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Isolation of *cis*- and *trans*-4-Methylcyclophosphamide and Antitumor Evaluation *in Vivo*[1]

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SUMMARY

4-Methylcyclophosphamide was synthesized and separated into *cis* and *trans* isomers by column chromatography. Isolation of these isomers permitted individual evaluation against murine leukemia L1210 *in vivo* and assessment of possible differences in antileukemic activity. Results indicate no appreciable difference in activity of the isomers, suggesting essentially equal facility for activation by mouse liver microsomes *in vivo*.

INTRODUCTION

A synthesis program in our laboratory has resulted in the preparation and antitumor evaluation of several "preactivated" derivatives of cyclophosphamide and isophosphamide (12, 13). Upon our initiation of synthesis of related analogs of 4-methylcyclophosphamide, Feil and Lamoureux (5) reported spectroscopic evidence for the detection of *cis* and *trans* isomers of this cyclophosphamide derivative. Consequently, we undertook the separation of the isomers in order to permit evaluation of each isomer separately against the experimental tumor L1210 leukemia. Such evaluation could reveal an enzymatic preference for 1 of the isomers, thereby suggesting structural features that might enhance or inhibit activation of this type of structure *in vivo* by the mixed-function oxidase of mouse liver cells. This is the 1st report of biological evaluation of 4- substituted isomers of cyclophosphamide *in vivo*.

MATERIALS AND METHODS

Thin-Layer Chromatography. Thin-layer chromatography was performed on Analtech (Newark, Del.) precoated silica gel G plates (250 μm thick) in acetone:chloroform (3:1). 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).

Instrumentation. Mass spectral analysis was performed with a Hitachi Model RMU-6D mass spectrometer (Perkin-Elmer Corp., Norwalk, Conn.) and NMR[2] (both ^{1}H and ^{13}C) measurements were performed with a Varian XL-100-15 spectrometer (Varian, Inc., Palo Alto, Calif.).

Evaluation against L1210 Leukemia. 4-Methylcyclophosphamide isomers were administered i.p. in 0.9% NaCl solution on the 1st day of innoculation of 10^5 leukemia cells in C57BL × DBA/2 (hereafter called $BD2F_1$) mice; 6 mice were used for each dose.

RESULTS

4-Methylcyclophosphamide was prepared as described by Feil and Lamoureux (5). The crude product was separated into its 2 isomers by column chromatography. Fractions containing a single isomer, as indicated by thin-layer chromatography, were combined and evaporated. Both isomers crystallized on standing at room temperature. Trituration of the faster-migrating isomer with cold ethanol and of the slower-migrating isomer with cold ether, followed by filtration, gave white crystalline solids with melting points of 72-74° and 102°, respectively. Thin-layer chromatography indicated that each isomer was uncontaminated with the other, R_F 0.57 and R_F 0.48, respectively. Elemental analysis (carbon, hydrogen, nitrogen) of each isomer was as follows: 35.14, 6.19, 10.24 (fast) and 35.03, 6.08, 10.08 (slow); 34.94, 6.23, 10.19 (theory).

The assignment of configuration to the 2 isomers (Chart 1) was based on a consideration of carbon-phosphorus coupling constants obtained from their ^{13}C NMR spectra. We assumed that the P → O bond is axial, and the bulky bis(2-chloroethyl)amino group is equatorial. This has, indeed, been shown to be the case in the solid state for cyclophosphamide (III) (7) and for 4-ketocyclophosphamide (2) and 4-peroxycyclophosphamide (8) by X-ray crystallography. Since an equatorial methyl group at C-4 would be expected to exert a smaller change on the ring

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[2] The abbreviations used are: NMR, nuclear magnetic resonance; LD_{50} and LD_{10}, doses of drug required to kill 50% and 10%, respectively, of test animals.

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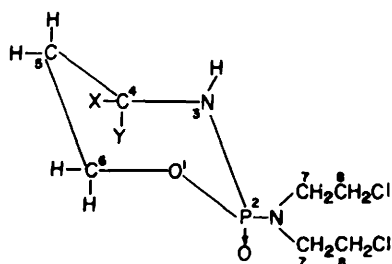

I (trans-) X = CH_3, Y = H
II (cis-) X = H, Y = CH_3
III X = H, Y = H

Chart 1. Cyclophosphamide and *cis-* and *trans*-4-methylcyclophosphamide.

bond angles than an axial 4-methyl, we expected the carbon-phosphorus couplings of C-4 and C-5 of the faster-migrating *trans* isomer, I, to be more like those of cyclophosphamide than the corresponding couplings of the slower-migrating *cis* isomer, II, would be. This appears to be substantiated by the data, and the assignments were made.

