOH to give **5b** as colorless plates, mp 115–117° (lit.[9] for **5a**, mp 119°). Since the mass spectrum of **5a** contained no intense signals above *m/e* 92/94, the deuterated analog was conventionally methylated with CH_2N_2 prior to mass spectral analysis as the methyl ester which gave *m/e* 279 (3%, $[M - OCH_3]^+$, d_4 analog), 273 (3, $[M - DCl]\cdot^+$), 261 (6, $[M - CH_2Cl]^+$, d_4 analog), 260 (15% of 261, d_3 analog), 166 (100, $[M - N(CD_2CH_2Cl)_2]^+$). For the mass spectrum of the methyl ester of **5a**, see ref 3. The ratio *m/e* 261/260 showed **5b** and its d_5 analog to be present in the ratio 87:13.

***N,N*-Bis(2-chloro-1,1-dideuterioethyl)phosphoroamidic Dichloride (8b).** A suspension of bis(2-chloro-1,1-dideuterioethyl)amine (**7b** hydrochloride, 0.87 g, 4.76 mmol) in $POCl_3$ (2.5 ml, 27.5 mmol) was heated under reflux until dissolution occurred (11 hr). Excess $POCl_3$ was removed in vacuo and a solution of the residue in $CHCl_3$ (3 ml) was washed through a column (5.5 × 1 cm) of dry silicic acid with $CHCl_3$. The colorless eluate (15 ml) was concentrated and the solid residue was recrystallized from Me_2CO–petroleum ether (bp 40–60°) to give 8b as colorless prisms (1.044 g, 83.5%): mp 51–52° (lit.[18] for **8a**, mp 54–56°).

2-[Bis(2-chloro-1,1-dideuterioethyl)amino]tetrahydro-2*H*-1,3,2-oxazaphosphorine 2-Oxide (Cyclophosphamide-d_4, 1b). To a stirred solution of *N,N*-bis(2-chloro-1,1-dideuterioethyl)phosphoramidic dichloride (**8b**, 0.46 g, 1.75 mmol) in dry 1,2-dichloroethane (1.1 ml) at 0° was added a solution in this solvent (1.1 ml) of 1-aminopropan-3-ol (0.162 g, 2.16 mmol) and Et_3N (0.52 ml) during 10 min. After 2 hr at room temperature, the mixture was cooled to 0°, filtered, and concentrated. Treatment of the residual oil with water (0.75 ml) and a trace of cyclophosphamide slowly yielded white crystals (0.204 g, 41%) of **1b**: mp 41–44° (lit.[19] for 1a monohydrate, mp 41–45°); mass spectrum *m/e* 264 (2.5%, $M\cdot^+$, d_4 analog), 217 (100, $[M - CH_2Cl]^+$), 216 (18, d_3 analog), 120 (55, $[M - N(CD_2CH_2Cl)_2]^+$). The ratio *m/e* 217/216 indicated that the product contained the d_4 and the d_3 isomers in the ratio 85:15.

2-[Bis(2-chloro-1,1-dideuterioethyl)amino]tetrahydro-2*H*-1,3,2-oxazaphosphorin-4-one 2-Oxide (4b). A solution of **1b** (0.2 g, 0.76 mmol) and $KMnO_4$ (0.4 g) in H_2O (8 ml) was stirred for 3 hr at room temperature (cf. ref 18). TLC (EtOAc) of the mixture on four grooved plates (20 × 20 cm) revealed a major product (detected by exposure to I_2 vapor) with an R_f value (0.3) corresponding to that of 4-ketocyclophosphamide (cf. cyclophosphamide R_f 0.1). The silicic acid in the appropriate areas was removed and extracted with EtOAc (250 ml). Concentration of the extract and crystallization of the residue from EtOH (1 ml) afforded **4b** as colorless crystals (0.041 g, 19%), mp 148–150° (lit.[3] for 4a, mp 148–149°). The product did not give the aldehyde reactions reported[20] for synthetic 4-hydroxycyclophosphamide (**2**) which is a possible intermediate in the formation of 4-ketocyclophosphamide by $KMnO_4$ oxidation. The product **4b** was therefore uncontaminated by the deuterated analog of **2**: mass spectrum *m/e* 278 (1%, $M\cdot^+$, d_4 analog), 229 (100, $[M - CH_2Cl]^+$), 228 (17, d_3 analog), 96 (33, $[CD_2{=}NCD_2CH_2Cl]^+$, d_4 analog), 95 (8, d_3 analog). The ratio *m/e* 229/228 indicated the product to contain **4b** and the d_3 analog in the ratio 85:15.

