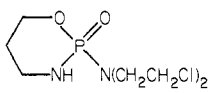
Stereospecific Synthesis of Chiral Metabolites of Ifosfamide and Their Determination in the Urine

K. Misiura,† A. Okruszek,† K. Pankiewicz, W. J. Stec,*,† Z. Czownicki,‡ and B. Utracka‡

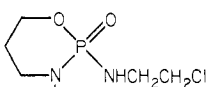
Polish Academy of Sciences, Center of Molecular and Macromolecular Studies, 90-362 Łódź, Boczna 5, Poland, and Institute of Oncology, 44-101 Gliwice, Poland. *Received July 16, 1982*

The stereospecific synthesis of two chiral metabolites of ifosfamide (2), 4-ketoifosfamide (5) and 2-amino-3-(2-chloroethyl)tetrahydro-2*H*-1,3,2-oxazaphosphorine 2-oxide (9), is reported. The absolute configuration of both compounds was assigned on the basis of chemical correlation. In addition, two other achiral metabolites of 2, carboxyifosfamide (6) and IPAM (7), were synthesized. These and other organophosphorus metabolites of ifosfamide were found, by ^{31}P NMR, in the urine of patients to whom racemic 2 was administered. The measurements performed in the presence of optically active lanthanide shift reagent [Eu(tfc)$_3$] showed considerable stereoselectivity of in vivo formation of some chiral metabolites of ifosfamide.

Ifosfamide (2) is a cyclophosphamide (1) analogue dif-

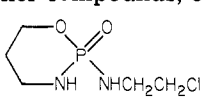


1

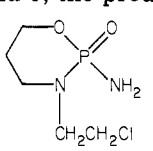


2

fering only in the position of the alkylating functionalities. It has been expected that its metabolism (Scheme I) resembles that of (1).[1] Indeed, 4-ketoifosfamide (5) and carboxyifosfamide (6) were found to be the major metabolites of 2 in both in vitro (mouse liver microsomes) and in vivo tests.[2] Although neither 4-hydroxyifosfamide (3) nor aldoifosfamide (4) were observed at that time, the importance of the C_4-hydroxylation step for the activation of ifosfamide was later proved by Takamizawa et al.[3] The evidence of participation of 3 and 4 in the metabolism of 2 has recently been provided by Hohorst et al.[4] Independently, Norpoth et al.[5] have shown that in addition to 5–7, two other compounds, 8 and 9, the products of de-



8

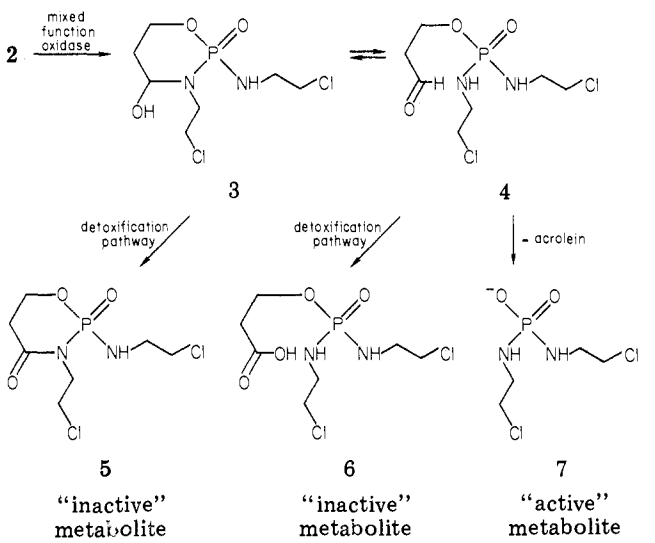


9

chloroethylation of 2, are present in the urine of patients treated with ifosfamide. It was assumed that the therapeutic effect of 2 depends partially on whether side-chain or ring oxidation is the predominant route of biotransformation. The latter conclusion emphasizes the major difference between the metabolism of ifosfamide (2) and cyclophosphamide (1) where side-chain oxidation is a minor process only.[1]

Despite the wealth of clinical and experimental knowledge accumulated during more than 10 years, one hitherto unexplored feature of compound 2 relates to its molecular structure. By virtue of the dissymmetric phosphorus atom, the molecule of 2 is chiral. Recently, three methods of synthesis of enantiomeric forms of 2 and the metabolite 8 were published.[6-8] The absolute configuration of 2 was established by means of both stereochemical correlation with that of 1[7] and X-ray crystallography.[9] It has been demonstrated that the levorotatory form of 2 has the *S* absolute configuration. The assignment of the enantiomeric purity of 2 was based on the measurement of the magnetic nonequivalence of phos-

Scheme I



phorus nuclei in the ^{31}P NMR spectra run in the presence of optically active lanthanide shift reagents.[10] Enantiomeric forms of 8 were also synthesized in this laboratory[7] as the key intermediates in the stereoselective synthesis of optically active 2. It was of interest to obtain the optically active forms of other chiral metabolites of ifosfamide, such as 5 and 9, and find the method of assignment of their absolute configuration and enantiomeric purity. Establishment of the stereochemistry of chiral metabolites of ifosfamide is the key point in studies of the stereose-

† Polish Academy of Sciences.
‡ Institute of Oncology.