Chemical shifts and the appropriate carbon-phosphorus coupling constants are given in Table 1 for the 2 4-methylcyclophosphamides and for cyclophosphamide itself. Assignments of the individual peaks were confirmed in all cases by the ^{1}H-coupled spectra.

Except in the methyl region, the ^{1}H NMR spectra were less informative because of very complex, overlapping multiplets. Our spectra of the separated isomers appear to be in fair agreement with the methyl data of Feil and Lamoureux (5) obtained on a mixture. Chemical shifts and methyl coupling constants are listed in Table 2.

Mass spectral analysis of the isomers indicated significant differences between the 2 (Table 3), thus permitting isomer differentiation by this method. The *trans* isomer exhibits a higher ratio of relative intensitites of the M—CH_3:M fragments than does the *cis* isomer. Application of this observation to the 4-ethoxy derivatives reported by Connors *et al.* (3) and Cox *et al.* (4) suggests the possibility that the fast product in each case may be of *trans* configuration.

Table 1

^{13}C NMR spectra of cis- and trans-4-methylcyclophosphamide

Spectra were determined in the pulsed Fourier transform mode on a Varian XL-100-15 NMR spectrometer operating at 25.16 MHz and equipped with a Digilab NMR-3 data system. Solutions were of 100 mg compound per 0.4 ml $CDCl_3$. Chemical shifts are in ppm downfield from internal tetramethylsilane on solutions in $CDCl_3$ and are correct to ±0.02 ppm.

	Compound					
	I		II		III	
Carbon	δ	J_{CP} (Hz)	δ	J_{CP} (Hz)	δ	J_{CP} (Hz)
4	48.35	2.7[a]	48.44	3.7	41.48	2.4
5	33.53	7.3	33.70	3.7	25.84	6.1
6	66.47	7.3	66.42	7.3	67.78	6.1
7	49.05	4.9	49.19	4.9	48.95	4.9
8	42.21	2.4	42.40	NR[b]	42.33	1.2
CH_3	23.19	7.3	24.11	12.2		

[a] Coupling constants are correct to ±0.3 for I and II and to ±0.6 for III.
[b] NR, not resolved.

Table 3

Relative abundance of selected fragments in the mass spectra of cis- and trans-4-methylcyclophosphamide

m/e	No. of Cl groups	Relative abundance: *cis*-isomer	Relative abundance: *trans*-isomer	Proposed structure
274	2	2	1	M
259	2	0.3	0.7	M—CH_3
239	1	3	4	M—Cl
225	1	100	54	M—CH_2Cl
211	1	2	2	M—CH_2CH_2Cl
197	1	9	5	*m/e*225—CH_2CH_2 or *m/e*239—CH_3CHCH_2
163	0	8	6	*m/e*225—CH_2CH_2Cl + H
134	0	60	65	M—$N(CH_2CH_2Cl)_2$
106	1	38	56	$CH_2CH_2NCH_2CH_2Cl$ + H
92	1	29	47	$CH_2NCH_2CH_2Cl$ + H
70	0	44	100	$NCH(CH_3)CH_2CH_2$

Table 2

^{1}H NMR spectra of cis- and trans-4-methylcyclophosphamide

Spectra were determined on a Varian XL-100-15 NMR spectrometer operating at 100 MHz. Solutions were of 20 mg compound per 0.4 ml $CDCl_3$. Chemical shifts are in ppm downfield from internal tetramethylsilane and are estimated to be accurate to ±0.01 ppm.

	Compound			
	I		II	
	δ	J (Hz)	δ	J (Hz)
CH_3 on C_4	1.29	$^4J_{PNCCH}$ = 1.95[a] $^3J_{HCCH}$ = 6.45	1.19	$^4J_{PNCCH}$ = 2.85 $^3J_{HCCH}$ = 6.25
H_5	1.5-2.0		1.5-1.9	
H_7, H_8, H_4	3.1-3.9		3.2-4.2	
H_6	4.0-4.6		4.0-4.6	

[a] Coupling constants are correct to ±0.1.

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Tentative identifications of certain fragments observed in the spectra are listed in Table 3.

