(120 ml), HNO_3 (*d* 1.42) (8 ml) was slowly run in at 45 ± 2°. After stirring at this temperature for a few minutes, H_2SO_4 (2 ml) was added to the reaction mixture in several portions. Stirring was continued at 55–60° for 15 min and cooled, and the product was filtered off giving 10.2 g (97%). Recrystallization from EtOH gave yellow needles, mp 308–309°. *Anal.* ($C_{15}H_8F_3N_3O_4$) C, H, N.

2-Amino-4-nitro-6(5H)-phenanthridinone (5).—A solution of KOH (1.1 g) in H_2O (5 ml) was added in one portion to a boiling suspension of **4** (3.1 g, 9 mmoles) in 95% EtOH (400 ml). The solution was boiled until crystallization of the product took place. The rest of the solvent was then driven off without heat by an air stream. The solid was triturated in H_2O and collected by filtration, 2 g (87%). Recrystallization from PhMe gave an analytical sample, mp 308–309° dec. *Anal.* ($C_{13}H_9N_3O_3$) C, H, N.

N-Acetyl derivative melted at 284–285° (AcOH). *Anal.* ($C_{15}H_{11}N_3O_4$) C, H, N.

4-Nitro-6(5H)-phenanthridinone (2b).—Deamination of **5** with H_3PO_2 (50%) gave yellow needles (C_6H_6–EtOH), mp 259–260° (lit.[9] mp 264–265°). *Anal.* ($C_{13}H_8N_2O_3$) C, H, N.

4-Amino-6(5H)-phenanthridinone (6). By Rearrangement of 9-Oxofluoren-1-amine.—Saturated aqueous NaN_3 (20 g) was added dropwise to a stirred and ice-cooled mixture of 9-oxofluoren-1-amine[10] (30 g) and H_2SO_4 (200 ml) over a period of 2.5 hr. After 22 hr of stirring at ambient temperature the reaction mixture was diluted with ice–water (200 ml). The amine sulfate was collected, treated with excess 5% NaOH, and the product, 28 g (87%), was recrystallized from EtOH giving lustrous crystals, mp 311.5–312.5°. *Anal.* ($C_{13}H_{10}N_2O$) C, H, N.

By Reduction of 2b.—A suspension of **2b** (1.4 g), 85% $N_2H_4 \cdot H_2O$ (3 ml), and 5% Pd-C (50 mg) in EtOH (100 ml) was gently refluxed for 5 hr and filtered, and the filtrate was concentrated giving 0.9 g, melting point and mixture melting point with the above compound showed no depression.

2,4-Diamino-6(5H)-phenanthridinone.—Reduction of **5** the same way as described above gave the diamine (70%), mp 310–311°. *Anal.* ($C_{13}H_{11}N_3O$) C, H, N.

Conversion of 1-Iodo-9-oxofluorene Oxime to 1-Iodo-9-oxofluorene in PPA.—1-Iodo-9-oxofluorene oxime[3] (0.5 g) was mixed with PPA (25 g). The mixture was heated at 125–130° for 15 min, cooled, and diluted (H_2O). The yellow solid was recrystallized from EtOH and then chromatographed in C_6H_6 through an alumina column giving 0.3 g of 1-iodo-9-oxofluorene[10,11] (melting point and mixture melting point).

Conversion of 1-Nitro-9-oxofluorene Oxime to 1-Nitro-9-oxofluorene in PPA.—Similarly 1-nitro-9-oxofluorene oxime (0.5 g) and PPA (25 g) were heated at 120–125° for 15 min and treated with H_2O. After chromatography on alumina (C_6H_6), 1-nitro-9-oxofluorene[12] (melting point and mixture melting point) was obtained.

4-Iodo-6(5H)-phenanthridinone (2a).—Saturated aqueous $NaNO_2$ (3.5 g, 0.05 mole) was added portionwise to a stirred mixture of **6** (6.3 g, 0.03 mole), H_2SO_4 (60 ml), and H_2O (120 ml) at 5–10° (15 min). After stirring at 0–5° for 1.5 hr, excess HNO_2 was destroyed by means of urea (1.2 g). A cold (5°) solution of KI (48 g), I_2 (24 g), and H_2O (50 ml) was then added all at once to the diazotization mixture, which was allowed to stand overnight, heated for 15 min on a steam bath, and diluted with H_2O. The product was filtered off and treated with dilute $Na_2S_2O_3$ giving 7.6 g (83.5%). Chromatography on alumina with C_6H_6 as eluent gave lustrous platelets, mp 243–244°. *Anal.* ($C_{13}H_8INO$) C, H, I, N.

