NH_4OH. The resulting yellow precipitate was isolated by filtration and then recrystallized from DMF–H_2O containing excess NH_4OH (charcoal). The crystals were collected on a filter, washed with acetone, and dried yielding 2.5 g: mp 332–334°. The filtrate was concentrated to 33% of the initial volume in vacuo and then made basic with concentrated NH_4OH. The resulting precipitate was separated and then recrystallized as above yielding 1.3 g: mp 332–334°. These two crops were combined yielding a total of 3.8 g (58%) (TLC in DMF). Anal. ($C_9H_{10}N_4O \cdot 1.5H_2O$) C, H, N.

Method A. Diethyl *N*-[α-(2-Amino-4-hydroxy-5-methylquinazol-6-ylimino)-*p*-toluyl]-L-glutamate (6a). A 2.85-g (0.015 mol) sample of **5a** and 5.53 g (0.0165 mol) of **4** were heated at reflux in DMSO for 6 hr in the presence of 1 g of 4Å molecular sieves. After cooling and filtering, H_2O was added to effect precipitation. The product was isolated by filtration, washed with H_2O, and then dried in vacuo, yielding 4.8 g (63%) (TLC in 1:4 DMF–EtOAc).

Method B (6b and 6d). Each of these was prepared in a similar manner except that in the case of **6b** only a 10% excess of aldehyde **4** was employed and a lower yield was obtained.

A mixture of 2.26 g (6.7 mmol) of **4**, 0.80 g (4.5 mmol) of 2,4,5-triaminoquinazoline[3] (**5d**), and 1 g of 4Å molecular sieves was boiled in 100 ml of EtOH for 18 hr. The TLC (DMF) showed that incomplete conversion to the anil had occurred. Therefore, 0.8 g (2.4 mmol) of additional **4** was added and the boiling continued for an additional 24 hr. After filtering the reaction mixture was refrigerated, causing the precipitation of a finely divided yellow solid. This was separated by filtration, washed with EtOH, and then dried in vacuo to yield 1.8 g (77%) of **6d** (TLC in DMF).

Method C (7a and 7b). These reductions were run in an identical manner except that heating was not required in the case of **7b**.

To a suspension of 2.54 g (5 mmol) of **6a** in 10 ml of glacial HOAc was added slowly with stirring 0.44 g (7.5 mmol) of dimethylamine borane in 10 ml of glacial HOAc. After stirring at ambient temperature for 2 days the reaction appeared to be incomplete by TLC so the mixture was heated to ca. 50° for 1 hr. Next, 10 ml of H_2O was added and the solution was neutralized to pH 8 with concentrated NH_4OH. The resulting solid was separated by filtration, washed with H_2O, and then dried in vacuo. Recrystallization from MeOH yielded 1.7 g (68%) of yellow crystalline **7a** (TLC in DMF–EtOAc, 1:4).

Method D (7c and 7d). A mixture of 1.5 g (8.5 mmol) of 2,6-diamino-4-hydroxyquinazoline[10] (**5c**) and 3.2 g (9.5 mmol) of **4** in 100 ml of 70% aqueous HOAc was hydrogenated in the presence of Raney nickel until H_2 uptake had ceased. TLC at this point showed that all of the quinazoline had been consumed. The solution was treated with charcoal, filtered through Celite, and then basified with concentrated NH_4OH to pH 8. The precipitate was separated by filtration, washed with H_2O, and then dried in vacuo yielding 3.9 g (93%) of **7c** as a yellow powder (TLC in DMF–MeCN, 1:3).

Method E (8a–d). Each of the hydrolysis reactions was conducted in a similar manner except that in the case of **7d**, EtOH (20%) was employed in order to improve solubility.