The *cis* isomer (NSC 241531) and the *trans* isomer (NSC 241532) were evaluated against an inoculum of 10^5 L1210 leukemia cells at doses of 200 and 100 mg/kg. The higher dose of both isomers was toxic ($>LD_{10}$), and the lower dose gave an increase in life-span of 45% and 48%, respectively. The average day of death of the control animals was 10.1 days. Cyclophosphamide administered at a dose of 300 mg/kg (LD_{10} = 312 mg/kg) routinely produces approximately 70% 30-day survivors and an increase in life-span of approximately 250% of the nonsurvivors under identical conditions (11).

DISCUSSION

Arnold *et al.* (1) synthesized numerous ring-alkylated analogs of cyclophosphamide many years ago, but only recently Feil and Lamoreux (5) demonstrated the existence of *cis* and *trans* isomers of this type of derivative. Because of the interest in the metabolism of cyclophosphamide and its analogs, biological evaluation of both *cis* and *trans* isomers of a representative of this type of structure could provide information on a stereochemical preference for activation by the mixed function oxidase of liver microsomes. Although evaluation of any of the alkyl isomers of both endo- and exocyclic-substituted analogs would be an aid to our understanding of the metabolic process of oxidation and consequent or subsequent generation, spontaneously or enzymatically, of the important alkylating product, analogs substituted on C_4 of the oxazaphosphorine ring, the important site of enzymatic oxidation, would be expected to influence the critical enzymatic reaction more directly. We, therefore, separated 4-methylcyclophosphamide into its *cis* and *trans* isomers. The existence of *cis*- and *trans*-4-ethoxycyclophosphamide (3) and *cis* and *trans*-4-ethoxy-6-methylcyclophosphamide (4) has been reported. The *cis*,-*trans*-designation in the latter case probably refers, by analogy with cyclophosphamide, to the 4-ethoxy substituents and not to the 6-methyl substituents; the added asymmetric center would allow for 4-*cis*-6-*cis*, 4-*cis*-6-*trans*, 4-*trans*-6-*cis*, and 4-*trans*-6-*trans* isomers, provided the P → O bond remains in the axial position.

Evaluation of *cis*- and *trans*-4-methylcyclophosphamide against L1210 leukemia *in vivo* failed to reveal any difference between the 2, thus indicating that both axial and equatorial configurations of the 4-methyl substituent have a similar effect on liver microsomal oxidation of C_4 in the cyclophosphamide ring. Both isomers are less active than cyclophosphamide against this experimental tumor system but are more toxic to the host. Cox *et al.* (4) have suggested that this increased toxicity results from the inability of normal tissue to detoxify the intermediate 4-hydroxy metabolite or its acyclic analog; resistance to further oxidation was nicely demonstrated by its stability in aqueous permanganate. Consequently, degradation *in vivo* to phosphoramide mustard (4, 6, 15) and methyl vinyl ketone (4, 15) would be anticipated. An interesting difference is noted between BALB/c mice used by Cox *et al.* (4) and $BD2F_1$ mice used in our experiments; whereas the toxic dose of the isomeric mixture of 4-methylcyclophosphamide is greater than that of cyclophosphamide in BALB/c mice, the reverse is true for either isomer of 4-methylcyclophosphamide in $BD2F_1$ mice. The same order of toxicity was observed in sheep (5) and in rats (1) as we observed in $BD2F_1$ mice. Administration of isolated 4-hydroxy-4-methylcyclophosphamide resulted in a smaller LD_{50}, suggesting less efficient oxidation of 4-methylcyclophosphamide *in vivo* (4).

The observation of similar activity against L1210 leukemia by both *cis*- and *trans*-4-methylcyclophosphamide is not only consistent with a similar ease of enzymatic oxidation but also of similar rates of degradation of the intermediate 4-hydroxy metabolites. Indeed, Cox *et al.* (4) observed a single product, the acyclic tautomer of 4-hydroxy-4-methylcyclophosphamide, from both chemical and enzymatic oxidation of 4-methylcyclophosphamide, indicating the extreme instability of the cyclic structure. Such a property appears to be in direct contrast to cyclophosphamide itself, where 4-hydroxycyclophosphamide can be isolated (14), but aldophosphamide, the acyclic isomer, apparently cannot, being characterized physically only transiently (10, 14) or as a stabilized derivative (9). A report to the contrary has appeared (16), but no definitive physical data (infrared, NMR, mass spectral) for the existence of aldophosphamide itself were included; consequently, it seems probable that the product described as aldophosphamide may be 1 of the *cis* or *trans* isomers of 4-hydroxycyclophosphamide, being analogous, therefore, to the recently reported ethoxy derivatives (3, 4).

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