

Irreversible Enzyme Inhibitors. CXXXIX.^{1,2} *p*-(4,6-Diamino-1,2-dihydro-2,2-dimethyl-*s*-triazin-1-yl)phenylpropionylsulfanyl Fluoride, an Active-Site-Directed Irreversible Inhibitor of Dihydrofolic Reductase. VI.³ Further Studies on Effects of Substitution on the Propionamide Bridge on Isozyme Specificity

B. R. BAKER AND GERHARDUS J. LOURENS⁴

Department of Chemistry, University of California at Santa Barbara, Santa Barbara, California 93106

Received July 24, 1968

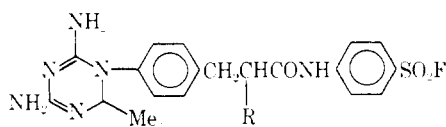
Thirteen candidate irreversible inhibitors of dihydrofolic reductase that have an alkyl, aryl, or aralkyl group on the propionamide bridge of the title compound (**1**) have been investigated with this enzyme from Walker 256 rat tumor, rat liver, three strains of L1210 mouse leukemia, and mouse liver. Substitution of an α -methyl (**6**), α -phenethyl (**7**), or N-methyl (**9**) eradicated the irreversible inhibition seen with **1**. Irreversible inhibition could be maintained with an α -aryl substituent such as phenyl, tolyl, anisyl, or naphthyl, but such a substitution was detrimental to the good reversible binding of **1** needed for irreversible inhibition at low concentration. The best compound emerging from the study was the β -methyl derivative (**8**), which showed specificity by inactivation of L1210 enzyme but not liver; however, **8** did not give complete irreversible inhibition of the L1210 enzyme due to the competition of enzyme-catalyzed hydrolysis of its SO₂F group.

In an earlier study on substitution⁵ on the propionamide bridge of **1**,⁶ it was observed that an α -phenyl substituent (**2**) gave an irreversible inhibitor that was more effective on rat liver dihydrofolic reductase than the enzyme from Walker 256 rat tumor. Furthermore, an *o*-tolyl (**3**) or *m*-tolyl (**4**) substituent gave an irreversible inhibitor more effective on the rat tumor enzyme than the rat liver enzyme; however, the separation of this irreversible inhibition on the enzymes from the two sources was considered insufficient to be chemotherapeutically useful. With the *p*-tolyl substituent, irreversible inhibition was abolished. Com-

tion⁸ of tumor *vs.* liver enzyme could be achieved. The results are the subject of this paper.

Enzyme Results.—The previous data^{5,6} with **1** and **2** on the dihydrofolic reductase from Walker 256, rat liver, L1210/FR8, and mouse liver are summarized in Table I for comparison purposes. The α -phenyl derivative (**2**) was near equally effective as an irreversible inhibitor of the dihydrofolic reductase from the three L1210 strains; however, **2** was not a good enough irreversible inhibitor⁹ of the L1210 enzymes to warrant assay on the mouse liver enzyme. Introduction of the *o*-methyl group (**3**) on **2** enhanced irreversible inhibition of the enzyme from the L1210 strains; irreversible inhibition of the liver enzyme was appreciably less. Similar results were observed with the *m*-tolyl derivative (**4**); furthermore, even though **4** was not a good irreversible inhibitor⁹ of the enzyme from L1210/DF8 and L1210/FR8, little irreversible inhibition of the mouse liver enzyme was observed with **4**. The *p*-tolyl derivative (**5**) showed no appreciable irreversible inhibition of the L1210 enzymes.

The alkyl and aralkyl derivatives (**6–9**) were then investigated as irreversible inhibitors of the dihydrofolic reductase from the various strains of L1210; the α -methyl (**6**), α -phenethyl (**7**), and N-methyl (**9**) derivatives failed to inactivate the enzyme. The β -methyl derivative (**8**) was previously reported⁶ to be a fair irreversible inhibitor of the enzyme from Walker 256 and L1210/FR8; **8** has now been observed to be an irreversible inhibitor of the L1210/DF8 and L1210/0 enzymes. Furthermore, **8** showed no irreversible



- 1, R = H 3, R = *o*-C₆H₄CH₃
 2, R = C₆H₅ 4, R = *m*-C₆H₄CH₃
 5, R = *p*-C₆H₄CH₃

pounds **2–5** were only poor irreversible inhibitors of L1210/FR8 mouse leukemia enzyme. In more recent studies,⁷ the dihydrofolic reductase from the parent L1210/0 strain and a different mutant, L1210/DF8, have been investigated. Therefore, compounds **2–5**, as well as the compounds with other substituents on the propionamide bridge described earlier,⁵ have now been assayed on the enzyme from L1210/0 and L1210/DF8; furthermore, some additional α -aryl analogs have now been synthesized to determine if a greater separation of active-site-directed irreversible inhibi-

(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 and N. M. J. Vermeulen, *J. Med. Chem.*, **12**, 89 (1969).