A suspension of 1.8 g (3.6 mmol) of **7c** in 144 ml of 0.1 *N* NaOH was stirred at ambient temperature for 3 days. Dissolution occurred gradually. The resulting solution was treated with charcoal, filtered through Celite, and then neutralized to pH 4.5 with 0.1 *N* HCl. The precipitate was isolated by filtration, washed with H_2O and acetone, and then dried in vacuo. There was obtained 1.1 g (72%) of **8c** as a light tan solid (TLC in DMF).

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Synthesis and Biologic Evaluation of Major Metabolites of *N*-(2-Chloroethyl)-*N*′-cyclohexyl-*N*-nitrosourea

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N-(2-Chloroethyl)-*N*′-(*cis*-4-hydroxycylohexyl)-*N*-nitrosourea, a major metabolite of *N*-(2-chloroethyl)-*N*′-cyclohexyl-*N*-nitrosourea (CCNU), and its trans isomer were prepared from the corresponding 4-aminocyclohexanols. A convenient and stereospecific precursor was found in 2-oxa-3-azabicyclo[2.2.2]oct-5-ene hydrochloride, hydrogenation giving pure *cis*-4-aminocyclohexanol hydrochloride. The metabolites were, at nontoxic levels, at least as active as CCNU in tests against murine leukemia L1210 implanted both intraperitoneally and intracerebrally and, on a weight basis, were more active and more toxic. These observations and previously reported metabolic studies suggest that the anticancer activity of CCNU is due primarily to its metabolites.

N-(2-Chloroethyl)-*N*′-cyclohexyl-*N*-nitrosourea (CCNU) is highly active against leukemia L1210 in mice and has proven to be a useful drug in man.[1] It has recently been shown that CCNU is rapidly metabolized by liver microsomal material from mice without loss of the cytotoxic nitrosoureido function,[2] raising the possibility that the anticancer activity of this agent is due primarily to its metabolites rather than to the drug itself. The major metabolite in mice has been identified as *N*-(2-chloroethyl)-*N*′-(*cis*-4-hydroxycyclohexyl)-*N*-nitrosourea[2] (**7**), although at least three other ring-hydroxylated derivatives are also formed.[3] Only two metabolites, **7** and the *trans*-4-hydroxy derivative **2**, have been identified in the urine of human patients treated with CCNU.[4] These results made the synthesis,

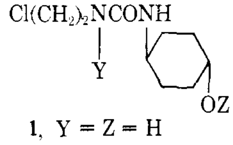
Table I. Activity of CCNU and Its 4-Hydroxy Metabolites against Leukemia L1210

Compd	Dose,[a] mg/kg	Inoculum		Median day of death	% ILS[b]	45-day survivors/ total no. of mice
		Cells	Route			
Control		10^5	ip	9		
CCNU	86	10^5	ip	18	100	7/10
	57			20	122	8/10
	38			22	144	9/10
7 (*cis*-4-OH)	90	10^5	ip	7		1/10
	60			12	33	3/10
	40			14	55	9/10
	26			15	66	8/10
	13			14	55	0/10
2 (*trans*-4-OH)	67	10^5	ip	6		0/10
	44			16.5	83	2/6
	30					6/6
	20					6/6
	13			15.5	72	4/6
Control		10^4	ic	9		
CCNU	86	10^4	ic	14	55	4/10
	57			19	111	7/10
	38			14	55	1/10
7 (*cis*-4-OH)	90	10^4	ic	7		1/10
	60			20	122	5/10
	40					10/10
	26			17	88	9/10
	13			13	44	1/10
2 (*trans*-4-OH)	67	10^4	ic	7		0/10
	44			24	166	3/10
	30					10/10
	20			17	88	8/10
	13			13	44	0/10

[a] Rx, day 1 only. [b] Of dying animals.

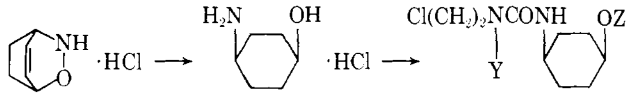
complete physicochemical characterization, and biologic evaluation of these compounds of great interest and importance.