(1) W. J. Stec, *Organophosphorus Chem.*, **13**, 145 (1982).
(2) D. L. Hill, W. Russell Laster, Jr., M. C. Kirk, S. El. Dareer, and R. F. Struck, *Cancer Res.*, **33**, 1016 (1973).
(3) A. Takamizawa, T. Iwata, and S. Matsumoto, *Chem. Pharm. Bull.*, **25**, 2900 (1977).
(4) T. Wagner, D. Heydrich, T. Jork, G. Voelcker, and H. J. Hohorst, *J. Cancer Res. Clin. Oncol.*, **100**, 95 (1981).
(5) K. Norpoth, G. Müller, and H. Raidt, *Arzneim.-Forsch.*, 26, 1376 (1976).
(6) (a) R. Kinas, K. Pankiewicz, W. J. Stec, P. B. Farmer, M. Jarman, and A. B. Foster, *Bull. Acad. Pol. Sci.*, **24**, 39 (1978). (b) S. M. Ludeman, D. L. Bartlett, and G. Zon, *J. Org. Chem.*, **44**, 1163 (1979).
(7) K. Pankiewicz, R. Kinas, W. J. Stec, A. B. Foster, M. Jarman, and J. M. S. Van Maanen, *J. Am. Chem. Soc.*, **101**, 7712 (1979).
(8) A. E. Wróblewski, S. M. Socol, A. Okruszek, J. G. Verkade, *Inorg. Chem.*, **19**, 3715 (1980).
(9) D. A. Adamiak, M. Gdaniec, K. Pankiewicz, W. J. Stec, *Angew. Chem., Int. Ed. Engl.*, **19**, 549 (1980).
(10) G. Zon, J. A. Brandt, and W. Egan, *J. Natl. Cancer Inst.*, **58**, 1117 (1977).

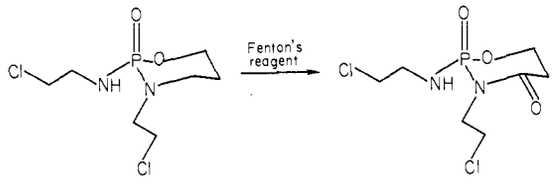
0022-2623/83/1826-0674$01.50/0

Table I. Conditions of Assignment of Optical Purity of 2 and Its Chiral Metabolites 5, 8, and 9 by ^{31}P NMR

compd	concn, mol/L	LSR[a]	compd/LSR ratio	solvent	^{31}P NMR chem shift $\delta(+)$	^{31}P NMR chem shift $\delta(-)$
2	0.02	$Eu(tfc)_3$	1:0.7	CCl_4	-67.2[b]	-68.0[b]
5	0.07	$Pr(tfc)_3$	1:0.5	C_6H_6	7.2	8.7
8	0.025	$Eu(tfc)_3$	1:0.5	C_6H_6	-39.3[b]	-36.8[b]
9	0.07	$Eu(tfc)_3$	1:0.4	$CHCl_3$	-31.7	-29.5

[a] Lanthanide shift reagent. [b] The opposite assignment of lanthanide-induced chemical shift values to a particular enantiomer of 2 and 8 given in our previous publication[7] was caused by the mistake made during the preparation of the manuscript.

Scheme II



(*S*)-2 (*S*)-5

lectivity of the metabolism of racemic 2 in humans. Such assignments should provide information about the stereoselectivity of the enzymatic activation of 2 and the validity of the molecular architecture of compounds like 2 with respect to their therapeutic function.

Results and Discussion

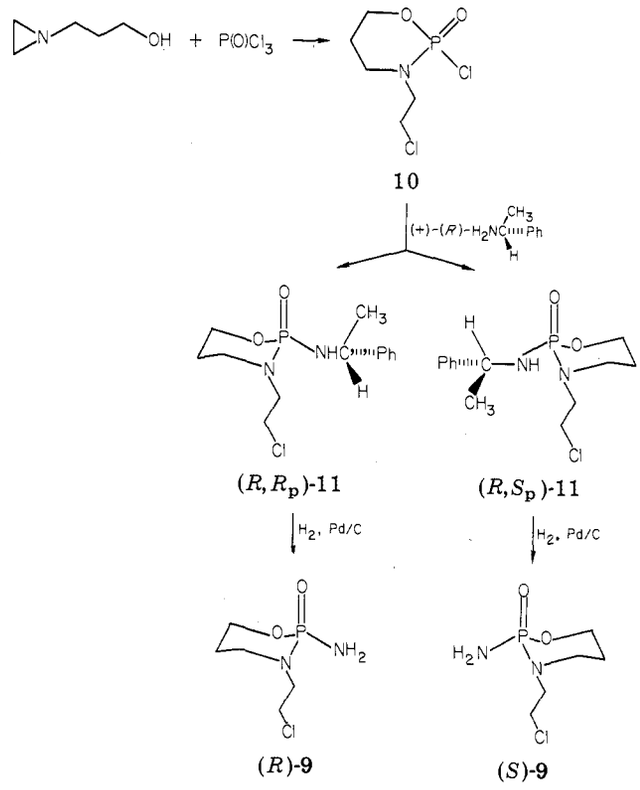
In this paper we report on the synthesis of enantiomers of 5 and 9, the synthesis of achiral 6 and 7, and on a new spectroscopic method of determination of 2 and its stable metabolites in the urine of patients treated with racemic 2.

The synthetic approach to 5 is based on earlier reports[2] on the oxidation of racemic 2 by means of Fenton's reagent. This procedure has been succesfully applied by us for the synthetis of optically active 4-ketocyclophosphamide.[11] Indeed, we have found, that the treatment of optically pure (−)-(*S*)-2 with $FeSO_4/H_2O_2$ in aqueous solution gave, after chromatographic separation, (−)-(*S*)-5: $[\alpha]^{20}_D$ −25.5° (Scheme II). The optical purity of enantiomeric 5 was checked by means of ^{31}P NMR/$Pr(tfc)_3$ spectroscopy. The data concerning the proportions of 1,3,2-oxazaphosphorine 2-oxide/shift reagent, solvent, molar concentrations, and induced chemical-shift values are given in Table I.

The preparation of enantiomers of 9 has been achieved by reaction of 3-(ethylenimino)-1-propanol with phosphorus oxychloride and subsequent condensation of racemic 2-chloro-3-(2-chloroethyl)tetrahydro-2*H*-1,3,2-oxazaphosphorine 2-oxide (10) with (+)-(*R*)-1-methylbenzylamine. Separation of diastereomeric 2-[(1-methylbenzyl)amino]-3-(2-chloroethyl)tetrahydro-2*H*-1,3,2-oxazaphosphorine 2-oxides (11), followed by catalytic debenzylation, gave optically active (*S*)-9 and (*R*)-9 (Scheme III). Assignment of the *S* absolute configuration to the levorotatory isomer of 9 was based on the conversion of 9 to 12 and then 12 to 2 without cleavage of bonds to the phosphorus atom (Scheme IV). The optical purity of 9 was checked as in the case of 5. Details are given in Table I.

