Irreversible Enzyme Inhibitors. CXVII.[1,2] Hydrophobic Bonding to Dihydrofolic Reductase. XI.[3] Comparison of the Enzyme from Walker 256, Rat Liver, and L1210 Mouse Leukemia

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Thirty-one 4,6-diamino-1,2-dihydro-*s*-triazines with varying substituents at the 1 and 2 positions have been compared as inhibitors of the dihydrofolic reductase from Walker 256 rat tumor, rat liver, L1210/FR8 mouse leukemia, and pigeon liver. It was demonstrated that a 1-phenyl group was complexed almost equally effectively to all four enzymes and that the interaction of the phenyl group with the enzyme was by hydrophobic bonding; similarly, all four enzymes showed about the same amount of hydrophobic bonding by a 1-(*n*-butyl) group. Differences in hydrocarbon interactions among the four enzymes were seen with larger hydrocarbon groups. The greatest differences in binding to the rat tumor and rat liver enzymes were seen with a 1-phenylbutyl group and 1-[*p*-(2,4-dichlorophenylbutyl)phenyl] group, the tumor enzyme being inhibited 100- and 40-fold better, respectively, than the rat liver enzyme. In no case was a compound strikingly more effective on the L1210 enzyme than the other three enzymes.

One of the key enzymes in cellular reproduction is dihydrofolic reductase which can reduce dihydrofolate and usually folic acid to the cofactor form, tetrahydrofolate; the latter is then involved in fifteen enzyme reactions catalyzing one-carbon transfer reactions including purine and pyrimidine biosynthesis.[4] After the discovery of a hydrophobic bonding region on dihydrofolic reductase,[5] an intense study of optimum conformation for binding to this region and the relative location on the enzyme surface was pursued.[6] Strong evidence was found that this hydrophobic bonding region was just adjacent to the active site and is located near the 4 or 8 position of dihydrofolate when it resides on the enzyme.[6] Since this hydrophobic region is not part of the active site, evolutionary changes of amino acids in this region would more easily occur without lethality than similar changes inside the active site.[7] Two studies have been previously made on species differences in reversible binding to the hydrophobic bonding region, the first on *Escherichia coli* B *vs.* pigeon liver[8] and the second on T_2 phage induced enzyme *vs. E. coli* B *vs.* pigeon liver.[3,9] In this paper are described the studies on comparison of the hydrophobic bonding region from four vertebrate sources, namely, Walker 256 tumor and liver from the rat, L1210 mouse leukemia, and pigeon liver.

(1) This work was generously supported by Grant CA-08695 from the National Cancer Institute, U. S. Public Health Service.

(2) For the previous paper in this series see B. R. Baker, P. C. Huang, and R. B. Meyer, Jr., *J. Med. Chem.*, **11**, 475 (1968).

(3) For the previous paper in this series see B. R. Baker, *ibid.*, **10**, 912 (1967).

(4) T. H. Jukes and H. P. Broquist in "Metabolic Inhibitors," R. M. Hochster and J. H. Quastel, Ed., Academic Press Inc., New York, N. Y., 1963, pp 481–534.

(5) B. R. Baker, B.-T. Ho, and D. V. Santi, *J. Pharm. Sci.*, **54**, 1415 (1965).

(6) For a review on the mode of binding to dihydrofolic reductase see B. R. Baker, "Design of Active-Site-Directed Irreversible Enzyme Inhibitors. The Organic Chemistry of the Enzymic Active-Site," John Wiley and Sons, Inc., New York, N. Y., 1967, Chapter X.

(7) For reviews on evolutionary changes in enzymes see (a) ref 6, Chapter IX, and (b) V. Bryson and H. J. Vogel, Ed., "Evolving Genes and Proteins," Academic Press Inc., New York, N. Y., 1965.

(8) B. R. Baker and B.-T. Ho, *J. Pharm. Sci.*, **55**, 470 (1966).

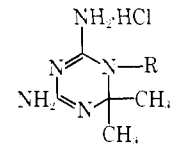
(9) Further examples of species differences in the ability of dihydrofolic reductase to bind 2,4-diaminoheterocycles that have varying hydrophobic groups[10] have been collated by Hitchings and Burchall; see (a) G. H. Hitchings and J. J. Burchall, *Advan. Enzymol.*, **27**, 417 (1965), and (b) J. J. Burchall and G. H. Hitchings, *Mol. Pharmacol.*, **1**, 126 (1965).