Procedures based on the reported[5] fractionation of *cis,-trans-N-*(4-hydroxycyclohexyl)acetamide and basic hydrolysis of the separated isomers sufficed for the preparation of *trans*-4-aminocyclohexanol, from which 2 was derived. Fractionation of the (2-chloroethyl)urea prepared by the reaction of commercial *cis,trans*-4-aminocyclohexanol with 2-chloroethyl isocyanate also provided satisfactory access to 2. Failure to achieve the necessary enrichment of



1, Y = Z = H
2, Y = NO; Z = H
3, Y = Z = NO

the cis acetamide, however, prompted a search for an alternative approach. A suitable precursor of 7 was found in 2-oxa-3-azabicyclo[2.2.2]oct-5-ene hydrochloride[6] (4), the product of the condensation of 1-chloro-1-nitrosocyclohexane with 1,3-cyclohexadiene in ether and ethanol. Catalytic hydrogenation of 4 afforded pure *cis*-4-aminocyclohexanol hydrochloride (5).



4

5

6, Y = Z = H
7, Y = NO; Z = H
8, Y = Z = NO

Formic acid and concentrated hydrochloric acid, media known to control the position of nitrosation of *N*-(2-chloroethyl)-*N'*-cyclohexylurea and similarly substituted *N*-(2-chloroethyl)ureas,[7,8] proved unsatisfactory for the nitrosation of the trans urea 1 with either sodium nitrite or dinitrogen trioxide. The impurity of isolated products was attributed to unwanted reaction of the hydroxyl group, possibly formylation in one case and oxidation in the other. It was somewhat unexpected, then, that the product isolated after nitrosation of 1 with dinitrogen trioxide in a limited amount of dilute hydrochloric acid appeared (TLC, ir, ^{1}H NMR) to be homogeneous with no evidence of nonselective nitrosation seen in the ^{1}H NMR spectrum. During the nitrosation, the reaction mixture was thinned with chloroform; the product was found in the separated chloroform layer. This method applied to the nitrosation of 1 on a larger scale afforded a product contaminated with the nitrous ester 3, which was removed by methanolysis,[9] and a second impurity, possibly the *N'*-nitroso isomer, which was removed by recrystallization. Nitrosation of the cis urea 6 by the same method provided the cis nitrosourea 7 as a thick yellow oil, which was free of the nitrous ester 8 after treatment with methanol and eventually became a pasty solid in cold storage. Even the analytical sample, however, contained a small amount of contaminant, whose identity as the *N'*-nitroso isomer was indicated by ^{1}H NMR and mass spectra.

Biologic Activity. CCNU and its 4-hydroxy metabolites were evaluated against leukemia L1210 implanted both intraperitoneally and intracerebrally in mice (Table I), and their single-dose LD$_{10}$ values were determined in normal mice: CCNU, >86 mg/kg;[10] *cis*-4-hydroxy (7), 33 mg/kg;

trans-4-hydroxy (**2**), 35 mg/kg. At approximately their LD_{10} values, both the *cis*- and *trans*-4-hydroxy derivatives cured essentially all treated animals of both the ip and the ic forms of the disease and were, at nontoxic levels, at least as active as CCNU in these test systems, but on a weight basis were more effective and more toxic. These results coupled with those of Reed[2] would appear to indicate that the anticancer activity of CCNU is due primarily to its metabolites.

