was precipitated by addition of water. The crude product (410 mg) was twice reprecipitated from DMF–ethanol to give the acyl octapeptide Ib (302 mg, 50.7% based upon initial glycine content of the resin): mp 206–208 °C dec; TLC R_f (E) 0.45, R_f (F) 0.63; $[\alpha]^{24}_D$ −43.1° (c 1, DMF). Anal. ($C_{73}H_{94}N_{14}O_{14}S_3$) C, H, N. Amino acid analysis:[27,29] Tyr, 0.79; Phe, 1.01; Glu, 1.03; Asp, 1.04; Cys(Bzl), 0.97; Pro, 1.03; Arg, 0.99; Gly, 1.00; NH_3, 2.95.

β-(*S*-Benzylmercapto)-β,β-cyclopentamethylenepropionyl-Tyr(Bzl)-Phe-Gln-Asn-Cys(Bzl)-Pro-Arg(Tos)-Gly-NH_2 (II). Boc-Tyr(Bzl)-Phe-Gln-Asn-Cys(Bzl)-Pro-Arg(Tos)Gly-resin[30] (1.46 g, 0.5 mmol) was converted to the acyl octapeptide resin (1.53 g, weight gain 70 mg, 95.9% of theory) in one cycle of deprotection, neutralization, and coupling with *p*-nitrophenyl β-(*S*-benzylmercapto)-β,β-cyclopentamethylenepropionate.[6] The resin was ammonolyzed[16] and the product extracted with dimethylformamide (DMF). The solvent was evaporated in vacuo, and the residue was precipitated by addition of water. The crude product (723 mg) was reprecipitated from DMF–ethanol and DMF–2% aqueous AcOH (488 mg; 62.4% based upon initial Gly content on the resin): mp 183–185 °C; TLC R_f (E) 0.38, R_f (D) 0.41; $[\alpha]^{23}_D$ −32.9° (c 1, DMF). Anal. ($C_{79}H_{98}N_{14}O_{14}S_3$) C, H, N. Amino acid analysis:[27] Tyr, 0.97; Phe, 1.02; Glu, 1.05; Asp, 1.01; Cys(Bzl), 0.98; Pro, 1.04; Arg, 0.98; Gly, 1.00; NH_3, 2.95.

[1-(β-Mercapto-β,β-cyclopentamethylenepropionic acid),2-(*O*-methyl)tyrosine]arginine-vasopressin (IIIa). A solution of the protected nonapeptide amide Ia (170 mg, 0.114 mmol) in sodium-dried and redistilled ammonia (400 mL) was treated at the boiling point and with stirring with sodium[22] from a stick of the metal contained in a small-bore glass tube until a light-blue color persisted in the solution for 30 s. Dry glacial acetic acid (0.4 mL) was added to discharge the color. The solution was evaporated and the residue was dissolved in aqueous acetic acid (0.2%; 800 mL), and this solution was treated with 2 M ammonium hydroxide solution to give a solution of pH 7.5. An excess of a solution of potassium ferricyanide (0.01 M, 11.4 mL)[4] was added gradually with stirring. The yellow solution was stirred for a further 90 min and for 1 h with anion-exchange resin (Bio-Rad AG-3, Cl^- form, 10 g damp weight). The suspension was slowly filtered through a bed of resin (80 g damp weight). The bed was washed with aqueous acetic acid (0.2%; 300 mL),[3] and the combined filtrate and washings were lyophilized. The resulting powder (1386 mg) was desalted on a Sephadex G-15 column (110 × 2.7 cm) eluting with aqueous acetic acid (50%)[23] with a flow rate 4 mL/h. The eluate was fractioned and monitored for absorbance of 280 mm. The fractions comprising the major peak were pooled and lyophilized, and the residue (55.5 mg) was further subjected to gel filtration on a Sephadex G-15 column (100 × 1.5 cm) eluting with aqueous acetic acid (0.2 M) with a flow rate of 2.5 mL/h.[23] The peptide was eluted in a single peak (absorbance 280 mm). Lyophilization of the pertinent fractions yielded the vasopressin analogue (49 mg, 37.3%): TLC R_f (E) 0.19, R_f (F) 0.30; $[\alpha]^{22}_D$ −59.6° (c 0.19, 1 M AcOH). Amino acid analysis:[27,29] Tyr, 0.81; Phe, 1.01; Glu, 1.04; Asp, 0.98; Pro, 1.04; Arg, 0.95; Gly, 1.00; NH_3, 3.10. Analysis following performic acid oxidation prior to hydrolysis[31] gave a Cys(O_3H)–Gly ratio of 1.03:1.00.