Carboxyifosfamide (6) has been prepared in the reaction of 2-(benzyloxycarbonyl)ethyl phosphorodichloridate (13) with aziridine in the presence of triethylamine, followed by the opening of both aziridine functions of intermediary 14 with hydrogen chloride and subsequent hydrogenolytic removal of the benzyl group from the carboxy function

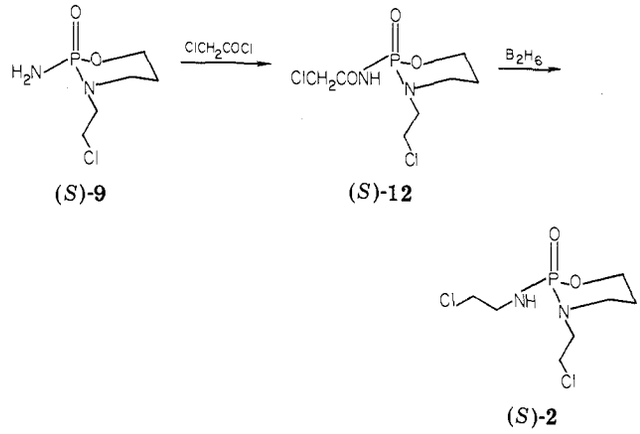
Scheme III



(*R*,*R*$_p$)-11 (*R*,*S*$_p$)-11

(*R*)-9 (*S*)-9

Scheme IV



(*S*)-9 (*S*)-12 (*S*)-2

(Scheme V). Another achiral metabolite, 7, was prepared in a similar manner. Benzyl phosphorodichloridate (16) was converted to benzyl *N*,*N*′-bis(2-chloroethyl)-phosphorodiamidate (18) by using aziridine in the presence of triethylamine. The reaction of 17 with hydrogen chloride, followed by hydrogenolysis of the intermediary compound (18), gave the "active" metabolite of ifosfamide, IPAM (7) (Scheme VI).

Knowledge the of ^{31}P NMR chemical shift of 2 and its "stable" metabolites 5–9 allowed us to determine the extent of metabolism and the quantities of the particular me-

(11) K. Misiura, K. Pankiewicz, W. J. Stec, and M. Jarman, *Experientia*, 37, 216 (1981).

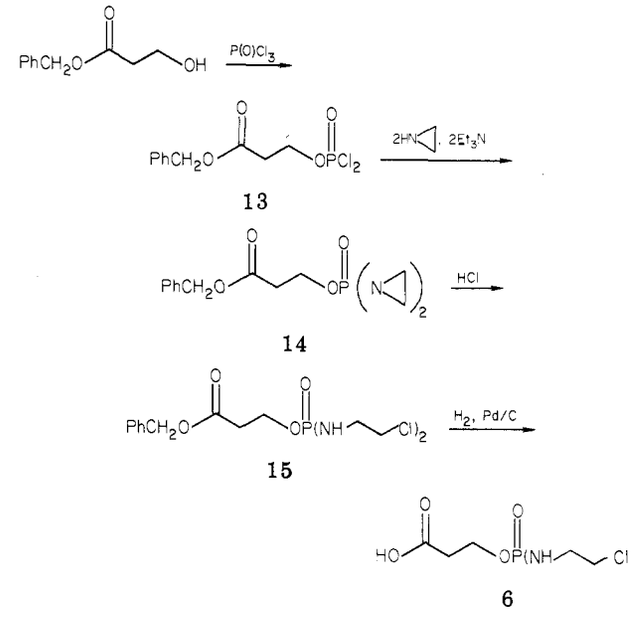
Table II. ^{31}P NMR Chemical-Shift Values of **2** and Its Organophosphorus Metabolites

compd	^{31}P NMR, δ conc urine	^{31}P NMR, δ ethanol (extract)
HMPT[a]	28.1	25.8
6	17.1	15.6
9	15.5	14.0
2	13.6	12.1
8	13.6	11.6
5	7.8	

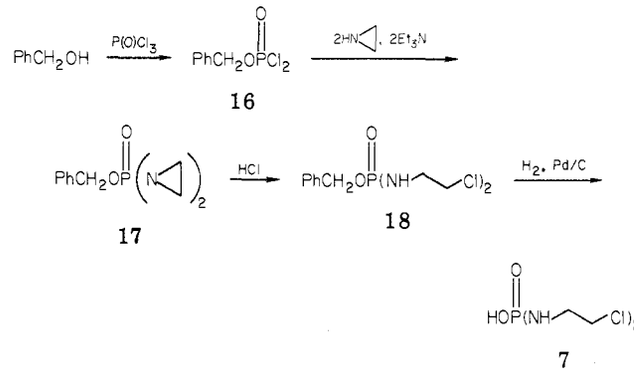
[a] Internal standard.

tabolite in the urine collected from two patients cured with racemic ifosfamide (Holoxan, Asta Werke, Germany). The patients were suffering from alveolar rhabdomyosarcoma (K.W., female, age 52) and embryonal rhabdomyosarcoma (K.D., female, age 19) and were given Holoxan intravenously in 2-g daily doses for 10 days. Urine was collected for 24 h after each bolus and stored in a refrigerator. The 1-L samples of urine were further concentrated, by using a vacuum rotatory evaporator with a bath temperature not exceeding 35 °C, to a volume of ~50 mL. Into each concentrated sample was introduced a known amount of hexamethylphosphoric triamide (HMPT) as an internal standard. Solids were centrifuged, and supernatants were examined by means of ^{31}P NMR spectroscopy. The quantitative results were obtained from electronically integrated spectra. Because the signals of **2** and **8** were not well separated in the spectra of crude, concentrated urine and, therefore, the relative ratios of **2** and **8** could not be calculated, samples of supernatant were extracted with chloroform and ethyl acetate. Extracts were evaporated and, after dissolving in a small volume of ethanol, examined by ^{31}P NMR. Since the extraction, as proved in a blank experiment, does not change the ratio of **2** to **8**, full quantitative analysis of the urine metabolites of **2** could now be performed. Chemical-shift values of organophosphorus metabolites of **2** obtained for both concentrated urine and extracts are collected in Table II. The correctness of quantitative assignments was further checked by introduction of known amounts of synthetically prepared **2** and its metabolites **5–9** to the samples. This