Experimental Section

Melting points with a range were determined with a Mel-Temp apparatus and are uncorrected; those without range with a Kofler Heizbank. Ir spectra were determined with Perkin-Elmer 521 and 621 spectrophotometers; ^1H NMR spectra with a Varian XL-100-15 spectrometer; and mass spectra with a Hitachi Perkin-Elmer RMU-6D-3 spectrometer. The ^1H NMR spectra of the nitrosoureas described here were consistent with assigned structures and were a useful indicator of the presence or absence of appreciable amounts of the corresponding *N'*-nitroso isomer.[7] GC analyses were performed on a Hewlett-Packard 5750 research chromatograph equipped with a flame ionization detector. Elemental analyses were performed, for the most part, by Galbraith Laboratories, Knoxville, Tenn.; elemental analysis of **1** and all spectral determinations were performed in the Molecular Spectroscopy Section of Southern Research Institute under the direction of Dr. W. C. Coburn, Jr.; and biological evaluation was performed in the Cancer Screening Division of Southern Research Institute under the direction of Dr. W. R. Laster, Jr. Analytical results indicated by element symbols were within ±0.4% of the theoretical values.

***N*-(*trans*-4-Hydroxycyclohexyl)acetamide.** A solution of *N*-(4-hydroxyphenyl)acetamide (25.0 g, 0.165 mol; Aldrich) in EtOH (150 ml) was hydrogenated over 5% Rh on Al_2O_3 (~5 g, Engelhard) at 50 psi and room temperature in a Parr apparatus; hydrogen absorption was complete after 4 hr. The catalyst was removed by filtration and washed with hot EtOH (50 ml); the filtrate and washings were combined and evaporated to dryness under reduced pressure. The residue (26 g, mp ~120°) was triturated in MeCN (65 ml); the air-dried insoluble solid (12.5 g, mp ~165°) was recrystallized from MeCN (75 ml) and dried in vacuo over P_2O_5: yield of the trans acetamide 8.7 g (34%), mp 165° (lit.[5,11,12] 165, 164, 165–167°); % trans 97.1 by GC [1.83 m × 6.35 mm o.d.) glass column, 10% Carbowax 20 M on 60–80 Gas Chrom Q, 195°, He flow 50 ml/min, retention times cis ~32 min, trans ~39 min]. [Successive recrystallizations of the enriched cis isomer (8.5 g, mp ~110°), which precipitated from the combined filtrates concentrated to ~45 ml, from MeCN (40 ml), acetone (45 ml), and then several times again from MeCN raised the melting point to ~130° (lit.[5,11] mp 135°); but the cis content according to GC analysis was only 74.4% and the yield impracticably low.]

***trans*-4-Aminocyclohexanol Hydrochloride.** A solution of *N*-(*trans*-4-hydroxycyclohexyl)acetamide (6.00 g, 38.2 mmol) in aqueous 10% KOH solution (50 ml) was refluxed for 5 hr, cooled, saturated with NaCl, and extracted with $CHCl_3$ (3 × 100 ml). The dried ($MgSO_4$) extracts were combined and evaporated under reduced pressure; a 2.00-g (17.4-mmol) portion of the residual *trans*-4-aminocyclohexanol [4.17 g (95%); mp ~110° (lit.[5,11] mp 110, 110–111°)] was dissolved in EtOH (10 ml)–Et_2O (20 ml) and treated with excess ethanolic hydrogen chloride solution. The precipitated hydrochloride collected from the chilled mixture and dried in vacuo over P_2O_5 (2.37 g; mp 225°) was reprecipitated from EtOH (25 ml) with Et_2O (100 ml): yield 2.02 g (74%, from the acetamide); mp 225° (lit.[11] mp 226–227°).

***N*-(2-Chloroethyl)-*N'*-(*trans*-4-hydroxycyclohexyl)urea (1). A. From *trans*-4-Aminocyclohexanol.** 2-Chloroethyl isocyanate [0.378 ml (465 mg), 4.40 mmol; Eastman] was added to a stirred solution of *trans*-4-aminocyclohexanol (507 mg, 4.40 mmol) in EtOH (20 ml) and the mixture stirred for 1 hr. The solvent was removed under reduced pressure, and the residue (960 mg, mp ~135°) was precipitated from EtOH (5 ml) with H_2O (25 ml). The product was dried in vacuo over P_2O_5: yield 350 mg (36%); mp 140°; ir (KBr) 3650–3150 (OH, NH), 1660 and 1645 (C=O), and 1560 cm^{-1} (CNH). Anal. ($C_9H_{17}ClN_2O_2$) C, H, N.