Cyclohexylammonium *N,N*-Bis(2-chloro-1,1-dideuterioethyl)phosphorodiamidate (Phosphoramide Mustard-d_4, 6b). (See ref 18 for a preparative route to 6a.)

(a) **Phenyl *N,N*-Bis(2-chloro-1,1-dideuterioethyl)phosphoramidochloridate.** To a boiling solution of *N,N*-bis(2-chloro-1,1-dideuterioethyl)phosphoramidic dichloride (**8b**, 0.26 g, 0.99 mmol) and PhOH (0.1 g, 1.06 mmol) in dry PhH (1.5 ml) was added dropwise a solution of Et_3N (0.16 ml, 0.117 g, 1.15 mmol) in PhH (0.5 ml). After further refluxing for 3 hr, the reaction mixture was kept overnight at room temperature and then filtered. The filtrate, which contained a single product (R_f 0.68, TLC, $CHCl_3$), was concentrated and the resulting pale yellow oil (0.325 g, 100%) was used for the next stage without further purification.

(b) **Phenyl *N,N*-Bis(2-chloro-1,1-dideuterioethyl)phosphorodiamidate.** Ammonia was passed for 40 min through a solution of the product from (a) in PhH (5 ml) initially with ice cooling. Excess NH_3 was removed under reduced pressure and NH_4Cl was removed by centrifugation. The supernatant was concentrated, the residue was dissolved in Me_2CO, and the solution was decolorized (charcoal) and concentrated. To a solution of the residue in PhH (2 ml) petroleum ether (bp 60–80°) was added to afford the product as white needles (0.23 g, 77.5% based on **8b**): mp 56–57° (lit.[18] for the nondeuterated analog, mp 57–59°); mass spectrum *m/e* 300 (8%, $M\cdot^+$), 251 (100, $[M - CH_2Cl]^+$).

(c) **Phosphoramide Mustard-d_4 (6b, Cyclohexylammonium Salt).** A solution in EtOH (1.5 ml) of the product (0.05 g, 0.168 mmol) from (b) was hydrogenated[21] over a Pt catalyst (0.05 g) for 1 hr at room temperature. The reaction mixture was centrifuged, and the supernatant was removed. The residue (precipitated free acid **6b** + catalyst) was resuspended in EtOH (3 ml), cyclohexylamine (0.022 ml, 0.018 g, 0.182 mmol) was added, and the mixture was stirred for 15 min. After removal of catalyst, the filtrate was concentrated to give the product as a white solid (0.027 g, 50%), mp 107–109° (lit.[21] for **6a**, mp 107–108°), which was recovered by suspension in ether and filtration.

3-(2-Chloro-1,1-dideuterioethyl)-4,4-dideuteriooxazolidone (9b). To a solution of $NaHCO_3$ (0.21 g, 2.5 mmol) and NaOH (0.1 g) in H_2O (2.5 ml) was added bis(2-chloro-1,1-dideuterioethyl)amine (**7b** hydrochloride, 2.5 mmol) (cf. ref 22 for a preparative route to **9a**). The mixture was kept at 37° for 20 min and then extracted with CH_2Cl_2 (5 × 10 ml). The dried (Na_2SO_4) extracts were concentrated and a solution of the resulting pale yellow oil in $CHCl_3$ was applied to a column (22 × 2.1 cm) of silicic acid (38 g) which was eluted with $CHCl_3$ (5-ml fractions). The required product appeared in fractions 18–40 and was isolated as a slightly yellow oil (0.285 g, 76%, R_f 0.18, TLC, $CHCl_3$): mass spectrum *m/e* 153 (19%, $M\cdot^+$, d_4 analog), 152 (3), 104 (100, $[M - CH_2Cl]^+$, d_4 analog), 103 (19, d_3 analog), 60 (55), 59 (11), 46 (15). 3-(2-Chloroethyl)oxazolidone (**9a**) gave *m/e* 149 (17%, $M\cdot^+$), 148 (1), 100 (100), 99 (1), 56 (41), 55 (1), 42 (15).