(10) For a similar study on the dihydrofolic reductase from *Trypanosoma equiperdum* see J. J. Jaffee and J. J. McCormack, Jr., *ibid.*, **3**, 359 (1967).

Enzyme Results.—The inhibition results with 31 1-substituted dihydro-*s*-triazines on the four sources of dihydrofolic reductase are collated in Tables I–III. It is again noteworthy that the 1-methyl-*s*-triazine (**1**) (Table I) binds almost the same to all the enzymes within a factor of 2.5 which also brackets the previous results with the enzyme induced by T_2 phage and the *E. coli* B enzyme;[3] these results further support the concept[3,8] that the 4,6-diamino-1,2-dihydro-*s*-triazine ring system complexes within the active site in the region that normally complexes the pteridine of dihydrofolate, a region unlikely to have undergone any mutation without lethality.

The increment in binding between the 1-methyl (**1**) and the 1-*n*-butyl (**2**) substituent due to hydrophobic interaction varies between 110- and 260-fold, depending upon the enzyme source. Similarly, the hydrophobic bonding increment between 1-methyl (**1**) and 1-phenyl (**5**) varies between 260- and 670-fold, indicating similar

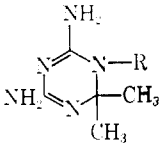
TABLE I
INHIBITION OF DIHYDROFOLIC REDUCTASE BY 1-ALKYL-, 1-ARYL-, AND 1-PHENYLALKYL-*s*-TRIAZINES



No.	R	Walker 256	Rat liver	L1210/FR8	Pigeon liver[b]
		——μM concn for 50% inhib[a]——			
1	CH_3	31	39	78	74
2	n-C_4H_9	0.12	0.37	0.39	0.36
3	i-C_5H_{11}	0.0061	0.078	0.070	0.058
4	n-C_8H_{17}	0.014	0.053	0.062	0.14
5	C_6H_5	0.12	0.15	0.21	0.11
6	$(CH_2)_3C_6H_5$	0.016	0.018	0.053	0.028
7	$(CH_2)_4C_6H_5$	0.00030	0.033	0.038	0.041
8	C_6H_4COOH-*p*			2200	1100
9	C_6H_4COOH-*m*	19	18	68	110
10	$C_6H_4COOC_2H_5$-*p*			27	42
11	C_6H_4CN-*p*	7.2	14	9.1	8.0
12	$C_6H_4CH_2NH_3^+$-*p*	6.3	3.7	14	10
13	$C_6H_4OCH_3$-*m*	0.67	0.78	0.79	0.54
14	$C_6H_4CF_3$-*m*	0.017	0.025	0.041	0.080
15	$C_6H_4NO_2$-*m*	0.084	0.16	0.22	0.072

[a] All assays were performed with 6 μM dihydrofolate and 30 μM TPNH in pH 7.4 Tris buffer as previously described.[16] The technical assistance of Sharon Lafler, Jean Reeder, and Diane Shea is acknowledged. [b] Data previously summarized.[3]

Table II
Inhibition of Dihydrofolic Reductase by Nonpolar Substituted 1-Aryl-s-triazines



No.	R	Walker 256	Rat liver	L1210/FR8	Pigeon liver[b]
		μM concn for 50% inhibn[a]			
5	C_6H_5	0.12	0.15	0.21	0.11
16	*p*-$C_6H_5C_6H_4$	20	22	51	160
17	*m*-$C_6H_5C_6H_4$	0.14	0.19	0.54	1.3
18	9-Fluorenon-2-yl	14	18	9.5	85
19	*m*-$C_6H_5CH_2C_6H_4$	0.010	0.0090	0.021	0.019
20	*p*-$C_6H_5CH_2C_6H_4$	0.0089	0.011	0.031	0.062
21	*p*-(*n*-C_4H_9)C_6H_4			0.054	0.064
22	*p*-$C_6H_4(CH_2)_4C_6H_3Cl_2$-2,4	0.00061	0.023	0.22	0.0053
23	*m*-$C_6H_4(CH_2)_4C_6H_3Cl_2$-2,4	0.035	0.018	0.0049	0.0080
24	*p*-$C_6H_5(CH_2)_3$-*m*-ClC_6H_3	0.011	0.010	0.0052	0.0093[c]
25	*m*-ClC_6H_4	0.017	0.030	0.010	0.0085
26	$C_6H_3Cl_2$-3,4	0.0029	0.010	0.013	0.015

[a] See footnote *a* in Table I. [b] Data previously summarized[3] unless otherwise indicated. [c] Data from ref 13.