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Potential Carcinolytic Agents. VII. Substituted Bis(2-methanesulfonoxyethyl)anilines[1a]

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New nuclear-substituted (3-acetamido, -amino, -carbethoxy, -chloro, -fluoro, -methyl, and -trifluoromethyl and 4-amino, -nitro, and -nitroso) bis(2-methanesulfonoxyethyl)anilines have been prepared by (1) N-hydroxyethylation of an appropriately ring-substituted aniline with ethylene oxide, (2) esterification of the hydroxyl groups with methanesulfonyl chloride, and (3) further ring substitution (nitrosation or nitration). The compounds were evaluated for antitumor activity and the pertinent results are reported. N,N-Bis(2-methanesulfonoxyethyl)-*p*-nitrosoaniline reported previously is still the most active compound in the series.

Earlier we reported[2,3] the high antitumor activity of N,N-bis(2-methanesulfonoxyethyl)-*p*-nitrosoaniline (**20**) against a variety of animal tumors. The most significant activity of **20** was shown against Walker carcinosarcoma 256 (intramuscular), Dunning leukemia (ascites), and against the cytoxan- and thiopurine-resistant strains of Dunning leukemia (ascites). It was also effective against intracerebral Dunning leukemia and had an ED_{50} in the order of 10^{-4} μg/ml in KB and L1210 cell cultures. In a mitotic index study using L1210 cell culture, the compound was found to be a potent inhibitor of cell division.[4] Preclinical toxicology studies of **20** unfortunately showed that dogs and monkeys developed leukopenia and congestive heart failure at doses of about 0.25 mg/kg.[5]

Chemistry.—Because of the interesting biological properties of **20** and in the hope of finding a compound of even higher activity, we undertook a program to synthesize a series of related compounds. These were prepared *via* the straightforward route illustrated in Scheme I. The substituted anilines I were hydroxyethylated with ethylene oxide[6,7] to the N,N-bis(2-

(1) (a) Presented in part at the First Northeast Regional Meeting of the American Chemical Society, Boston, Mass., Oct 13–15, 1968. The work was sponsored by the Cancer Chemotherapy National Service Center, National Cancer Institute, National Institutes of Health, Contract No. PH-43-66-502. Paper VI: Z. B. Papanastassiou, R. J. Bruni, and E. White, V, *Experientia*, **24**, 325 (1968). (b) Deceased. (c) To whom inquiries should be addressed.

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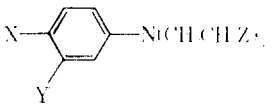
TABLE I