[1-(β-Mercapto-β,β-cyclopentamethylenepropionic acid),2-(*O*-methyl)tyrosine]arginine-vasopressin (IIIb). Treatment of the acyl octapeptide Ib (160 mg, 0.107 mmol) as detailed for IIIa yielded the analogue IIIb (64 mg, 51.7%), indistinguishable from preparation IIIa by TLC: $[\alpha]^{23}_D$ −59.1° (c 0.5, 1 M AcOH). Amino acid analysis:[27,29] Tyr, 0.80; Phe, 1.02; Glu, 1.02; Asp, 0.98; Pro, 1.03; Arg, 0.96; Gly, 1.00; NH_3, 3.05. Analysis following performic acid oxidation prior to hydrolysis[31] gave a Cys(O_3H)–Gly ratio of 1.02:1.00.

[1-(β-Mercapto-β,β-cyclopentamethylenepropionic acid)]arginine-vasopressin (IV). Treatment of the acyl octapeptide II (173 mg, 0.111 mmol) as detailed above for IIIa yielded the analogue IV (66 mg, 52.5%): TLC R_f (E) 0.19, R_f (F) 0.43; $[\alpha]^{23}_D$ −58.7° (c 0.5, 1 M AcOH). Amino acid analysis:[27] Tyr, 0.96; Phe, 0.98; Glu, 1.01; Asp, 1.01; Pro, 1.05; Gly, 1.00; NH_3, 2.95. Analysis following performic acid oxidation prior to hydrolysis[31] gave a Cys(O_3H)–Gly ratio of 1.01:1.00.

Measurement of Duration of Antivasopressor Action of d(CH_2)$_5$Tyr(Me)AVP (Figure 1). Rats were prepared as for routine vasopressor assays[32] with urethane anesthesia and phenoxybenzamine. After repeated vasopressor responses to intravenous injections of 2 milliunits of the USP posterior pituitary reference standard were found to remain stable, the antagonist, d(CH_2)$_5$Tyr(Me)AVP, was injected in the "effective dose" corresponding to the previously estimated pA_2. The 2 milliunit dose of standard was injected 1, 10, and 30 min later and each 30 min thereafter over a total period of 4 h. This dose of standard is equivalent in vasopressor activity to 5 ng of AVP or 25 ng/kg.

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Synthesis of Syn and Anti Isomers of 6-[[(Hydroxyimino)phenyl]methyl]-1-[(1-methylethyl)sulfonyl]-1*H*-benzimidazol-2-amine. Inhibitors of Rhinovirus Multiplication

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The synthesis and antirhinovirus activity of syn and anti isomers of 6-[[(hydroxyimino)phenyl]methyl]-1-[(1-methylethyl)sulfonyl]-1*H*-benzimidazol-2-amine (**4** and **5**) are reported. The structural assignments of **4** and **5** are based upon ^{13}C NMR spectra of both isomers and also X-ray analysis of **5**. The anti-isomer **5** was more potent than the syn-isomer **4** when compared as an inhibitor of rhinovirus multiplication in vitro. Both isomers inhibited multiplication of 15 different serotypes of rhinovirus.

Early reports by Thompson[1] and Brown[2] of the inhibition of virus multiplication by benzimidazole stimulated the synthesis and evaluation of a wide variety of benzimidazole analogues. Many of these compounds had no