Scheme V



Scheme VI



also gave us an additional proof of recognition of a particular metabolite. It should be mentioned that the presented method did not allow us to detect[12] the presence

Table III. Quantities of **2** and Its Metabolites Found in the Urine of Patient K.D. Treated with Ifosfamide[a]

day	quantity of urine, mL	quantities of metabolites, mg: **6**	**9**	**2**	**8**	**5**	portion of Holoxan excreted in the form of **2**, **5**, **6**, **8**, or **9**, (%)
3[b]	1200	70	64	348	198	44	39.8
4	2310	62	163	442	432	34	65.5
5	1700	70	95	200	258	81	40.2
6	1300	348	243	500	434	136	91.5
7	1200	312	180	432	348	84	74.0
8	1300	56	130	494	338	78	61.7
9	1500	186	231	1100	310	24	100.0
10	1600	130	160	421	320	66	61.0

[a] Two grams per day for 10 days. [b] Although urine for days 1 and 2 was collected, the storage at room temperature caused its elimination from further studies.

Table IV. Enantiomeric Composition of **2** and Its Chiral Metabolites 8 and 9 Extracted from the Urine of Patients K.D. and K.W.

patient	**2**: ratio of (−)-*S*, %	**2**: ratio of (+)-*R*, %	**8**: ratio of (−)-*R*, %	**8**: ratio of (+)-*S*, %	**9**: ratio of (−)-*S*, %	**9**: ratio of (+)-*R*, %
K.D.	45	55	55	45	73	27
K.W.	43	57	28	72	87	13

of metabolite 7 in the urine. This may be due to known chemical lability of 7 in aqueous solution. The results of the quantitative analysis of urine metabolites of 2 for patient K.D. are collected in Table III. Independently, samples of extract were chromatographed on silica gel to give particular components 2, 8, and 9 as chemically pure compounds. The use of ^{31}P NMR/Eu(tfc)$_3$ spectroscopy let us determine the enantiomeric composition of chiral metabolites 2, 8, and 9. The data for both patients are collected in Table IV. The lack of data for compound 5 results from our inability to extract it from the concentrated urine in NMR-detectable amounts. This is probably due to its excellent solubility in water and relatively low abundance in the analyzed samples of urine.

The quantitative results shown in Tables III and IV, being limited to the analysis of urine collected from two patients only, do not permit any generalization regarding the correlation of the metabolite spectrum with the type of cancer disease and results of particular treatment but may rather serve as an illustration of the possibilities offered by the presented method. Two conclusions could be made, however, on the basis of the presented data.

Inspection of Table III shows that a rather high portion of administered Holoxan, ranging from 40 to 100%, is excreted in the urine in the form of nonmetabolized 2 or its "inactive" metabolites 5, 6, 8, and 9. The second conclusion is related to the enantiomeric composition of nonmetabolized 2 and chiral metabolites 8 and 9 present in the urine. The side-chain hydroxylation, followed by dechloroethylation, leading to metabolites 8 and 9 is a highly stereoselective enzymatic process producing considerable excess of one enantiomer. This finding is consistent with our earlier observation of stereodifferentiated metabolism of 4-methylcyclophosphamide in mice.[13] We have found that of four possible stereoisomers, three are metabolized in a way analogous to cyclophosphamide itself while the other one undergoes preferential enzymatic side-chain α-hydroxylation, followed by elimination of chloroacetaldehyde.

The relatively low enantiomeric enrichment of ifosfamide excreted in the urine may be explained by the fact that 2 is involved in at least three different metabolic pathways, each of them not necessarily "consuming" the same enantiomer. The differences in the enantiomeric composition of compound 8 in the urine of two patients may be speculatively regarded as a result of the action of subject-differentiated oxygen-carrying proteins associated with cytochrome P-450. In order to evaluate the presented ^{31}P NMR method for quantitative studies of the metabolism of 2 in humans, it seems suitable to compare it with an alternative method used previously for studies on the metabolism of cyclophosphamide based on mass spectrometry (MS) measurements.[14] The MS method seems to be much more laborious and complicated, since it would require the use, as quantitative standards, of samples of enantiomers of 2 and its metabolites specifically labeled with deuterium or other isotopes. In our method, based on ^{31}P NMR spectroscopy, the only quantitative standards are commercially available HMPT and lanthanide shift reagents. However, this method requires much more voluminous samples of body fluids than the MS method and, hence, is practically limited to the analysis of organophosphorus urine metabolites.

The "active" metabolites of ifosfamide, such as 3, 4, or 7, which were not found in urine by the ^{31}P NMR method, can be, after specific derivatization, determined in human plasma, blood, or urine by gas chromatography[15] or fluorimetry.[4]

Experimental Section

All solvents were reagent grade and were dried over molecular sieves before use. Melting points and boiling points are uncorrected. ^{31}P NMR spectra were recorded at 24.3 MHz with a JEOL C-60H spectrometer working in the FT mode equipped with hetero-spin decoupler SNH-SD-HC. Positive chemical-shift values (parts per million) are reported for compounds absorbing at lower fields than the external standard, 85% H_3PO_4. Mass spectra were obtained on a LKB 2091 spectrometer at 70-eV ionizing energy. Optical activity measurements were made with a Perkin-Elmer 241 MC photopolarimeter. Product purities were determined from integrated ^{31}P NMR spectra and TLC (silica gel 60, F_{254}). The R_f values refer to solvent mixtures used for chromatographic separation. The accuracy of ^{31}P NMR measurements was calculated to be ±5%.