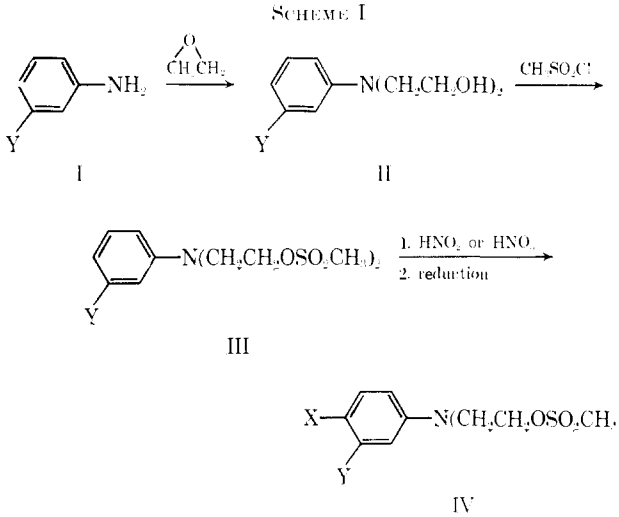
N,N-BIS(2-HYDROXYETHYL)- AND -2-METHANESULFONOXYETHYL)ANILINES



Compd	X	Y	Z	Over-all yield, %	Mp or bp (mm), °C	Recrystn solvent[a]	Formula	Analyses
1	H	F	OH	74	162 (0.2)		$C_{10}H_{14}FNO_2$	H; C[b]
2	H	$COOC_2H_5$	OH	90	213 (0.4)		$C_{13}H_{19}NO_4$	C, H
3	H	Cl	OMs	46	60–61.5	D–H	$C_{12}H_{18}ClNO_6S_2$	C, H, N
4	H	F	OMs	74	Oil		$C_{12}H_{18}FNO_6S_2$	H; C[c]
5	H	$NHCOCH_3$	OMs	36	95–97	D–H	$C_{14}H_{22}N_2O_7S_2$	C, H; N[d]
6	H	NO_2	OMs	82	110.5–111.5	D	$C_{12}H_{18}N_2O_8S_2$	C, H
7	H	CF_3	OMs	41	54–56	C–F	$C_{13}H_{18}F_3NO_6S_2$	C, H
8	H	$COOC_2H_5$	OMs	72	80–81.5	C–H	$C_{15}H_{23}NO_8S_2$	C, H
9	H	$NH_2 \cdot HCl$	OMs	68	113–117	B	$C_{12}H_{21}ClN_2O_6S_2$	C, H, Cl
10	NO	CH_3	OMs	38	95–99 dec	C	$C_{13}H_{20}N_2O_7S_2$	C, H, N
11	NO	Cl	OMs	28	100–102	G–H	$C_{12}H_{17}ClN_2O_7S_2$	H, N; C[e]
12	NO	F	OMs	25	113.5 dec	G–H	$C_{12}H_{17}FN_2O_7S_2$	C, H, N
13	NO	$NHCOCH_3$	OMs	20	131–132 dec	G–F	$C_{14}H_{21}N_3O_8S_2$	C, H, N
14	NO_2	H	OMs	11	134.5–136.5	G	$C_{12}H_{18}N_2O_8S_2$	C, H
15	NO_2	Cl	OMs	29	121.5–123[f]	E	$C_{12}H_{17}ClN_2O_8S_2$	H, N; C[g]
16	NO_2	F	OMs	32	143–143.5	A–F	$C_{12}H_{17}FN_2O_8S_2$	C, H, N
17	NO_2	CF_3	OMs	15	105.5–107	E	$C_{13}H_{17}F_3N_2O_8S_2$	C, H, N
18	NO_2	$COOC_2H_5$	OMs	47	95–96	G–F	$C_{15}H_{22}N_2O_{10}S_2$	C, H
19	$NH_2 \cdot HCl$	$COOC_2H_5$	OMs	36	99–103 dec	A	$C_{15}H_{25}ClN_2O_8S_2$	C, H, Cl, N

[a] Recrystallized from A, Me_2CO; B, MeCN; C, C_6H_6; D, $CHCl_3$; E, EtOH; F, Et_2O; G, EtOAc; H, petroleum ether (bp 37–48°). [b] C: calcd, 60.29; found, 59.50. [c] C: calcd, 40.55; found, 40.05; ash, 0.30. [d] N: calcd, 7.10; found, 6.63. [e] C: calcd, 35.95; found, 36.41. [f] Polymorphic form mp 105–106.5°. [g] C: calcd, 34.57; found, 35.09.

SCHEME I



X = H, NH_2, NO, NO_2

Y = H, $COOC_2H_5$, NH_2, $NHCOCH_3$, CH_3, F, Cl, CF_3, NO_2

hydroxyethyl)anilines II. Most hydroxy compounds were available commercially or had been reported earlier.[8] Physical properties of the two new ones are listed in Table I.

The methanesulfonates III were prepared by treating II with $MeSO_2Cl$.[9a] Some of the methanesulfonates III were nitrosated in HCl by the addition of aqueous $NaNO_2$ or nitrated in AcOH by the addition of concentrated HNO_3 to give the substituted anilines IV. Some of the nitro groups were reduced catalytically (Pd–C) to amino groups. The methanesulfonate esters (Table I) prepared by this scheme were identified by ir (1350- and 1175-cm^{-1} bands for OSO_2CH_3) and nmr (δ 3.0 s, 4.0 t, 4.5 t, and 6.5–8.1 m ppm for SO_2CH_3, NCH_2, OCH_2, and aromatic H, respectively) spectra and elemental analysis. In general they were stable crystalline solids, although their stability appeared to depend on the electron-donating and -withdrawing ability of the nuclear substituents X and Y. The greater the donating power, the lesser the stability; *e.g.*, the phenylenediamine **9** was shown by nmr to solvolize (with the formation of $MeSO_3H$) at a faster rate than the nitrosoaniline **20**. Methanesulfonation of N,N-bis(2-hydroxyethyl)-*p*-anisidine, *m*-[N,N-bis(2-hydroxyethyl)amino]benzoic acid, *m*-[N,N-bis(2-hydroxyethyl)amino]phenol, *p*-[N,N-bis(2-hydroxyethyl)-amino]acetanilide, and N,N-(2-hydroxydiethyl)aniline gave only impure oils. The desired products could not be purified due to their instability, attributed to the higher basicity of the amino nitrogen of these compounds. The nitroso compounds were relatively stable, but they appeared to be slightly sensitive to light and heat.