B. From *cis,trans*-4-Aminocyclohexanol. 2-Chloroethyl isocyanate (15.1 ml, 0.176 mol) was added slowly to a cold, stirred solution of *cis,trans*-4-aminocyclohexanol (20.3 g, 0.176 mol; Pfalz and Bauer) in MeCN (150 ml); precipitation started after the addition. The mixture was stirred at room temperature for 1 hr and at 5° for 15 min. The precipitate was collected, washed with cold MeCN (15 ml), dried in vacuo over P_2O_5 (wt 14.1 g, mp ~130°), recrystallized three times from minimal volumes of MeCN (with heat), and dried again in vacuo over P_2O_5: yield 7.1 g (18%); mp 140°; infrared spectrum identical with that of the authentic trans urea described above.

***N*-(2-Chloroethyl)-*N'*-(*trans*-4-hydroxycyclohexyl)-*N*-nitrosourea (2).** A stirred suspension of **1** (250 mg, 1.13 mmol) in 1 *N* HCl (1.2 ml) was diluted with H_2O (3.0 ml) and chilled to 0–5°. N_2O_3 (Matheson) was bubbled into the cold mixture intermittently during 2 hr—1 hr before and 1 hr after addition of $CHCl_3$ (5 ml), which dissolved the suspended solid. The $CHCl_3$ layer was separated, dried (Na_2SO_4), and evaporated under reduced pressure. The light-yellow residue was dissolved in EtOH (2 ml), recovered by evaporation under reduced pressure, and dried in vacuo over P_2O_5: yield 240 mg (85%); mp 120°; ir (KBr) 3650–3050 (OH, NH), 1730 and 1695 (C=O), 1540 (CNH), and 1465 cm^{-1} (N=O); ir ($CHCl_3$) 1720 cm^{-1} (C=O); mass spectrum (70 eV) *m/e* (rel intensity) 249 (0.1, M^+), 142 (9), 124 (7), 81 (100). Anal. ($C_9H_{16}ClN_3O_3$) C, H, N. The product was homogeneous by TLC (silica gel, 9:1 $CHCl_3$–MeOH, uv or ninhydrin), and the ^1H NMR spectrum ($CDCl_3$–TMS) showed no evidence of the *N'*-nitroso isomer.

Nitrosation on a larger scale [**1** (5.0 g), 1 *N* HCl (24 ml), H_2O (60 ml)] required dilution with $CHCl_3$ (100 ml) after 30 min. The product (5.5 g, mp ~100°) recovered from $CHCl_3$ had two contaminants according to TLC (silica gel, 9:1 $CHCl_3$–MeOH, ninhydrin). Purification was effected by (1) dissolving in MeOH (60 ml), stirring until gas evolution ceased (bubble counter), and evaporation under reduced pressure and (2) recrystallization of the residue (5.3 g, mp ~115°) from MeCN (20 ml): yield after drying in vacuo over P_2O_5 3.3 g (58%); mp 121°. The product was identical (ir, TLC) with the analytical sample.

***cis*-4-Aminocyclohexanol Hydrochloride (5).** A stirred solution of **4**[6] [11.0 g, 74.5 mmol; mp 149–150° (lit. mp 147–147.5°)] in MeOH (400 ml) was hydrogenated over PtO_2 (5.0 g, 84.4%; Engelhard) at atmospheric pressure until the calculated amount of H_2 had been absorbed (~1.5 hr). The catalyst was removed and washed with MeOH (2 × 25 ml). The filtrate and washings were combined and, after the addition of a saturated solution of dry HCl in Et_2O (50 ml), evaporated under reduced pressure; the residue (10.8 g, mp ~170°) was dissolved in EtOH (80 ml) and the solution filtered and diluted with MeCN (300 ml). The mixture was stirred at 0° for 20 min; the product was collected and dried in vacuo over P_2O_5: yield 7.3 g (65%); mp 180–181°. A second crop (0.5 g, mp ~175°) was obtained from the filtrate by concentration and dilution with MeCN. The two crops were homogeneous by TLC (silica gel, H_2O, ninhydrin) and identical (ir) with the thrice-recrystallized analytical sample, mp 180–182°, obtained from a pilot run. Anal. ($C_6H_{13}NO{\cdot}HCl$) C, H, N.