(2*S*)-2-[(2-Chloroethyl)amino]-3-(2-chloroethyl)tetrahydro-2*H*-1,3,2-oxazaphosphorin-4-one 2-Oxide [4-Ketoifosfamide, (*S*)-5]. Compound (−)-(*S*)-2 (200 mg, 0.77 mmol; $[\alpha]^{25}_D$ −45.2°) was dissolved in 18 mL of water containing $FeSO_4$ (2.9 mmol). The solution was cooled in an ice bath and treated dropwise with stirring under an argon atmosphere with a solution of H_2O_2 (3.6 mmol) in water (2 mL). The mixture was stirred for 1 h at 0 °C. The solution (pH 3–4) was extracted four times with chloroform (20 mL). The extract was dried over $MgSO_4$ and concentrated. The residue (75 mg) was purified by preparative TLC with chloroform–acetone (1:1, v/v) as a developing system. Compound (*S*)-5 was isolated as a thick, colorless oil in 5% yield (12.1 mg): $[\alpha]^{25}_D$ −25.5° (*c* 1.2, MeOH); TLC R_f 0.52; ^{31}P NMR δ 6.5 ($CHCl_3$); MS, *m/z* 274 ($M^{+\cdot}$, 1.5), 239 (15), 225 (100). The product was contaminated with ~5% of an unidentified substance: ^{31}P NMR ($CHCl_3$) δ 8.8.

The ^{31}P NMR spectrum of racemic 5 run in the presence of Pr(tfc)$_3$ [molar ratio of 5/Pr(tfc)$_3$, 1:0.5] in C_6H_6 solution (2 mL, 0.07 mol/L) revealed the presence of two signals at $\delta_{(-)}$ 8.7 and $\delta_{(+)}$ 7.2 in a 1:1 ratio. The ^{31}P NMR spectrum of (−)-(*S*)-5 (0.04 mol/L, other conditions as above) indicated the presence of a single peak at δ 8.5. Addition of racemic 5 caused an increase of intensity of the peak at δ 8.5 and the appearance of a second signal at δ 7.4.

2-Chloro-3-(2-chloroethyl)tetrahydro-2*H*-1,3,2-oxazaphosphorine 2-Oxide (10). Into a solution of 3-(ethylenimino)-1-propanol (5.0 g, 0.049 mol) and triethylamine (5.0 g, 0.049 mol) in benzene (75 mL) was added dropwise with stirring a solution of phosphoryl chloride (7.8 g, 0.051 mol) in benzene (25 mL). Stirring was continued for 4 h, precipitated triethylamine hydrochloride was filtered off, and the filtrate was concentrated. The residue (7.0 g) was purified by column chromatography on silica gel with benzene–chloroform–acetone (4:3:1, v/v) as the eluent. The product 10 (4.6 g, 41%) was obtained as thick oil: TLC R_f 0.42; ^{31}P NMR (benzene) δ 7.9; MS, *m/z* 217 ($M^{+\cdot}$, 2), 168 (100), 128 (14).

(1′*R*,2*S*)-2-[(1′-Methylbenzyl)amino]-3-(2-chloroethyl)-tetrahydro-2*H*-1,3,2-oxazaphosphorine 2-Oxide [(*R*,S_P)-11] and Its Diastereomer [(*R*,R_P)-11]. Into a solution of (*R*)-1-methylbenzylamine [2.42 g, 0.02 mol; $[\alpha]^{25}_D$ +39.8° (neat)] and triethylamine (2.02 g, 0.02 mol) in benzene (30 mL) was added dropwise, with stirring, 10 (4.36 g, 0.02 mol) dissolved in benzene (20 mL). After 15 h, the precipitated triethylamine hydrochloride was filtered off, and the filtrate was concentrated. The residue (6.8 g) was crystallized from ethyl ether. The product (4.1 g) appeared to be an equimolar mixture of (*R*,R_P)-11 and (*R*,S_P)-11 (^{31}P NMR assay), which was separated by means of column chromatography on silica gel. The mixture of hexane–chloro-

(12) The detectable amounts of 2 and its metabolites in 1 L of urine by ^{31}P NMR are at the level of 5 mg.

(13) R. W. Kinas, K. Pankiewicz, W. J. Stec, P. B. Farmer, A. B. Foster, and M. Jarman, *J. Org. Chem.*, **42**, 1650 (1977).

(14) P. J. Cox, P. B. Farmer, A. B. Foster, L. J. Griggs, M. Jarman, R. Kinas, K. Pankiewicz, and W. J. Stec, *Biomed. Mass Spectrom.*, **4**, 371 (1977).

(15) B. M. Bryant, M. Jarman, M. H. Baker, J. E. Smith, and J. F. Smyth, *Cancer Res.*, **40**, 4734 (1980).

form–*tert*-butyl alcohol (4:2:1, v/v) was used as eluent. The "faster" migrating isomer (R,S_P)-**11** [1.2 g (39% yield); mp 97–98 °C; TLC R_f 0.22; ^{31}P NMR (benzene) δ 10.2; $[\alpha]^{25}_D$ +0.8° (*c* 3.3, MeOH); MS, *m/z* 302 ($M^{+\cdot}$, 4), 287 (21), 253 (20), 225 (100), 120 (57)], as well as the "slower" migrating isomer (R,R_P)-**11** [1,2 g (39% yield); mp 93–94 °C; TLC R_f 0.18; ^{31}P NMR (benzene) δ 9.8; $[\alpha]^{25}_D$ +72.8° (*c* 2.2, MeOH); MS, *m/z* 302 ($M^{+\cdot}$, 0.6), 287 (4.5), 253 (4), 225 (100), 120 (15)] were obtained in the form of white crystals.