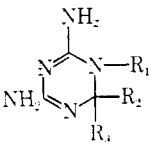
of this group is more effective on rat liver than on rat tumor. Although no comparison between L1210 mouse leukemia enzyme and mouse liver has been made, the pattern with the L1210 enzyme is strikingly similar to the pigeon and rat liver enzymes with **1**–**7**.

The main line of evidence that the 1-phenyl group of **5** was interacting with the pigeon liver enzyme by hydrophobic bonding was the large decrease in binding observed when the phenyl group was substituted with either an anionic group (**8**, **9**) or a cationic group (**12**);[11] other polar groups such as CN (**11**) and OCH_3 (**13**) also led to a loss in binding. There was no correlation with the Hammet σ constant showing the benzene binding had no charge-transfer character. Similar results were obtained with **8**–**15** on the dihydrofolic reductase from Walker 256 rat tumor, rat liver, and L1210 mouse leukemia. In no case with compounds **8**–**15** was binding more than fivefold different between enzyme sources.

Substitution on the 1-phenyl group of **5** with an in-plane *p*-phenyl group (**19**) (Table II) was previously shown to cause a 1500-fold decrease in binding to the pigeon liver enzyme;[11] similarly, when the phenyl group of **5** was replaced by the large flat 9-fluorenon-2-yl group (**18**), an 800-fold loss in binding occurred.[12] This hindrance to binding to the other three enzymes was less severe with **16**, being only 150–240-fold; similarly, with **18**, the loss was only 45-fold with the L1210 enzyme and 120-fold with the two rat tissue enzymes. More hindrance to binding to the pigeon liver enzyme was also seen with the *m*-phenyl substituent (**17**) on **5** than with the other three enzymes; the loss in binding to the pigeon liver enzyme was 12-fold, to the L1210 enzyme about twofold, but no change in binding occurred with the two rat enzymes. It was previously noted[3] that the *m*-phenyl substituent of **17** gave an increase in binding to the T_2 phage induced and *E. coli* B enzymes.

The *m*-benzyl (**19**)[12] and *p*-benzyl (**20**)[11] substituents (Table II) on the 1-phenyl group of **5** were previously observed to give six and twofold increments, respectively, in binding to the pigeon liver enzyme. The binding increment to the other three enzymes was

Table III
Inhibition of Dihydrofolic Reductase by Dihydro-s-triazines with Variants at the 2 Position



No.	R_1	R_2	R_3	Walker 256	Rat liver	L1210/FR8	Pigeon liver[b]
				μM concn for 50% inhibn[a]			
5	C_6H_5	CH_3	CH_3	0.12	0.15	0.21	0.11
7	$(CH_2)_4C_6H_5$	CH_3	CH_3	0.00030	0.033	0.038	0.041
25	*m*-ClC_6H_4	CH_3	CH_3	0.017	0.030	0.010	0.0085
27	*m*-ClC_6H_4	C_6H_4NHAc-*p*	H	130	110	230	190
28	*m*-ClC_6H_4	$CH_2C_6H_5$	H	0.24	0.34	0.17	1.1
29	$C_6H_5(CH_2)_4$	C_6H_4NHAc-*p*	H	0.50	0.50	0.13	0.62
30	*o*-ClC_6H_4	CH_3	CH_3	70	77		160
31	$C_6H_3(CH_3)_2$-2,6	CH_3	CH_3	110	150	82	150

[a] See footnote *a* in Table I. [b] Data previously summarized.[3]

binding; these differences should be compared with the much smaller 23-fold increment in binding between **1** and **5** on the *E. coli* enzyme.[3]

By increasing the *n*-butyl group (**2**) to *n*-octyl (**4**), a 3–9-fold increment in binding is seen, the higher increment with Walker 256 enzyme and the lower increment with the pigeon liver enzyme; this increment is about the same for Walker 256 and the rat liver enzyme. In contrast, terminal substitution of the *n*-butyl group (**2**) with phenyl (**7**) gives a 400-fold increment in binding with the rat tumor enzyme, but only a tenfold increment with the enzyme from rat liver and the other two sources; about a tenfold increment was seen with the T_2 phage induced and *E. coli* B enzymes.[3]

The branching of the *n*-butyl group (**2**) to isoamyl (**3**) gives a 20-fold increment in binding to the rat tumor enzyme, but only about a sixfold increment with the other three enzymes.