A solution of the second crop (480 mg, 3.17 mmol) was basified with 50% NaOH (0.5 ml) and extracted with $CHCl_3$ (2 × 35 ml). The dried (Na_2SO_4) extract was evaporated under reduced pressure and the residue recrystallized by dissolving in hot toluene (10 ml) and diluting with petroleum ether (25 ml): yield of free base, 350 mg (95%); mp 79–81° (lit.[5,11] mp 79–80, 78–80°).

***N*-(2-Chloroethyl)-*N'*-(*cis*-4-hydroxycyclohexyl)urea (6).** A stirred solution of **5** (6.00 g, 39.6 mmol) in H_2O (20 ml) was made basic with 50% NaOH solution (6 ml) and extracted with $CHCl_3$ (3 × 60 ml). The extracts were combined, dried (Na_2SO_4), and evaporated under reduced pressure. A cold (0–5°), stirred solution of the residual free base [4.32 g (95%); mp 79–80° (lit.[5] mp 79–80°); 37.5 mmol] in MeCN (100 ml) was treated with 2-chloroethyl isocyanate (3.26 ml, 38.0 mmol). Stirring was continued at 0° for 30 min and at room temperature for 1 hr. The precipitated product was collected (after further chilling) and dried in vacuo over P_2O_5: yield 7.05 g (85%); mp 111–112°. A second crop (345 mg; mp 108–110°) was obtained from the concentrated filtrate. The infrared spectra of these crops were identical with the spectrum of an analytical sample, mp 111–112°, produced by a pilot run: ir (KBr) 3650–3100 (OH, NH), 1650 (C=O), 1560 cm^{-1} (CNH). Anal. ($C_9H_{17}ClN_2O_2$) C, H, N.

***N*-(2-Chloroethyl)-*N'*-(*cis*-4-hydroxycyclohexyl)-*N*-nitrosourea (7).** A stirred suspension of **6** (5.00 g, 22.6 mmol) in 1 *N* HCl (22.6 ml) was diluted with H_2O (50 ml), chilled to 0–5°, and nitrosated with N_2O_3 as described above for the trans isomer. After 30 min, the mixture was thinned with $CHCl_3$ (75 ml) and nitrosation continued for 1 hr. The dried (Na_2SO_4) $CHCl_3$ layer was evaporated under reduced pressure. The residue was further dried in vacuo over P_2O_5, but remained a yellow oil (5.30 g): ir (KBr) 1715 (C=O), 1635 (ON=O), 1520 (CNH), and 1485 cm^{-1}