(3) For the previous paper in this subseries see B. R. Baker and G. J. Lourens, *ibid.*, **11**, 677 (1968), paper CXXXIX of this series.

(4) G. J. L. wishes to thank the Council for Scientific and Industrial Research, Republic of South Africa, for a tuition fellowship.

(5) B. R. Baker and G. J. Lourens, *J. Med. Chem.*, **11**, 672 (1968), paper CXXVIII of this series.

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

(7) B. R. Baker, G. J. Lourens, R. B. Meyer, Jr., and N. M. J. Vermeulen, *ibid.*, **12**, 67 (1969), paper CXXXIII of this series.

(8) 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.

(9) A good irreversible inhibitor is defined as one that at an I₅₀ > 6K_i concentration gives greater than 70% inactivation of an enzyme; an excellent irreversible inhibitor gives greater than 70% inactivation at I₅₀ = K_i; a fair irreversible inhibitor shows >70% inactivation at 30K_i ≈ 5I₅₀. The dependence of the extent of irreversible inhibition on concentration of SO₂F-type irreversible inhibitors is due to the competition between enzyme inactivation and enzyme-catalyzed hydrolysis of the SO₂F group within the enzyme-inhibitor complex;¹⁰ the ratio of these two rates is sensitive to structural change.^{3,3,4}

(10) (a) B. R. Baker and J. A. Hurlbut, *J. Med. Chem.*, **11**, 233 (1968), paper CXIII of this series; (b) B. R. Baker and E. H. Erickson, *ibid.*, **11**, 245 (1968), paper CXV of this series.

(11) B. R. Baker and G. J. Lourens, *ibid.*, **11**, 666 (1968), paper CXXVII of this series.

TABLE I
 INHIBITION^a OF DIHYDROFOLIC REDUCTASE BY

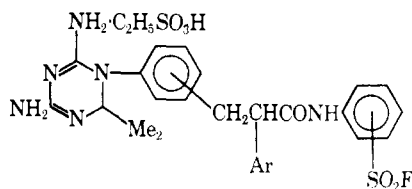
No.	R	Enzyme source	Reversible ^b		Irreversible ^c			
			I ₅₀ , ^d μM	K _i , ^e μM	Inhib. μM	% EI ^f	Time, min	% inactvn
1	H	Walker 256 ^g	0.020	0.003	0.020	87	1, 3	50, 90 ^h
		Rat liver ^g	0.006	0.001	0.020	95	8 ⁱ	50 ^h
		L1210/FR8 ^g	0.080	0.01	0.070	84	<2, 60	70, 70 ^h
		L1210/DF8 ^j	0.025	0.004	0.070	94	<2, 10	84, 84 ^h
		L1210/0 ^j	0.012	0.002	0.070	98	60	73 ^k
		Mouse liver ^g			0.070		60	53 ^k
2	α-C ₆ H ₅	Walker 256 ^g	0.074	0.01	0.074	87	2, 60	38, 38 ^h
		Rat liver ^g	0.18	0.03	0.074	84	4, 9, 60	50, 71, 71 ⁱ
		L1210/FR8	0.20	0.03	1.2	96	2, 8, 30	50, 88, 94 ^h
		L1210/DF8	0.045	0.007	0.16	86	<2, 60	65, 65 ^h
		L1210/0			0.48	99	60	27
		L1210/0			0.48		60	34 ^k
3	α-(<i>o</i> -C ₆ H ₄ CH ₃)	L1210/DF8	0.35 ⁿ		0.70		60	68 ^k
		L1210/0			0.70		60	73 ^k
		Mouse liver			0.70		60	19 ^k
4	α-(<i>m</i> -C ₆ H ₄ CH ₃)	L1210/DF8			0.42		60	65 ^k
		L1210/0			0.42		60	52 ^k
5	α-(<i>p</i> -C ₆ H ₄ CH ₃)	Mouse liver	0.20	0.03	0.42	92	60	20 ^k
		L1210/FR8	0.63	0.1	3.1	97	60	13
6	α-Me	L1210/0			1.2		60	0
		L1210/DF8	0.050 ⁿ		0.10		60	0
7	α-(CH ₂) ₂ C ₆ H ₅	L1210/DF8			0.10		60	0
		L1210/0			0.10		60	0
8	β-Me	L1210/0	1.9 ⁿ		3.8		60	0
		L1210/DF8			3.8		60	0
9	N-Me	L1210/DF8 ^j			0.16		60	40 ^k
		L1210/0 ⁱ	0.044	0.007	0.16	97	60	25 ^k
		Mouse liver ⁱ	0.080	0.01	0.40	97	60	0 ^k
		Mouse spleen ⁱ			0.16		60	38 ^k
10	α-C ₆ H ₅ ^l	L1210/0	0.041 ⁿ		0.082		60	0
		Walker 256	0.54	0.09	2.7	97	60	0
		L1210/FR8	0.48	0.06	2.4	97	60	0
11	α-C ₆ H ₅ ^m	L1210/0			0.96		60	0
		Walker 256			0.65		60	10 ^k
		L1210/DF8	0.13	0.02	0.65	97	60	0
12	H ⁿ	L1210/0			0.65		60	10 ^k
		Rat liver	0.012	0.002	0.060	96	2, 20	72, 71
		L1210/DF8	0.018	0.003	0.10	98	60	53 ^k
13	α-(<i>o</i> -C ₆ H ₄ OCH ₃)	Mouse liver			0.10		60	49 ^k
		Walker 256			0.20		60	26 ^k
		Rat liver			1.0		60	83
		L1210/DF8	0.47		1.0	97	60	75
14	α-(<i>m</i> -C ₆ H ₄ OCH ₃)	L1210/0			2.4		60	76 ^k
		Walker 256			2.4		60	57 ^k
		Rat liver			0.70		60	73
15	α-(α-Naphthyl)	L1210/DF8	0.35		0.70		60	57
		Walker 256			1.8	97	60	71 ^k
		Rat liver			1.1		60	78
		L1210/DF8	0.57		1.1		60	54
					2.8		60	61 ^k