The compound in the series **1**–**7** showing the greatest specificity toward inhibition of the Walker 256 enzyme over the rat liver enzyme is the phenylbutyltriazine (**7**) where the difference is 100-fold; the next most specific compound is the isoamyltriazine with a 20-fold difference between rat tumor and liver. No compound

(11) B. R. Baker and B.-T. Ho, *J. Heterocycl. Chem.*, **2**, 335 (1965).
(12) B. R. Baker and B.-T. Ho, *ibid.*, **2**, 72 (1965).

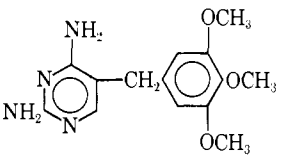
larger, a 10–17-fold increment being observed with **19** and a 7–14-fold increment with **20**. Longer phenylalkyl substituents such as phenylbutyl were observed[13] to give even better binding than benzyl to the pigeon liver enzyme; the 2,4-dichlorophenylbutyl substituent on the *meta* position (**23**) gave a 14-fold increment and on the *para* position (**22**) a 21-fold increment. There was considerable difference in binding to the four enzymes with these two compounds (**22**, **23**). The *para* compound (**22**) gave a 190-fold increment in binding to the rat tumor enzyme, but only a tenfold increment in binding to the rat liver enzyme, and no increment in binding to the L1210 enzyme. In contrast, this substituent at the *meta* position (**23**) gave only a three fold binding increment to that rat tumor enzyme, an eightfold increment with rat liver enzyme, a 14-fold increment with pigeon liver enzyme, but a 43-fold increment in binding to the L1210 enzyme. Little difference in binding among the four enzymes was seen with the 3-chloro and 4-phenylbutyl substituents (**24**) on the 1-phenyl of **5**. Also, little difference in binding among the four enzymes was observed with the *m*-chloro (**25**) and 3,4-dichloro (**26**) substituents, although the chlorines did increase binding.

The greatest difference in binding to the two rat enzymes with the compounds in Table II was observed with the *p*-(2,4-dichlorophenylbutyl) substituent of **22** where the tumor enzyme was inhibited 40-fold better. No compound in Table II was more than twice as effective on rat liver enzyme than rat tumor enzyme. The pattern observed with the L1210 enzyme was quite similar to the pigeon and rat liver enzymes except for **22**, which was tenfold more effective on the rat liver enzyme and 40-fold more effective on the pigeon liver enzyme; no compound in Table II was more than threefold more effective on L1210 enzyme than on the two liver enzymes.

Introduction of larger groups at the 2 position of the dihydro-*s*-triazine system was observed to be more detrimental in binding to the enzyme from pigeon liver than from *E. coli* B.[3,8] Similar losses in binding to the three mammalian enzymes have now been observed (Table III). Introduction of a *p*-acetamidophenyl substituent (**27**) at the 2 position of **25** gave a 22,000-fold loss in binding to the pigeon liver and L1210 enzymes; the loss in binding to the two rat enzymes was also large, being 3700–7700-fold. The loss in binding by the *p*-acetamidophenyl substituent (**29**) was less when the *s*-triazine was substituted with a 1-phenylbutyl group (**7**); the least effect was on the L1210 enzyme where only a threefold loss in binding occurred, but was most dramatic on the rat tumor enzyme where a 1700-fold loss in binding occurred. The 2-benzyl substituent (**28**) showed much less steric hindrance to binding than the 2-(*p*-acetamidophenyl) substituent (**27**), **28** causing only a 7–27-fold loss in binding to the four enzymes, in contrast to the 3700–22,000-fold loss with **27**. Of the compounds **27**–**31** in Table III none showed more than a fourfold difference in binding to the four enzymes.

Conclusions.—It has been shown that the 1-phenyl-*s*-triazine (**5**) has essentially the same ability to bind to the four vertebrate enzymes and that the 1-phenyl group is complexed by hydrophobic interaction with these enzymes. However, substitution of additional nonpolar groups on the 1-phenyl moiety of **5** showed differences in binding to the four enzymes. Many of these differences were due to a stronger hydrocarbon interaction to the rat tumor enzyme than the other enzymes.