(2*R*)-2-Amino-3-(2-chloroethyl)tetrahydro-2*H*-1,3,2-oxazaphosphorine 2-Oxide [(*R*)-9] and Its Enantiomer [(*S*)-9]. Compound (R,S_P)-**11** (1.0 g, 3.3 mmol; $[\alpha]^{25}_D$ +0.8°) was dissolved in EtOH (40 mL) and, after addition of 10% Pd/C (0.5 g), then hydrogenated at room temperature under normal pressure. The progress of C–N bond splitting was followed by means of TLC. When (R,S_P)-**11** disappeared (3–4 days), the catalyst was filtered off, solvent was evaporated, and the residue was purified on a silica gel column with chloroform–ethanol (9:1, v/v) as eluent. Compound (*S*)-**9** was obtained in 47% yield (0.3 g): mp 103–104 °C; $[\alpha]^{25}_D$ −18.0° (*c* 3.0, MeOH); ^{31}P NMR (chloroform) δ 12.4; TLC R_f 0.20; MS, *m/z* 198 ($M^{+\cdot}$, 2), 149 (100), 120 (6).

Hydrogenolysis of (R,R_P)-**11** (0.8 g, 2.6 mmol; $[\alpha]^{25}_D$ +72.8°) was performed in analogous manner, giving compound (*R*)-**9** (0.25 g, 48%): $[\alpha]^{25}_D$ +16.4° (*c* 3.0, MeOH); other parameters identical with those described for (*S*)-**9**.

The optical purity of enantiomeric **9** was checked by means of ^{31}P NMR spectroscopy. Spectra were taken in $CDCl_3$ at a concentration of 0.07 mol/L in the presence of Eu(tfc)$_3$. The proportions (*S*)-**9** or (*R*)-**9**/shift reagent were 1:0.4. Induced chemical-shift values were as follows: $\delta_{(-)}$ −29.5, $\delta_{(+)}$ −31.7 ppm. The enantiomer (−)-(*S*)-**9**, $[\alpha]^{25}_D$ −18.0°, was optically pure, and the purity of (+)-(*R*)-**9**, $[\alpha]^{25}_D$ +16.4°, was about 95%. Racemic **9** was obtained in 62% yield by the procedure described by Takamizawa et al.[3]

(2*S*)-2-[(Chloroacetyl)amino]-3-(2-chloroethyl)tetrahydro-2*H*-1,3,2-oxazaphosphorine 2-Oxide [(*S*)-12]. Into a solution of (*S*)-**9** (198.5 mg, 1 mmol; $[\alpha]^{25}_D$ −18.0°) in 8 mL of THF was added dropwise a solution of chloroacetyl chloride (0.17 g, 1.5 mmol) in 5 mL of THF. The solution was stored for 12 h at room temperature and concentrated. The residue was dissolved in 10 mL of $CHCl_3$ and washed with a 5% solution of Na_2CO_3 (5 mL) and then twice with water (5 mL). After the solution was dried over anhydrous $MgSO_4$, the solvent was evaporated. The colorless, oily liquid [140 mg; $[\alpha]^{25}_D$ −16.6° (*c* 2.1, MeOH); TLC R_f 0.32 (chloroform–ethanol, 18:1); ^{31}P NMR ($CHCl_3$) δ −0.2; MS, *m/z* 274 ($M^{+\cdot}$, 1.7), 225 (6), 150 (100), 42 (34)] was obtained in 50% yield.

(2*S*)-2-[(2-Chloroethyl)amino]-3-(2-chloroethyl)tetrahydro-2*H*-1,3,2-oxazaphosphorine 2-Oxide [Ifosfamide, (*S*)-2]. Into a solution of (*S*)-**12** (80 mg, 0.29 mmol; $[\alpha]^{25}_D$ −16.6°) in THF (2 mL) was added dropwise, under argon, at 0 °C a solution of B_2H_6 (0.3 mmol) in 1 mL of THF. The solution was left for 12 h at room temperature, and methanol (0.5 mL) was added. The solvent was then evaporated, and the residue was purified by preparative TLC (silica gel) with chloroform–ethanol (18:1) as the eluent. The oily residue was crystallized from ethyl ether to give (*S*)-**2** (21 mg, 28%) in the form of white crystals: mp 61–62 °C; $[\alpha]^{25}_D$ −45.0° (*c* 1.2, MeOH); ^{31}P NMR (MeOH) δ 13.8; MS, *m/z* 260 ($M^{+\cdot}$, 1), 211 (100), 134 (36)].

Benzyl 3-[[Bis(aziridinyl)phosphinyl]oxy]propanoate (14). A solution of benzyl 2-*O*-(phosphorodichloro)propionate (**13**; 2.97 g, 10 mmol) in 25 mL of ether was added dropwise with stirring at 5–10 °C into a solution of aziridine (0.86 g, 20 mmol) and Et_3N (2.4 g, 24 mmol) in 20 mL of ether. The mixture was stirred at room temperature for 2 h, the precipitated Et_3N·HCl was filtered off, and filtrate was concentrated. The residue (3.1 g) was chromatographed on a column filled with silica gel with chloroform–ethanol (18:1) as eluent, giving the product in the form of an oily liquid: yield 2.3 g; TLC R_f 0.35 (chloroform-ethanol, 18:1); ^{31}P NMR (ether) δ 29.0; MS, *m/z* 310 ($M^{+\cdot}$, 5), 149 (15), 91 (100).

2-(Benzyloxycarbonyl)ethyl *N,N'*-Bis(2-chloroethyl)-phosphorodiamidate (15). Compound **14** (1.55 g, 5 mmol) was dissolved in 20 mL of ether. A solution of HCl in ether was added dropwise with stirring to the point where excess HCl could be detected with a wet pH paper. The solvent was evaporated, and crude product (1.7 g) was purified by column chromatography with chloroform–ethanol (18:1) as eluent to give **15** as an oily liquid: yield 1.55 g (81%); TLC R_f 0.27; ^{31}P NMR (MeOH) δ 15.4; MS, *m/z* 382 ($M^{+\cdot}$, 7.7), 384 ($M^{+\cdot}$ + 2, 5.5), 304 (14), 306 (5), 91 (100).