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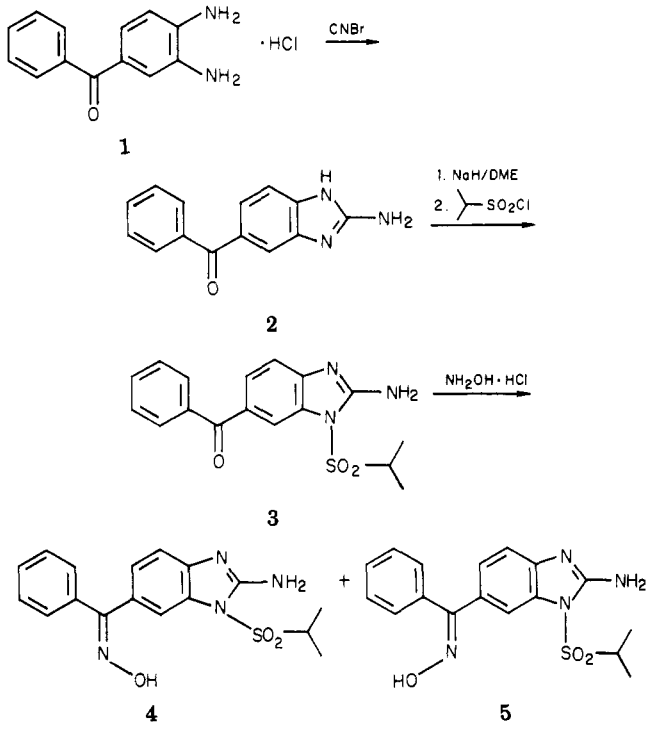
0022-2623/80/1823-0368$01.00/0

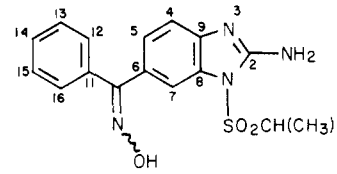
Scheme I



effect on virus multiplication. Tamm et al.[3] in 1954 reported the inhibition of influenza virus by 5,6-dichloro-1-(α-D-ribofuranosyl)benzimidazole, but later studies by Diwan et al.[4] indicated that this activity was due to a direct effect on the host cell. Hollinshead and Smith[5] discovered the activity of 2-(α-hydroxybenzyl)benzimidazole (HBB) which has been proven to be a virus-specific inhibitor[6,7] and to induce or to select resistant mutants also indicating a virus-specific action.[8] O'Sullivan and Wallis[9] reported the evaluation of 1,2-bis(2-benzimidazolyl)-1,2-ethanediol. This was followed by the synthesis and evaluation of (*S,S*)-1,2-bis(5-methoxy-2-benzimidazolyl)-1,2-ethanediol by Akihama et al.[10] and Schleicher et al.[11] This compound was found to have a broad spectrum of activity against picornaviruses and to be a good inhibitor of rhinovirus multiplication in tissue culture and in chimpanzees.[12]

The present study reports the preparation, identification, and evaluation of the syn and anti isomers of 6-[[(hydroxyimino)phenyl]methyl]-1-[(1-methylethyl)-sulfonyl]-1*H*-benzimidazol-2-amine, **4** and **5** (LY122771 and LY122772), which are virus-specific inhibitors of picornavirus multiplication.[13-15] The compounds show a high degree of activity in tissue culture. Tissue levels exceeding the amount required for virus inhibition have been achieved in experimental animals after oral administration.[16] Separation of the isomers has achieved a six- to tenfold difference between the isomers in the tissue culture therapeutic ratios for rhinovirus-infected cells. The anti isomer had the higher therapeutic ratio. The compounds described here appear to be unique from previously described antiviral benzimidazole analogues, since all three substitutions in the 1, 2, and 6 positions were required for activity.[13]

Table I. ^{13}C NMR Assignments for the *syn*- and *anti*-Oximes[a]



carbon	syn	anti	
2	154.9	155.2	Ss[b]
4	115.2	115.2	Ds
5	124.9	120.0	Dd
6	125.7	128.2	Sd
7	113.0	109.9	Dd
8	128.6	129.1	Sd
9	142.4	143.1	Sq
11	137.3	133.7	S-
12, 16	128.1	128.8	D-
13, 15	127.3	127.9	D-
14	130.8	131.4	D-
C=N	153.4	153.5	S-
CH	55.7	55.6	D-
CH_3	15.6	15.6	Q-

[a] The samples were run in Me_2SO using Me_4Si as an internal reference. [b] Gated decoupling patterns with capital letters representing one-bond coupling pattern and small letters representing three-bond coupling patterns. A hyphen means the long-range couplings were too complex to be usable or not observed because of overlap.