From the relative intensities of *m/e* 104 and 103 the ratio between **9b** and its d_3 analog was found to be 85:15.

Methyl *N,N*-Bis(2-chloroethyl)phosphorodiamidate (10a) and Its Trideuteriomethyl Analog (10b). A solution of *N,N*-bis(2-chloroethyl)phosphorodichloridate (**8a**, 1 g, 3.9 mmol) in MeOH (20 ml) was kept at room temperature for 8 hr. TLC ($CHCl_3$) then revealed two mobile products which were dimethyl *N,N*-bis(2-chloroethyl)phosphoroamidate (mass spectrum $M\cdot^+$ at *m/e* 249) and methyl *N,N*-bis(2-chloroethyl)phosphoroamidochloridate ($M\cdot^+$, *m/e* 253). The mixture was concentrated and reconcentrated with CH_2Cl_2 (3 × 10 ml), and the residue was dissolved in 0.4 *M* NH_3 in CH_2Cl_2 (20 ml). After 16 hr, the solution was filtered and the filtrate was applied to a column (25 × 2 cm) of silicic acid (40 g), which was eluted (10-ml fractions) with $CHCl_3$ (600 ml) and then with $CHCl_3$–MeOH (19:1). The required product (**10a**) was isolated from fractions 9–30 and crystallized from CCl_4 as colorless plates (0.27 g, 30%): mp 82–83°; mass spectrum *m/e* 234 (2%, $M\cdot^+$), 185 (100, $[M - CH_2Cl]^+$); ^1H NMR data ($CDCl_3$) τ 6.30 (*s*, 3 H, OMe), 6.25–6.54 (m, 8 H, $ClCH_2CH_2$), 6.69 (*d*, J = 6 Hz, 2 H, NH_2 coupled with P). Anal. ($C_5H_{13}Cl_2N_2O_2P$) C, H, Cl, N, P.

The crystalline trideuteriomethyl ester (**10b**, 0.16 g, 35%) was prepared from 0.5 g of the dichloride 8a by using CD_3OD in place of CH_3OH as the reaction medium: mass spectrum *m/e* 237 (2%, $M\cdot^+$), 188 (100, $[M - CH_2Cl]^+$); ^1H NMR data ($CDCl_3$) τ 6.25–6.53 (m, $ClCH_2CH_2$), 6.67 (d, J = 6 Hz, NH_2 coupled with P).

Investigations into the Quantitation of Cyclophosphamide (1a), 4-Ketocyclophosphamide (4a), and Carboxyphosphamide (5a) in Human Plasma and Urine. Blood and urine samples were obtained from a patient at various times (see below) after intravenous injection of cyclophosphamide (1 g). To aliquots (1 ml) of urine and of plasma recovered by centrifugation of blood samples was added 10 μl of an aqueous solution containing cyclophosphamide-d_4 (**1b**), 4-ketocyclophosphamide-d_4 (**4b**), and carboxyphosphamide-d_4 (**5b**) each at a concentration of 1.00 mg/ml, followed by 0.1 *N* HCl (1 ml). Each sample was applied to a column (7 × 1 cm) of Amberlite XAD-2 resin, prewashed[5] to remove contaminants which would interfere with subsequent mass spectral analysis. The column was eluted with water (25 ml), drained under mild suction, and then eluted with MeOH (5 ml). The MeOH extract was concentrated to dryness and the residue dissolved in MeOH followed by CH_2N_2–Et_2O (1 ml of each). After 10 min, the solution was concentrated and applied to the direct insertion probe prior to mass spectrometry (source temperature 100°). Representative mass spectra are shown in Figure 1 (for a structural assignment of the designated ions, see the text).

The relative heights of *m/e* 211 and 215 (for 1a and **1b** in plasma and urine) and of *m/e* 239 and 243 (for 4a and **4b** in urine) were determined from scans of limited mass range covering these regions of the spectra, as previously described for the quantitation of cyclophosphamide in human blood.