2-(Benzyloxycarbonyl)ethyl Phosphorodichloridate (Carboxyifosfamide, 6). Compound **15** (0.96 g, 2.5 mmol) was dissolved in 30 mL of 96% ethanol. Palladium on charcoal (10%, 100 mg) was added, and the mixture was stirred under an H_2 atmosphere for 1.5 h. The catalyst was filtered off, and the solvent was evaporated to give **6** as a colorless oil: yield 0.7 g (96%); TLC (silica gel) R_f 0.16 (chloroform–ethanol, 18:1, v/v); TLC (cellulose) R_f 0.50 (2-propanol–concentrated aqueous ammonia, 7:1, v/v); ^{31}P NMR (H_2O) δ 17.5. The structure of **6** was further proved by its benzylation, leading to **15**.

Compound **6** (146 mg, 0.5 mmol) was dissolved in 2 mL of methanol. A solution of phenyldiazomethane in ether was added dropwise with stirring until a permanent, orange color was observed. The solvent was evaporated, and the residue (0.4 g) was chromatographed on a short silica gel column, eluting with chloroform–ethanol (18:1), to give benzyl ester **15** as an oily liquid: yield 130 mg (70%); TLC R_f 0.27; ^{31}P NMR (MeOH) δ 15.4.

Benzyl Phosphorodichloridate (16). A solution of $P(O)Cl_3$ (15.3 g, 0.1 mol) in 100 mL of benzene was added dropwise, with stirring, at 5–10 °C to a solution of benzyl alcohol (10.8 g, 0.1 mol) and Et_3N (10.1 g, 0.1 mol) in 50 mL of benzene. The mixture was stirred at room temperature for 1 h and the precipitated Et_3N·HCl was filtered off. After evaporation of the solvent, crude **16** was obtained as a pale, oily liquid: ^{31}P NMR (benzene) δ 7.7; MS, *m/z* 126 (18), 128 (6), 91 (100). An attempt to distill crude **16** at 0.01 mmHg caused its decomposition.

Benzyl *N,N'*-Bis(ethylene)phosphorodiamidate (17). Into a solution of freshly prepared **16** (0.05 mol) in 100 mL of benzene was added dropwise, with stirring, at 5–10 °C a solution of aziridine (4.73 g, 0.11 mol) in 50 mL of benzene. The mixture was stirred at room temperature for 0.5 h. The precipitated Et_3N·HCl was filtered off, and the filtrate was washed twice with H_2O (100-mL portions). The solution was dried over anhydrous $MgSO_4$ and evaporated. The crude product was purified by means of column chromatography with chloroform–ethanol (18:1, v/v) as eluent. Compound **17** was obtained as a colorless liquid: yield 8.4 g (71%); TLC R_f 0.38; ^{31}P NMR ($CDCl_3$) δ 30.4; MS, *m/z* 238 ($M^{+\cdot}$, 2), 126 (16), 128 (5), 91 (100).

Benzyl *N,N'*-Bis(2-chloroethyl)phosphorodiamidate (18). Compound **17** (2.38 g, 0.01 mol) was dissolved in 30 mL of ether. A solution of HCl in ether was added dropwise with stirring to the point where the excess HCl could be detected by a wet pH paper. The solvent was evaporated, and crude product was purified by means of column chromatography with chloroform–ethanol (18:1, v/v) as eluent. Compound **18** was obtained as a colorless liquid: yield 2.8 g (90%); TLC R_f 0.31; ^{31}P NMR ($CDCl_3$) δ 15.4; MS, *m/z* 311 ($M^{+\cdot}$, 5.6), 313 ($M^{+\cdot}$ + 2, 4.3), 261 (8), 263 (3), 91 (100).

***N,N'*-Bis(2-chloroethyl)phosphorodiamidic Acid (IPAM, 7).** Compound **18** (0.93 g, 3 mmol) was dissolved in 60 mL of 96% ethanol. Palladium on charcoal (10%, 60 mg) was added, and the mixture was stirred under H_2 atmosphere for 0.5 h. The catalyst was filtered off, and the solvent was evaporated. A white, crystalline residue was washed with ether–methanol (5:1, v/v) and dried under vacuum. Compound **7** was obtained in the form of tiny, white crystals: yield 0.57 g (86%); mp 106–107 °C; ^{31}P NMR (D_2O) δ 4.3; TLC (cellulose) R_f 0.48 (2-propanol–concentrated aqueous ammonia, 7:1, v/v). The structure of **7** was additionally proved by its transformation into benzyl ester **18**. Compound **7** (110 mg, 0.5 mmol) was suspended in MeOH (2 mL). A solution of phenyldiazomethane in ether was added dropwise with stirring until a permanent orange color was observed. The solvent was evaporated, and the residue (0.3 g) was chromatographed on a short column with chloroform–ethanol (18:1, v/v) as eluent to give **18** (79 mg, 50%) as a colorless liquid: ^{31}P NMR ($CDCl_3$) δ 15.4. The identity of **7** was further confirmed by comparison with a sample of IPAM prepared by different procedure[16] and kindly delivered by Professor Struck, of the Southern

(16) R. F. Struck, private communication.

Research Institute, Birmingham, AL.

Acknowledgment. This project was supported by a grant to the Polish Academy of Sciences, Center of Molecular and Macromolecular Studies, from the National Cancer Program (PR-6, 2204). The authors are indebted to Professor N. Brock of Asta-Werke AG, Bielefield, Federal Republic of Germany, for the generous gift of Holoxan.

Registry No. (±)-**2**, 84711-20-6; (*S*)-**2**, 66849-33-0; (*R*)-**2**, 66849-34-1; (*S*)-**5**, 84681-42-5; **6**, 53459-52-2; **7**, 31645-39-3; (*R*)-**8**, 72578-69-9; (*S*)-**8**, 72578-70-2; (*R*)-**9**, 83802-21-5; (*S*)-**9**, 83802-22-6; (±)-**10**, 81485-04-3; (R,S_P)-**11**, 84681-43-6; (R,R_P)-**11**, 84681-44-7; (*S*)-**12**, 84681-45-8; **13**, 84681-46-9; **14**, 84681-47-0; **15**, 66046-57-9; **16**, 52692-02-1; **17**, 84681-48-1; **18**, 84681-49-2; 3-(ethylenimino)-1-propanol, 31190-87-1; phosphoryl chloride, 10025-87-3; (*R*)-1-methylbenzylamine, 3886-69-9; chloroacetyl chloride, 79-04-9; aziridine, 151-56-4.