Chemistry. Compounds **4** and **5**, designated *syn*- and *anti*-oxime isomers, respectively, were synthesized from the ketone **3** and hydroxylamine hydrochloride (Scheme I). The synthesis of the ketobenzimidazole was accomplished by the method of Leonard et al.[17] from the diamine monohydrochloride **1** and cyanogen bromide. The resultant benzimidazole was sulfonated by a modification of the method described by Price et al.[18] using isopropylsulfonyl chloride and sodium hydride in dimethoxyethane.

The oxime isomers **4** and **5** were separable by either high-performance liquid chromatography or by fractional crystallization. The latter method was preferred to yield large quantities of the isomers. Isomer separation from the crude product was accomplished from a mixture of

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Table IV. Detection of the Quantity of 4 and 5 to Reduce Plaque Formation by Rhinovirus Type 20 in HeLa Cells

compd		level of compd mcg/mL of agar overlay								
		3.0	1.5	0.75	0.35	0.17	0.08	0.04	0.02	0.00
4	no. of plaque	0	0	0	49	87	127	ND[a]	ND	153
	% reduction	100	100	100	67	50	16	ND	ND	0
5	no. of plaque			0	0	0	0	18	68	153
	% reduction			100	100	100	100	87	55	0

[a] Not done.

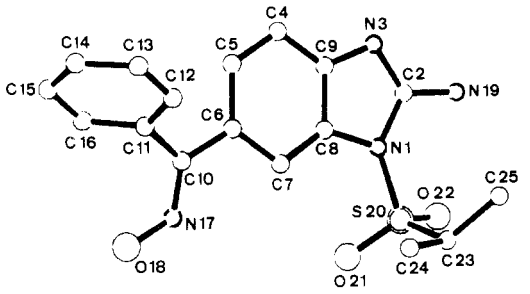

Figure 1. ORTEP plot of 5.

acetone and water. The anti isomer 5 was recrystallized from acetonitrile for final purification, whereas the syn isomer 4 was recrystallized from methanol.

Structure Identification. NMR Results. The differences in the ^1H NMR spectra were not conclusive in determining which isomer was syn and which isomer was anti. However, there were differences in the ^{13}C NMR spectra which lead to the configurational assignments of the two benzimidazoles. The ^{13}C NMR chemical shifts, assignments, and coupling patterns are summarized in Table I.

The ability to distinguish between *syn*- and *anti*-oximes lies in the shielding effects of the oxime oxygen on the α carbon when the oxygen is syn to that carbon.[20] In the situation reported here, carbon-6 in the syn isomer appeared at 125.7 ppm, upfield by 2.5 ppm from carbon-6 in the anti isomer. For the anti isomer, carbon-11 appeared at 133.7 ppm, which was 3.6 ppm upfield from carbon-11 in the syn isomer. There were also significant differences in chemical shifts for carbon-5 and -7; however, these differences are not fully understood at present and may be due in part to slight differences in conformations of the two compounds.

X-ray Results. The crystal structure of 5 was determined by X-ray diffraction. As shown in Figure 1, the oxime is in the designated anti configuration. The atomic coordinates and temperature factors (Table II) and the bond distances and bond angles (Table III) are included in the supplementary material.

Biological Evaluation. Plaque-Reduction Assays. The results obtained with 4 and 5 in a plaque-reduction assay with rhinovirus type 20 are summarized in Table IV. Both compounds completely inhibited plaque production at higher concentrations, and then at lower levels there was a dose-related reduction in activity. With rhinovirus type 20, compound 5, the anti isomer, inhibited plaque formation by 50% (ID_{50}) at a level which was tenfold lower than the amount required for 4, the syn isomer.

Similar studies were made to compare the activity of the two isomers against 14 additional types of rhinovirus. The results are summarized in Table V. The ID_{50} values summarized were determined by a plaque-reduction assay with the indicated rhinovirus type. Average values of 0.18 μg/mL of 4 and about 0.02 μg/mL of 5 were obtained with the 15 types of rhinovirus. There was at least a sixfold difference in the amount of each isomer required to inhibit plaque formation with different types of rhinovirus. A portion of this difference may have been due to test and biological variation, but additional studies indicated that some types of rhinovirus were more sensitive than others. Although there was a difference in ID_{50} values, the data indicated that all 15 types were inhibited by both 4 and 5.