For the preparation of **12** it was necessary to use isoamyl nitrite in AcOH with a small amount of concentrated HCl, since the usual nitrosation procedure proved unsuccessful. Furthermore, we were not able to repeat the synthesis. The nitrosation of **7** and **8** was also unsuccessful.

The nitrocarbethoxy compound **18** was prepared by the usual nitration of **8** but **18** was also the only compound isolated during the attempted nitrosation of **8**. The nitrochloro compound **15** was obtained as two

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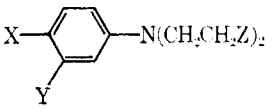
TABLE II
SCREENING DATA[a]



Compd	X	Y	Z	KB ED_{50}, μg/ml	LE[b] mg/kg	LE[b] T/C, %	WM[c] mg/kg	WM[c] T/C, %
3	H	Cl	OMs	2.6×10^{-2}	8.00	150	16.0	2
4	H	F	OMs	1.6×10^{-1}	20.0	151		
5	H	$NHCOCH_3$	OMs	8.9	3.00	186	5.00	0
6	H	NO_2	OMs	6.8	100	148	100	0
7	H	CF_3	OMs	7.6	25.0	156	17.0	0
8	H	$COOC_2H_5$	OMs	2.9×10^{-1}	200	132	128	0
9	H	NH_2	OMs	6.6	8.00	209[d]		
10	NO	CH_3	OMs	5.9×10^{-5}	0.50	149	5.00	3
11	NO	Cl	OMs	1.3×10^{-2}	0.50	138	0.20	6
12	NO	F	OMs	1.3×10^{-2}	7.00	151	5.00	12
13	NO	$NHCOCH_3$	OMs	1.3×10^{-2}	0.40	130	3.40	17
14	NO_2	H	OMs	3.6	400	121	400	3
15	NO_2	Cl	OMs	6.7	400	130	50.0	24
16	NO_2	F	OMs	6.2	400	105		
17	NO_2	CF_3	OMs	1.6×10	400	93		
18	NO_2	$COOC_2H_5$	OMs	1.7	400	104	400	79
19	NH_2	$COOC_2H_5$	OMs	6.0	5.00	154	5.00	3
20	NO	H	OMs	1.7×10^{-4}	0.64	140	2.00	0
21	NO	H	OTs	1.4×10^{-1}	100	124	50.0	5
22	NO	H	OH	8.3×10^{-1}	20.0	101		

[a] Only the most effective doses below lethal levels and the resulting T/C percentages are presented for each compound. [b] LE = L1210 lymphoid leukemia. [c] WM = Walker carcinosarcoma 256 (intramuscular). [d] Nonreproducible results.

polymorphs of different melting points, as proven by microscopical fusion analysis, in two identical preparations.

Biological Results.—The compounds were screened for antitumor activity against KB cell culture, L1210 lymphoid leukemia, and Walker carcinosarcoma 256 (intramuscular) under the auspices of the CCNSC.

Our results (presented in part in Table II) indicated that the addition of almost any nuclear substituent imparted higher activity to the parent N,N-bis(2-methanesulfonoxyethyl)aniline.[9b] Of the groups investigated the *p*-NO produced the highest activity; **20** stands as the most active compound in the series. The addition of a *p*-NO group to the *meta*-substituted methanesulfonoxyethylanilines (**10–13**) failed to increase the activity above that of **20** even though some of the *meta*-substituted methanesulfonoxyethylanilines themselves (**3–9**) showed considerable activity. The corresponding nitro compounds (**14–18**) were less active than the unnitrated compounds. Both nuclear substitution and the methanesulfonate alkylating moiety seemed to be required for attaining carcinolytic activity; *e.g.*, **22** (a nuclear-substituted hydroxyethylaniline) and N,N-bis(2-methanesulfonoxyethyl)aniline (a methanesulfonate with no nuclear substituent) were both inactive, whereas **20** (a nuclear-substituted methanesulfonate) was very active. Two examples (chlorambucil and sarcolysin) of nuclear substitution enhancing the activity of the parent aromatic mustard were cited by Ross.[10]