The biggest differences in reversible inhibition of the enzymes from Walker 256 rat tumor and rat liver are observed with the 1-phenylbutyl (**7**) and the *p*-(2,4-dichlorophenylbutyl)phenyl (**22**) substituents, being 100- and 40-fold, respectively. These differences are not sufficient to expect chemotherapeutic effectiveness on the tumor; note that the antibacterial agent, trimethoprim (**32**), shows 50,000–70,000-fold more ef-



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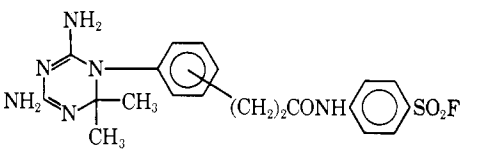
fectiveness on bacterial dihydrofolic reductases than on mammalian dihydrofolic reductases.[9] Of the 31 compounds listed in Tables I–III, **22** shows the greatest spread in effectiveness between the pigeon liver and *E. coli* B enzymes, being only 230-fold. Since trimethoprim resulted from years of study in the pyrimidine area by Hitchings' laboratory,[9] it is clear that our limited studies on seeking a reversible inhibitor of dihydrofolic reductase that is 50,000-fold more effective on Walker 256 than rat liver enzyme has been minimal in comparison. From the evolutionary standpoint, it is unlikely that such large differences between the two rat enzymes will be found, though some additional attempts have been made.[14] However, it is clear that small differences exist in binding to the hydrophobic bonding region of dihydrofolic reductase from Walker 256 rat tumor, rat liver, and pigeon liver. It was predicted[3] that it should be possible to greatly amplify these relatively small differences in hydrophobic bonding by use of the bridges principle of specificity[15] with active-site-directed irreversible inhibitors.[6,15] This prediction was first borne out with the dihydro-*s*-triazine (**33**) bearing a terminal sulfonyl fluoride group; even though differences in reversible binding were small, **33** was a rapid irreversible inhibitor of the pigeon liver dihydrofolic reductase, but showed no inactivation of the



33, *meta* isomer
34, *para* isomer

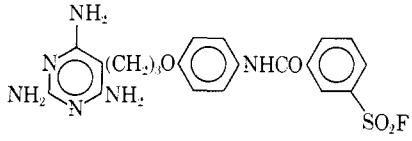
enzyme from Walker 256 or L1210.[16] The *para* isomer showed rapid inactivation of the enzyme from Walker 256 and liver from the rat, L1210 mouse leukemia, and

(13) B. R. Baker, B.-T. Ho, and G. J. Lourens, *J. Pharm. Sci.*, **56**, 737 (1967).

(14) B. R. Baker and M. A. Johnson, *J. Med. Chem.*, **11**, 486 (1968); paper CXVIII of this series.

(15) See ref 6, Chapter IX.

(16) B. R. Baker and G. J. Lourens, *J. Med. Chem.*, **10**, 1113 (1967), paper CV of this series.



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pigeon liver. Further studies led to compound **35** which showed little difference in irreversible inhibition of mammalian dihydrofolic reductases, but showed selective irreversible inhibition; a 1 μM concentration of **35** could rapidly inactivate the Walker 256 rat tumor enzyme and the L1210/FR8 mouse leukemia enzyme but did not inactivate the rat or mouse liver enzymes at this concentration.[17]

(17) B. R. Baker and R. B. Meyer, Jr., *J. Med. Chem.*, **11**, 489 (1968), paper CXIX of this series.

Irreversible Enzyme Inhibitors. CXVIII.[1,2] Hydrophobic Bonding to Dihydrofolic Reductase. XII.[2] Further Comparisons with the Enzyme from Walker 256 Rat Tumor and Rat Liver

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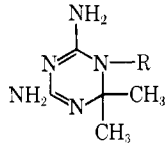
Fourteen *ortho*-substituted 1-phenyl-4,6-diamino-1,2-dihydro-*s*-triazines have been measured as inhibitors of the dihydrofolic reductase from Walker 256 rat tumor and rat liver: no appreciable difference in binding to the enzyme from the two sources was found. The 600-fold loss in binding when 4,6-diamino-2,2-dimethyl-1,2-dihydro-1-phenyl-*s*-triazine (**4**) is substituted with an *o*-chloro group (**5**) can be recouped by further substitution of a 3-chloro group (**16**) or 4-phenylbutyl group (**20**).