Table V. Activity of 4 and 5 in Preventing Rhinovirus Plaque Formation in HeLa Cells

serotype of rhinovirus	amount to prevent plaque formation by 50% mcg/mL of agar overlay	
	4	5
3	0.37	0.04
14	0.36	0.03
15	0.14	0.02
16	0.16	0.02
20	0.19	0.02
32	0.13	0.01
33	0.10	0.01
37	0.25	0.04
48	0.28	0.03
50	0.12	<0.01
61	0.07	0.01
65	0.07	<0.01
69	0.08	0.01
70	0.06	<0.01
72	0.30	0.03

Therapeutic Ratio in Tissue Culture. When the two isomers were compared in assays to determine their effect on HeLa cell colony plating efficiency, both isomers allowed plating efficiency equal to the controls when present at concentrations of 8 μg/mL and lower. Levels of 24 μg/mL and higher reduced plating efficiency. The fact that the anti isomer required less compound to inhibit rhinovirus replication but had essentially equivalent toxicity indicated an increase in therapeutic ratio due to separation of the isomers.

Discussion

Compounds 4 and 5 are potent inhibitors of a broad selection of types of rhinovirus in vitro. In addition to the inhibition of the 15 rhinovirus types detailed in this paper, DeLong reported inhibition of virus multiplication with 43 of 43 rhinovirus types tested.[13] It is highly probable that these compounds may be effective against all known types of rhinovirus. Since clinical diagnosis of type and strain of rhinovirus is difficult, this broad effectiveness would be an important factor in a useful drug. In addition to inhibiting a broad spectrum of rhinoviruses, the compounds were reported to inhibit all other picornaviruses tested.[13]

Comparison of the antiviral activity of the syn and anti isomers indicated consistent maximum activity with the anti configuration. We believe compounds 4 and 5 possess

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unique structural characteristics with respect to other antiviral benzimidazoles, since proper substitution was required in the 1, 2, and 6 positions of the benzimidazole nucleus.[13]

In summary, these compounds have the following desirable traits: (1) a broad spectrum of antiviral activity in vitro against picornavirus; (2) a virus-specific mode of action;[14] (3) a high therapeutic index in vitro; (4) oral absorption to produce blood and lung levels of compound greater than the ID_{50} values in mice and dogs;[16] and (5) activity in rhinovirus-infected human organ culture.[21] We believe these compounds represent potentially useful antiviral agents in human chemotherapy. In depth studies with each isomer are currently in progress.

Experimental Section

Melting points were obtained in a capillary tube using a Mel-Temp apparatus and were uncorrected. ^{13}C NMR spectra were recorded on a JEOL PFT 100 spectrometer. ^1H NMR spectra were recorded on a Varian HA 100 spectrometer with Me_4Si as an internal standard. UV spectra were recorded on a Cary Model 15 spectrometer in MeOH solutions. Mass spectra were recorded on a Hitachi Perkin-Elmer RMU-6 interfaced with System 150 data handling system (Systems Industries). Analytical high-pressure LC separation was performed using a Waters Model 6000A solvent delivery system with a Varian Vari-chrom detector. The analytical high-pressure LC column was a Waters μBondapak C_{18}, 3.9 mm × 30 cm. Elemental analyses were performed as indicated, and the results were within ±0.4% of the theoretical values.

(2-Amino-1*H*-benzimidazol-5-yl)phenylmethanone (2). Compound **2** was prepared by the method of Leonard et al.[17] from cyanogen bromide and 3,4-diaminobenzophenone, mp 174–176 °C. Anal. ($C_{14}H_{11}N_3O$) C, H, N.

2-Amino-1-(isopropylsulfonyl)-6-benzimidazolyl Phenyl Ketone (3). To a stirred solution of 1 L of dimethoxyethane and 127 g (0.53 mol) of **2** was added portionwise 26 g of NaH as a 50% oil dispersion. After the solution was stirred for 1 h at room temperature, 80 g (0.56 mol) of $(CH_3)_2CHSO_2Cl$ in 200 mL of solvent was added, and the reaction mixture was heated to reflux for 4 h. The mixture was cooled to room temperature and filtered, and the filtrate was concentrated under reduced pressure to dryness. The residue was slurried with a mixture of H_2O and hexane. The resulting solid was collected by filtration and dried to yield 156 g of crude material. Recrystallization from EtOAc (carbon), twice, yielded 36 g (20%) of **3**: mp 185–186 °C; NMR (Me_2SO-d_6) δ 1.4 (6 H, d, CH_3 isopropyl), 4.0 (1 H, m, methine isopropyl), 7.3–8.1 (10 H, m, aromatics and NH_2); UV (MeOH) λ_{210} (ε 34 000), λ_{245} (ε 17 654), λ_{316} (ε 19 016). Anal. ($C_{17}H_{17}N_3O_3S$) C, H, N.