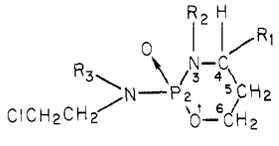
Activated Cyclophosphamide Anticancer Drugs: Molecular Structures of *cis*- and *trans*-4-Hydroperoxyisophosphamides[1]

Arthur Camerman,*[2a] H. Warren Smith,[2a] and Norman Camerman[2b]

Departments of Medicine (Neurology) and Pharmacology, University of Washington, Seattle, Washington 98195, and Department of Biochemistry, University of Toronto, Toronto, Ontario, M5S 1A8 Canada. *Received August 4, 1982*

Molecular structures of two stereoisomers of 4-hydroperoxyisophosphamide (HPIPA) have been determined by single-crystal X-ray diffraction. These isomers are active cytostatic agents closely related to an active metabolite of the antitumor drug isophosphamide, an analogue of cyclophosphamide. Both isomers crystallize in monoclinic space group $P2_1/c$ with cell dimensions for *cis*-HPIPA of a = 8.999 (2), b = 8.743 (2), c = 17.078 (4) Å; β = 107.91 (2)°, and Z = 4 molecules per unit cell, and cell dimensions for *trans*-HPIPA of a = 15.184 (3), b = 10.345 (3), c = 18.205 (3) Å, β = 114.15 (1)°, and Z = 8. The structures were solved by direct methods and refined by anisotropic least squares to a discrepancy index R = 0.048 for *cis*-HPIPA and R = 0.065 for *trans*-HPIPA. In both isomers the 4-hydroperoxy group is situated axial to the ring. The phosphoryl oxygen atom is situated axial to the ring and, thus, cis to the C(4) oxygen in the *cis*-HPIPA isomer. In the *trans*-HPIPA isomer, the phosphoryl oxygen is equatorial to the ring and trans to the C(4) oxygen.

Cyclophosphamide (CPA, 1), one of the most extensively



1 (CPA), $R_1 = R_2 = H$; $R_3 = CH_2CH_2Cl$
2 (IPA), $R_1 = R_3 = H$; $R_2 = CH_2CH_2Cl$
3 (HPIPA), $R_1 = OOH$; $R_2 = CH_2CH_2Cl$; $R_3 = H$
4 (HPCPA), $R_1 = OOH$; $R_2 = H$; $R_3 = CH_2CH_2Cl$

used chemotherapeutic agents in the treatment of many types of cancer, has been the focus of research efforts aimed at understanding its mode of action and at developing analogues with improved function. One of the potentially clinically useful drugs that has resulted from this search is isophosphamide (IPA, 2), which has one of the alkylating groups moved from the exocyclic nitrogen to the ring nitrogen.

The first step in the activation of CPA (and its analogues, including IPA) is monooxidation by the mixed-function oxidases of liver microsomes to form the 4-hydroxy derivative. The 4-hydroxy derivative is further metabolized by one of two competing pathways: (1) toxification, apparently by spontaneous chemical decomposition, to yield acrolein and phosphoramide mustard,[3] which is likely the ultimate cytostatic agent, or (2) detoxification by the enzymatic formation of 4-ketocyclophosphamide or carboxyphosphoramide. Thus, both formation of active metabolites of CPA's and detoxification (important for normal cells) involve enzymatic reactions at C(4); the stereochemistry at this position may therefore be an important consideration for improved specificity and selectivity in the design of cyclophosphamide analogues.

The chemistry and biochemistry of the 4-hydroxy and 4-hydroperoxy derivatives of IPA have been shown to be analogous to the corresponding derivatives of CPA: both compounds are cytostatically active; as with CPA, 4-hydroperoxy isophosphamide is readily converted in vivo to the 4-hydroxy derivative, but the 4-hydroperoxy compound is chemically the more stable of the two. It has been found that 4-hydroperoxyisophosphamide (HPIPA, 3) can exist in two epimeric forms in acidic solution; the two forms have been identified as the major and minor products obtained in the ozonalysis synthesis of HPIPA.[4] Both epimers display in vitro and in vivo cytotoxic activity. We have previously determined the crystal structure of 4-hydroperoxycyclophosphamide (HPCPA, 4)[5] and found that the mustard group in that molecule is situated equatorial and the hydroperoxy is situated axial on the heterocyclic ring. It is therefore of interest to determine the configuration at the phosphorus and C(4) atoms in the two active epimers of HPIPA. We now report the crystal and molecular structure determination of these two isomers as a further step toward understanding the relationship between stereochemistry and activity in this important group of drugs.

Experimental Section

The two IPA derivatives are hereafter referred to as *cis* and *trans*-HPIPA; this nomenclature describes the relationship between the phosphoryl oxygen and oxygen atoms attached to C(4), as revealed by the structure determination.

Crystals of *cis*- and *trans*-HPIPA were supplied by Dr. A. Takamizawa. The *cis*-HPIPA crystals were irregularly shaped, thick tablets, which were found by photographic methods to be

(1) Presented in part at the Symposium on the Metabolism and Mechanism of Action of Cyclophosphamide, London, July 10–12, 1975.
(2) (a) University of Washington. (b) University of Toronto.
(3) T. A. Connors, P. J. Cox, P. B. Farmer, A. B. Foster, and M. Jarman, *Biochem. Pharmacol.*, **23**, 115–129 (1974).
(4) A. Takamizawa, T. Iwata, K. Yamaguchi, O. Shiratori, M. Harada, Y. Tochina, and S. Matsumoto, *Cancer Treat. Rep.*, **60**, 361–368 (1976).
(5) A. Camerman, H. W. Smith, and N. Camerman, *Acta Crystallogr., Sect. B*, **33**, 678–683 (1977).

0022-2623/83/1826-0679$01.50/0