(NN=O); TLC (silica gel, 9:1 $CHCl_3$–MeOH, ninhydrin) showed two components, neither of which was the unnitrosated urea; ^1H NMR ($CDCl_3$–TMS) detected $ClCH_2CH_2NH$ attributable to the *N′*-nitroso isomer but showed much less *O*H than expected. A solution of this oil (5.00 g) in MeOH (100 ml) was stirred for 6 hr and left at 0° for 30 hr. Removal of the solvent under reduced pressure left a yellow oil, which was further dried in vacuo over P_2O_5 and eventually became a pasty solid when stored at ~0°: yield 4.52 g (85%, corrected for MeOH treatment); absence of nitrous ester shown by TLC and ir, which were identical with an analytical sample prepared similarly, but on a smaller scale; ^1H NMR showed the presence of some $ClCH_2CH_2NH$[13] but was otherwise consistent with the expected structure; ir (film) 3650–3050 (OH, NH), 1710 (C=O), 1520 (CNH), and 1485 cm^{-1} (N=O); mass spectrum (70 eV) *m/e* (rel intensity) 250 (0.02, $M^+ + 1$), 171 (0.1), 144 (1), 142 (20), 126 (0.1), 124 (17), 81 (100) (*m/e* 171, 144, and 126 could not reasonably derive from M, but could from iso-M, the *N′*-nitroso isomer—$[\text{iso-M} - Cl(CH_2)_2NH]^+$, $[\text{iso-M} - Cl(CH_2)_2NHCO + H]^+$, $[\text{iso-M} - Cl(CH_2)_2NHCO + H - H_2O]^+$). Anal. ($C_9H_{16}ClN_3O_3$) C, H, N.

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3-Substituted 2-Formylquinoxaline 1,4-Dioxides

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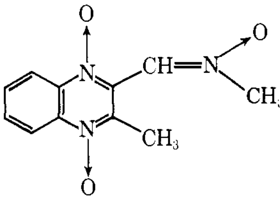
The methylnitrone of 3-methyl-1,4-dioxidoquinoxaline-2-carboxaldehyde (1) has shown exceptional antibacterial activity in vivo. Derivatives of 3-hydroxymethyl-1,4-dioxidoquinoxaline-2-carboxaldehyde and 3-acetoxymethyl-1,4-dioxidoquinoxaline-2-carboxaldehyde were prepared. Several of these compounds were found to be antibacterial agents of the same order of activity as 1.

The methylnitrone of 3-methylquinoxaline-2-carboxaldehyde (1) has shown exceptional activity against *Proteus mirabilis* and *Salmonella schottmeulleri* in experimental infections in mice.[1] The in vitro activity for 1 and its analogs is less than one would expect from their in vivo activity, suggesting the possible existence of an active metabolite.



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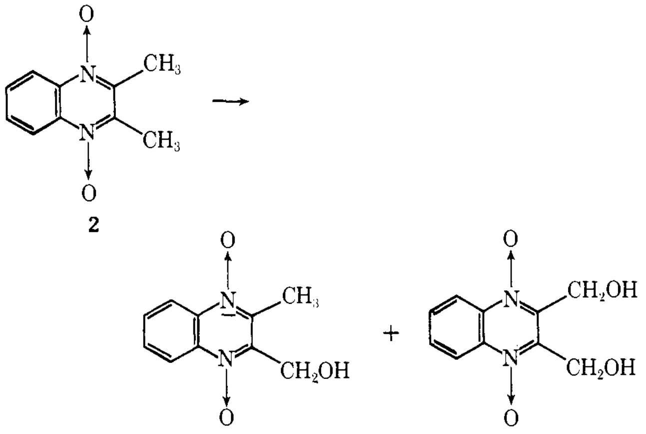
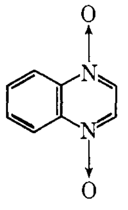


Precedent for such an active metabolite in this type of series was found in a report on 2,3-dimethylquinoxaline 1,4-dioxide.[2] As with our compounds, the in vitro activity of 2 did not correlate well with the in vivo results. An investigation of the metabolism of 2 found 3 and 4 to be active metabolites.[3] Therefore, we felt the 3-hydroxymethyl analog of 1, if not a metabolite, was likely to be an active antibacterial and its synthesis was undertaken. The 3-acetoxymethyl analogs were also prepared as it was felt that in vivo these might be hydrolyzed to the hydroxymethyl compounds. A more likely explanation for the discrepancy between the in vivo and in vitro data for 1 appeared after this work was complete.[4] The in vitro antibacterial activity of quinoxaline 1,4-di-*N*-oxide (17) (Quidoxin, Imperial Chem-



17