***syn*- and *anti*-6-[[(Hydroxyimino)phenyl]methyl]-1-[(1-methylethyl)sulfonyl]-1*H*-benzimidazol-2-amine (4 and 5).** A solution of 72 g (0.2 mol) of the benzimidazole ketone **3** and 42 g (0.6 mol) of hydroxylamine hydrochloride in 3400 mL MeOH was heated at reflux for 24 h. The solvent was distilled at atmospheric pressure to 25% of the original volume. Heating was stopped and to the distillation residue was added with vigorous stirring 1000 mL of H_2O, 1000 mL of pH 7 buffer (Fisher reagent), and 1000 mL of 50% pH 7 buffer/H_2O. This mixture was allowed to cool to room temperature. After the solution was stirred overnight, the product was collected by filtration and washed with H_2O to yield 36.5 g (48%) of a mixture of **4** and **5** as a white solid: mp 121–126 °C; NMR (Me_2SO-d_6) δ 1.30 (6 H, d, CH_3 isopropyl), 3.88 (1 H, m, methine isopropyl), 7.0–8.0 (10 H, m, aromatics and NH_2), 11.21 and 11.35 (1 H, br d, OH); UV (MeOH) λ_{210} (ε 34 400), λ_{254} (ε 14 200), λ_{295} (ε 15 700); EIMS 358, 342, 253, 252, 235, 103, 76, 64; high-pressure LC isomer ratio 45:55 anti/syn.

High-Pressure LC Methods. Analytical separation was achieved using a μBondapak C_{18} silica gel column with a mobile phase consisting of a mixture of 56% MeOH and 44% H_2O. The flow rate was 200 mL/h. The isomer ratio was obtained by setting the variable-wavelength detector at 272 nm, at which point the isomers exhibited equimolar absorptivities. Direct comparison of the computerized peak area of each isomer yielded the ratio. The retention times of the syn and anti isomers were 9.1 and 10.6 min, respectively.

The preparative separation was accomplished using a Waters Prep 500 high-pressure LC with a Prepack C_{18} cartridge. The mobile phase consisted of a mixture of 56% MeOH and 44% H_2O. Typically, 1.5 g of the isomeric mixture was applied to the column in a solution of the eluant; then, by means of recycling, cutting, and shaving the eluting material, a separation of the isomers was achieved. The solvent volume of the collected fractions was reduced under vacuum, and the precipitated solid was collected by filtration. Analysis by high-pressure LC of the collected anti isomer indicated an isomer ratio of 94:6 anti/syn and of the collected syn isomer a ratio of 9:91 anti/syn. Crystallization of the anti isomer from CH_3CN yielded material containing an isomer ratio by high-pressure LC of 98.1:1.9 anti/syn: mp 198–199 °C; NMR (Me_2SO-d_6) δ 1.30 (6 H, d, CH_3 isopropyl), 3.88 (1 H, m, methine isopropyl), 7.0–8.0 (10 H, m, aromatics and NH_2), 11.21 (1 H, br s, OH); UV (MeOH) λ_{218} (ε 45 600), λ_{290} (ε 27 100). Anal. ($C_{17}H_{18}N_4O_3S$) C, H, N.

Crystallization of the syn isomer from MeOH yielded material having an isomer ratio of 0.4:99.6 anti/syn: mp 182–183 °C; NMR (Me_2SO-d_6) δ 1.30 (6 H, d, CH_3 isopropyl), 3.88 (1 H, m, methine isopropyl), 7.0–8.0 (10 H, m, aromatics and NH_2), 11.35 (1 H, br s, OH); UV (MeOH) λ_{254} (ε 20 800), λ_{285} (ε 13 200). Anal. ($C_{17}H_{18}N_4O_3S$) C, H, N.