In the previous paper of this series a comparison of the binding to the hydrophobic bonding region of the dihydrofolic reductase from Walker 256 rat tumor and rat liver were made with 31 substituted 4,6-diamino-1,2-dihydro-*s*-triazines. The largest difference in binding between the two enzymes was observed[2] with **1** and **2**, the tumor enzyme being inhibited better by 100- and 40-fold, respectively. Even though *ortho* substituents on the 1-phenyl group of dihydro-*s*-triazines give a great loss in biological activity,[3,4] this area has now been further studied particularly since it was possible that

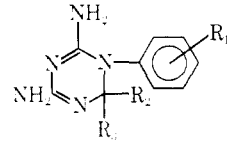


1, R = $(CH_2)_4C_6H_5$
2, R = p-$C_6H_4(CH_2)_4C_6H_3Cl_2$-2,4
3, R = o-R′C_6H_4

the lowered activity could be recouped by additional substituents on the benzene ring[5] and it was possible that tissue specificity might be uncovered. The results of this study on binding of **3** and related compounds to Walker 256 and liver dihydrofolic reductase of the rat is the subject of this paper.

The inhibition of dihydrofolic reductase from two sources with 14 *ortho*-substituted 1-phenyl-*s*-triazines and three related compounds are collated in Table I. No specificity in binding to the two enzymes was observed; nevertheless, some interesting correlations on the *ortho* effects can be made. For discussion purposes only the Walker 256 data will be used.

Table I
Inhibition of Dihydrofolic Reductase by *ortho*-Substituted 1-Phenyl-*s*-triazines



No.	R_1	R_2	R_3	μM conen for 50% inhibr Walker 256	Rat liver
4[a]	H	CH_3	CH_3	0.12	0.15
5[b]	*o*-Cl	CH_3	CH_3	70	77
6[c]	*o*-Br	CH_3	CH_3	56	87
7	*o*-I	CH_3	CH_3	24	26
8	*o*-F	CH_3	CH_3	18	15
9[c]	*o*-CH_3	CH_3	CH_3	100	93
10[c,d]	*o*-CH_3O	CH_3	CH_3	210	210
11[c]	H	C_6H_5	H	4.5	10
12	*o*-Cl	C_6H_5	H	150	130
13	*o*-F	C_6H_5	H	140	88
14	*o*-Br	C_6H_5	H	2500	2700
15[b]	*m*-Cl	CH_3	CH_3	0.017	0.030
16	2,3-Cl_2	CH_3	CH_3	0.30	0.41
17[c]	2,5-Cl_2	CH_3	CH_3	370	350
18[c]	2,4-Cl_2	CH_3	CH_3	150	150
19	2,4,5-Cl_3	CH_3	CH_3	42	90
20	*o*-Cl-*p*-$C_6H_5(CH_2)_4$	CH_3	CH_3	0.29	0.46

[a] Assayed with 6 μM dihydrofolate and 30 μM TPNH in pH 7.4 Tris buffer as previously described.[13] The technical assistance of Sharon Lafler, Jean Reeder, and Diane Shea is acknowledged. [b] Data from ref 2. [c] Prepared by the method of Modest.[3b] [d] See M. Furukawa, Y. Seto, and S. Toyoshima, *Chem. Pharm. Bull.* (Tokyo), **9**, 914 (1961).

(1) This work was generously supported by Grant CA-08695 from the National Cancer Institute, U. S. Public Health Service.

(2) For the previous paper of this series, see B. R. Baker, *J. Med. Chem.*, **11**, 483 (1968).

(3) (a) E. J. Modest, S. Farber, and G. E. Foley, *Proc. Am. Assoc. Cancer Res.*, **1**, 33 (1954); (b) E. J. Modest, *J. Org. Chem.*, **21**, 1 (1956).

(4) B. R. Baker and B.-T. Ho, *J. Pharm. Sci.*, **53**, 1137 (1964).

(5) B. R. Baker, B.-T. Ho, and G. J. Lourens, *ibid.*, **56**, 737 (1967), paper LXXXVI of this series.