An attempted correlation between the estimated pK_a values of the substituted methanesulfonoxyethylanilines and the cytotoxicity in KB cell culture was unrewarding. The values were too scattered to indicate a trend. Thus the compounds of the methanesulfonate series did not follow quantitatively what Everett and Ross[11] had demonstrated for the aryl-2-haloalkylamines, *i.e.*, that "substitution in the aromatic nucleus tending to reduce the chemical reactivity of the halogen atoms (as measured by the rate of hydrolysis in aqueous acetone) also reduces the biological activity."

N,N-Bis[2-(*p*-toluenesulfonoxyethyl)]-*p*-nitrosoaniline (**21**) was found to be less active than the corresponding methanesulfonoxy compound **20**.

Experimental Section[12]

Except for the following examples, the usual synthesis techniques referred to above were followed for the preparation of the compounds mentioned.

3-[Bis(2-methanesulfonoxyethyl)amino]acetanilide (5).—$MeSO_2Cl$ (14 ml, 0.18 mole) was added with stirring to a solution of 17.6 g (0.075 mole) of 3-[bis(2-hydroxyethyl)amino]acetanilide in 105 ml of dry (BaO) pyridine at −10°. The mixture was stirred at −10° for 30 min, then poured into petroleum ether (bp 37–48°) to precipitate the crude product as an oil. The solvent was decanted and the oil was triturated several times with petroleum ether. The oil was dissolved in $CHCl_3$ and the solution was washed (ice-cold 5% H_2SO_4, H_2O, saturated NaCl). The dried (Na_2SO_4) $CHCl_3$ solution was warmed and clouded with petroleum ether and allowed to crystallize. Properties of the product are listed in Table I.

N,N-Bis(2-methanesulfonoxyethyl)-3-fluoro-4-nitrosoaniline (12).—To a solution of 1.43 g (0.004 mole) of N,N-bis(2-methanesulfonoxyethyl)-3-fluoroaniline in 5.0 ml of AcOH and 0.8 ml of concentrated HCl was added 0.7 ml (0.006 mole) of *i*-AmONO.

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(11) J. L. Everett and W. C. J. Ross, *J. Chem. Soc.*, 1972 (1949).

(12) All melting points were taken on a Mel-Temp capillary melting point apparatus and are uncorrected. Ir spectra were determined as Fluorolube and Nujol mulls and were recorded on a Perkin-Elmer 237 spectrophotometer. The nmr spectra were obtained on a Varian Associates A-60 spectrometer equipped with a V-6040 variable-temperature controller and probe. The microanalyses were performed by Dr. S. M. Nagy of the Massachusetts Institute of Technology. Where analyses are indicated only by symbols of the elements, analytical results obtained for these elements were within ±0.4% of the theoretical values.

The solution was stirred for 10 min with slight cooling and then a solution of 0.4 g (0.006 mole) of urea in 1 ml of H_2O and 3 drops of concentrated HCl was added. The reaction solution was immediately poured into 150 ml of cold H_2O and extracted (EtOAc). The EtOAc solution was partially dried by filtering through $CaSO_4$ and enough petroleum ether was added to force H_2O and a brown tar out of solution. The EtOAc–petroleum ether solution was decanted from H_2O and tar and clouded with more petroleum ether and allowed to crystallize at Dry Ice temperature. Properties of the product are listed in Table I.

N,N-Bis[2-(*p*-toluenesulfonoxy)ethyl]-*p*-nitrosoaniline (21). N,N-Bis[2-(*p*-toluenesulfonoxy)ethyl]aniline[9a] was nitrosated in a mixture of AcOH and 6 *N* HCl and worked up in the usual way.[5] The crude product was recrystallized from absolute EtOH to give a 75% yield of pure **21** as a green powder, mp 117–118° dec. *Anal.* ($C_{24}H_{26}N_2O_7S_2$) C, H, N.