Cyclophosphamide levels (given in parentheses in μg/ml) were determined for plasma obtained from blood samples taken at the following times after injection: 30 min (36), 1 hr (36.5), 2 hr (23), 4 hr (20), 8 hr (14.5), 12 hr (10.5), and 24 hr (4.3). Urine samples

were taken at 4 hr and at 24 hr. Levels of cyclophosphamide were respectively 157 and 34 μg/ml and of 4-ketocyclophosphamide, 5.4 and 4.7 μg/ml.

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Synthesis and Antitumor Activity of 6-Trifluoromethylcyclophosphamide and Related Compounds

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In an attempt to increase the combined toxicity of the metabolic end-products [acrolein (4) and phosphoramide mustard (3)] from cyclophosphamide (1), the analog 2-[bis(2-chloroethyl)amino]tetrahydro-6-trifluoromethyl-2*H*-1,3,2-oxazaphosphorine 2-oxide (2, 6-trifluoromethylcyclophosphamide) was synthesized and its metabolism and antitumor activity studied. Following metabolism of 2 by rat liver microsomes the predicted formation of 4,4,4-trifluorocrotonaldehyde (5) was confirmed by isolation and identification, by mass spectrometry, of its dinitrophenylhydrazone. The therapeutic indices (LD_{50}/ID_{90}) for 2 against the ADJ/PC6 mouse tumor and the Walker 256 tumor in the rat were 28.6 and 7.7, respectively, and were lower than the corresponding values for 1 (91.8 and 33.2, respectively) although the toxicities toward Walker cells in a bioassay system of 1 and 2 following microsomal metabolism were similar. In order to study the toxicities of 4 and 5 released under drug metabolizing conditions independently of the production of a toxic mustard the analogs 18 [2-(diethylamino)tetrahydro-2*H*-1,3,2-oxazaphosphorine 2-oxide] and 6 [2-(diethylamino)tetrahydro-6-trifluoromethyl-2*H*-1,3,2-oxazaphosphorine 2-oxide] were also synthesized. The release of 5 from 6 following metabolism was confirmed and shown by use of the bioassay system to be an event of similar toxicity to release of 4 from 18; in vivo, however, 6 (LD_{50} 330 mg/kg) was more toxic to mice than 18 (LD_{50} >500 mg/kg).

Since cyclophosphamide [2-[bis(2-chloroethyl)amino]tetrahydro-2*H*-1,3,2-oxazaphosphorine 2-oxide (1)] was first synthesized[1] in 1958 and found to be an effective antitumor agent, many analogs and derivatives have been prepared,[2] but none has activity superior to that of the parent drug. However, recent advances[3-5] in the understanding of the metabolism and possible mode of action of the drug may permit a more rational design of agents and investigation of structure–activity relationships than has been possible hitherto. We now report on the synthesis and biological activity of 6-trifluoromethylcyclophosphamide [2-[bis(2-chloroethyl)amino]tetrahydro-6-trifluoromethyl-2*H*-1,3,2-oxazaphosphorine 2-oxide (2)].

The metabolism of cyclophosphamide is believed[3-5] to proceed via initial formation of 4-hydroxycyclophosphamide, the open-chain tautomer (aldophosphamide) of which yields the cytotoxic products phosphoramide mustard (3) and acrolein (4). Other metabolites of cyclophosphamide, in particular the major urinary excretion products, carboxyphosphamide and 4-ketocyclophosphamide, are not significantly more toxic than the parent drug.[5] The relatively selective toxicity of cyclophosphamide toward tumor cells in vivo has been tentatively attributed[5] to the preferential release of the alkylating agent 3 therein, and the concomitant production of an equimolar amount of acrolein may also be of importance with regard to the antitumor activity.[6,7] On a molar basis acrolein is 40-fold less toxic than phosphoramide mustard (see Results) and it is thus probable that it plays only a minor role in the antitumor effect of cyclophosphamide. Indeed the analog of cyclophosphamide where the bis(2-chloroethyl)amino group is replaced by a diethylamine residue also liberates acrolein in a microsomal system[7] but is inactive as an antitumor agent,[2] showing acrolein to be ineffective when formed in isolation. However, if an α,β-unsaturated carbonyl compound of far greater toxicity than acrolein were released in association with