Crystallization Method. A slurry of 100 g of the oxime mixture **4** and **5** in 2000 mL of CH_3COCH_3 was warmed on a steam bath to obtain a complete solution. The solution was treated with carbon, filtered, and allowed to cool to room temperature. After the solution was cooled to room temperature, 2800 mL of H_2O was added to the filtrate with vigorous stirring for 30 min. The resultant precipitate was collected by suction filtration and dried. Analysis by high-pressure LC of this material indicated an isomer ratio of 93.6:6.4 anti/syn (49 g). Concentration under reduced pressure of the aqueous filtrate to remove the CH_3COCH_3 resulted in the precipitation of a second crop, which was collected by filtration. Analysis by high-pressure LC indicated an isomer ratio of 22.2:77.8 anti/syn (50 g).

Recrystallization of 25 g of the anti isomer from 1600 mL of CH_3CN yielded 18.2 g (98.3:1.7 anti/syn). Recrystallization of 50 g of the syn isomer from 500 mL of MeOH yielded 16.5 g (3.9:96.1 anti/syn).

Plaque-Reduction Tests. Rhinovirus-susceptible HeLa cells were grown in 25-cm^2 Flacon flasks at 37 °C in Medium 199 with 5% inactivated fetal bovine serum (FBS), penicillin (150 units/mL), and streptomycin (150 μg/mL). When confluent monolayers were formed, growth medium was removed and 0.3 mL of an appropriate dilution of virus was added to each flask. After absorption for 1 h at room temperature, the infected cell sheet was overlaid with equal parts of 1% Agarose and 2× minimum essential medium with Earle's salts with 2.5% FBS, penicillin, and streptomycin containing various concentrations of **4** or **5**. The compounds were dissolved in Me_2SO at 10 000 μg/mL and then an aliquot was diluted to the desired concentration with the agar medium mixture. All flasks were incubated at 32 °C until control flasks indicated optimum plaque size (2–10 mm). A 10% formalin–2% sodium acetate solution was added to each flask to inactivate the virus and fix the cell sheet to the plastic surface. The plaques were counted after staining the surrounding cell areas with Crystal violet. Results from duplicate flasks at each concentration were averaged. The inhibition of plaque formation by 50% (ID_{50}) was estimated by plotting all results from 10 to 90% inhibition.

Cell Colony Plating Efficiency. Rhinovirus-susceptible HeLa cells were diluted in media containing varying amounts or no compound to a dilution producing distinct colonies of cells. Microtiter trays were used and the trays allowed the examination of a wide range of cell numbers and compound concentrations. Cells and compounds were mixed in suspension before cells were allowed to attach to the plastic surface. The compounds remained in contact with cells throughout the experiment. Cell growth was studied at 37 °C using Medium 199 with 5% FBS, penicillin, and streptomycin. Compound concentrations which allowed colony formation equivalent to controls were used to evaluate therapeutic

(21) Donald C. DeLong and Sylvia E. Reed, unpublished results.

ratios. We feel this is the most critical evaluation of cell toxicity available.

Viruses. Rhinovirus types were obtained from the Research Resources Branch of the NIAID. The viruses were passed in WI-38 cells in our laboratory and titered in HeLa cells using plaque formation.

X-ray Structure Determination. The monohydrate of **5** crystallizes from methanol–water as colorless, highly refractive polyhedra in the centrosymmetric monoclinic space group, $P2_1/n$, with four molecules in a unit cell having the dimensions: a = 16.400 ± 0.003 Å; b = 10.045 ± 0.003 Å; c = 11.187 ± 0.003 Å; β = 106.32 ± 0.02. The density calculated for $C_{17}H_{18}N_4O_3S\cdot H_2O$ (M_r 376.4) is 1.41 g cm^{-3}, and the density observed by flotation is 1.42 g cm^{-3}. The intensities of 2576 reflections, of which 170 were considered unobserved, were measured on a four-angle automated diffractometer, using monochromatic copper radiation. The structure was solved by direct methods, using the program MULTAN. All 26 of the nonhydrogen atoms (including the oxygen of the previously unsuspected water of hydration) showed up on the first E map. The structure was refined to an R factor of 0.080 using anisotropic temperature factors for the heavy atoms and isotropic temperature factors for the hydrogen atoms, which were placed at assumed positions.

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Supplementary Material Available: Table II, atomic coordinates and U_{ij} values; Table III, bond distances and bond angles (4 pages). Ordering information is given on any current masthead page.