Charge–Spatial Models. *cis*- and *trans*-3- and -4-Substituted Cyclohexyl Phosphates as Analogs of 2′-Deoxyuridine 5′-Phosphate[1]

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The isomers of 3- and 4-substituted cyclohexyl phosphates were synthesized for examination of the binding sites of thymidylate synthetase. *cis*-3-Hydroxycyclohexanecarboxylic acid treated with diphenylphosphorochloridate gave *cis*-3-diphenylphosphorylcyclohexanecarboxylic acid (**5a**). Subsequent treatment with $SOCl_2$ followed by NH_4OH gave *cis*-3-carbamoylcyclohexyl diphenyl phosphate (**5b**). Acetylation of **5b** gave *cis*-3-N-acetylcarbamoylcyclohexyl diphenyl phosphate (**5c**). The synthesis of *cis*-3-allophanoylcyclohexyl diphenyl phosphate (**5d**) was accomplished by treating the acid chloride of **5a** with urea. Pt-catalyzed hydrogenolysis of the diphenyl phosphates **5b–d** yielded the respective phosphates **1b–d**. The corresponding *trans*-3 (**2b–d**), *cis*-4 (**3b–d**), and mixed *cis-trans*-4 (**4b–d**) phosphates were prepared by the same sequence. The phosphates were found to be weak inhibitors of thymidylate synthetase; highest activity resided in *cis*-4-N-acetylcarbamoylcyclohexyl phosphate (**3c**) and *cis*-4-allophanoylcyclohexyl phosphate (**3d**).

The commonest approach to the design of enzymatic inhibitors is based on analogs of the natural metabolites of the particular enzyme. While there is no question of the productivity of this approach, the metabolite variations that can be designed as potential inhibitors are limited and in many cases the synthesis becomes extremely laborious.

Another approach to the design of enzyme inhibitors that is unlimited in scope can be built on the premise that the most important features in specific enzymatic binding reside in a maximum of two or three portions of a molecule, and the remainder of the molecule is a template structure holding the correct charge sites in the proper spatial arrangement. Binding to the enzyme through these charge sites of the molecule relies on attractive forces such as exist between unlike charges, either dipoles or ion–ion pairs, hydrophobic bonding, or other physicochemical cohesive forces.[2] An essential to binding is that these sites of the molecule are held in the correct steric–spatial relationship without steric interference of approach to the enzyme.

In an attempt to examine these postulates initial studies were directed to the inhibition of thymidylate synthetase, the enzyme catalyzing the conversion of 2′-deoxyuridine 5′-phosphate to thymidine 5′-phosphate.[3] Previous studies by many investigators have demonstrated that the phosphate group is essential for binding. In addition, studies reported on azapyrimidine nucleotides and 5-fluoro- or 5-trifluoro-2′-deoxyuridine 5′-phosphates suggest a requirement for an acidic function (pK_a = 9.5 to ~7.0) at the N_3H–C_4O portion of the pyrimidine ring.[3c,4] The correct spatial relationship of these two moieties for maximum binding has not been studied. Assuming the *syn* or *anti* configuration for the pyrimidine ring and the fact that the 5′-phosphate is theoretically freely rotating, several possible spatial arrangements can be formulated. Using the substrate 2′-deoxyuridine 5′-phosphate as the model, with a fully extended phosphate the *syn* and *anti* pyrimidine ring configurations show a range of 6.4–7.6 Å between the center of the P atom and the center of N-3.

For the preliminary studies this range of 6.4–7.6 Å between the acidic function and a phosphate was selected. The models used were cyclohexyl phosphates substituted *cis* or *trans* at positions 3 or 4: compounds **1b–d**, **2b–d**, **3b–d**, and **4b–d**. The substituents used at these positions were the amide which is neutral and should be inactive, the N-acetylamide, and the acylcarbamate group, both of which are weakly acidic. Although the 1,3-*trans* and the 1,4-*cis* systems are flexible, the distances between the ionizable NH and the P atom in these models, assuming extended phosphate and amide groups, are estimated to be 6.0 Å (1,3-*trans*), 6.4 Å (1,3-*cis*), 6.8 Å (1,4-*cis*), and 7.2 Å (1,4-*trans*).

Catalytic hydrogenation of *m*-hydroxybenzoic acid[5] has afforded mainly *cis*-3-hydroxycyclohexanecarboxylic acid. On the basis of more recent favorable results

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(2) For further consideration of attractive forces between molecules particularly as applied to enzymatic binding see: (a) B. R. Baker, "Design of Active-Site-Directed Irreversible Enzyme Inhibitors," John Wiley and Sons, Inc., New York, N. Y., 1967; (b) J. Leyden Webb, "Enzyme and Metabolic Inhibitors," Vol. I, Academic Press, New York, N. Y., 1963.

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