Synthesis and Antitumor Activity of Cyclophosphamide Analogues. 3.[1] Preparation, Molecular Structure Determination, and Anticancer Screening of Racemic *cis*- and *trans*-4-Phenylcyclophosphamide

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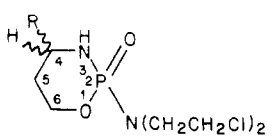
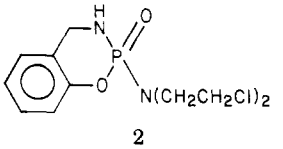
Cyclization of racemic 3-amino-3-phenyl-1-propanol with bis(2-chloroethyl)phosphoramidic dichloride gave a diastereomeric mixture of 4-phenylcyclophosphamide (**3**), which was chromatographically separated into the faster and slower eluting components. A combination of $^1H/^{31}P$ NMR and IR spectral data indicated that the faster and slower racemates correspond to *cis*-**3** (mp 129–130 °C) and *trans*-**3** (mp 112–114.5 °C), respectively. The molecular structure of the former compound was determined by X-ray crystallography and thereby unambiguously established the cis relationship between equatorially disposed phenyl and P=O substituents in a chair conformation. These results confirm the stereochemical assignments for *cis*- and *trans*-**3** which have been independently deduced by Y. E. Shih, J. S. Wang, and C. T. Chen [*Heterocycles*, **9**, 1277 (1978)]. Anticancer screening tests against L1210 lymphoid leukemia in mice have revealed that, while both diastereomers of **3** afford toxic metabolites, *trans*-**3** led to therapeutic activity and *cis*-**3** did not. The relevance of these findings to results reported for 4-methylcyclophosphamide and cyclophosphamide is briefly discussed.

The clinical utility of racemic cyclophosphamide (**1**)



1, R = H
3, R = Ph
4, R = Me



2

against a relatively broad spectrum of human cancers has prompted numerous investigations regarding the metabolism, mechanism of action, and influence of structural modification upon the therapeutic efficacy of this drug.[2-4] Metabolic details for **1** are not fully understood at present; however, knowledge that enzymatic C-4 oxidation ("activation") is followed by competing toxification, detoxification, and delayed toxicity processes has allowed the conception of diverse strategies for predictably altering and/or improving chemotherapeutic activity. The consequences of lowering the oxidation potential of the C-4 position by modifying the structure of **1** has been of interest to us and led to the synthesis of 5,6-benzocyclophosphamide (**2**) as a candidate system;[5] however, lack of activity for **2** against L1210 lymphoid leukemia in mice diverted our attention to its exocyclic cognate, 4-phenylcyclophosphamide (**3**), 2-[bis(2-chloroethyl)amino]-4-phenyl-2*H*-1,3,2-oxazaphosphorinane 2-oxide.

Monosubstitution at C-4 in racemic **1** generates a second chiral center, and the resultant diastereomeric racemates, which may simply be referred to as *cis*- and *trans*-**3** (*cis* = *RS*/*SR*; *trans* = *RR*/*SS*),[6] were of further interest relative to stereochemical studies with enantiomers of **1**[7,8] and

(1) For paper 2, see S. M. Ludeman, G. Zon, and W. Egan, *J. Med. Chem.*, **22**, 151 (1979).
(2) D. L. Hill, "A Review of Cyclophosphamide", Charles C. Thomas, Springfield, Ill., 1975.
(3) O. M. Friedman, A. Myles, and M. Colvin in "Advances in Cancer Chemotherapy", A. Rosowsky, Ed., Marcel Dekker, New York, 1979, pp 143–204.
(4) M. Colvin in "Clinical Pharmacology of Anti-Neoplastic Drugs", H. M. Pinedo, Ed., Elsevier/North-Holland Biomedical Press, 1978, pp 245–261.
(5) S. M. Ludeman and G. Zon, *J. Med. Chem.*, **18**, 1251 (1975).
(6) The cis and trans relationships refer to the P=O and phenyl substituents, in accordance with IUPAC rules for nomenclature [*J. Org. Chem.*, **35**, 2849 (1970)].
(7) M. Jarman, R. A. V. Milsted, J. F. Smyth, R. W. Kinas, K. Pankiewicz, and W. J. Stec, *Cancer Res.*, **39**, 2762 (1979), and references to earlier work cited therein.

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