^a The technical assistance of Sharon Laffer, Diane Shea, and Carolyn Wade with these assays is acknowledged. ^b Assayed with 6 μM dihydrofolate and 30 μM TPNH in pH 7.4 Tris buffer containing 0.15 M KCl as previously described.⁷ ^c Incubated at 37° in pH 7.4 Tris buffer in the presence of 60 μM TPNH as previously described.⁷ ^d I₅₀ = concentration for 50% inhibition. ^e Estimated from $K_i = K_m[I_{50}]/[S]$ which is valid since $[S] = 6K_m = 6\mu\text{M}$ dihydrofolate; see ref 8, p 202. ^f Calculated from $[EI] = [E]_t/(1 + K_i/[I])$ where $[EI]$ is the amount of the total enzyme (E_t) reversibly complexed; see ref 8, Chapter 8. ^g Data from ref 5. ^h From a time-study plot; see ref 6. ⁱ Half-time of reaction. ^j Data from ref 7. ^k Zero point obtained by adding inhibitor to assay cuvette prior to addition of enzyme aliquot.^{6,7} ^l Propionamide bridge connected *meta* to 1-phenyl-*s*-triazine moiety. ^m SO₂F connected *meta* to propionamide bridge. ⁿ I₅₀ on L1210/FR8 enzyme.⁵

inhibition of the mouse liver enzyme at a concentration of 5I₅₀.

From this evaluation of 2-9 on the dihydrofolic reductases from L1210/0 emerged two studies worthy

of pursuit: (1) further structural variations of the β-methyl derivative (8) should be pursued, and (2) further α-aryl derivatives related to 2-5 should be synthesized to see if better and more selective ir-

TABLE II
 PHYSICAL PROPERTIES OF


No. ^a	Bridge position	SO ₂ F position	Ar	% yield	Mp, °C dec	Formula ^d
10	<i>meta</i>	<i>para</i>	C ₆ H ₅	32 ^b	235-236	C ₂₆ H ₂₇ FN ₆ O ₃ S · C ₂ H ₅ SO ₃ H
11	<i>para</i>	<i>meta</i>	C ₆ H ₅	47 ^c	215-216	C ₂₆ H ₂₇ FN ₆ O ₃ S · C ₂ H ₅ SO ₃ H
13	<i>para</i>	<i>para</i>	<i>o</i> -C ₆ H ₄ OMe	60 ^b	208-209	C ₂₇ H ₂₉ FN ₆ O ₄ S · C ₂ H ₅ SO ₃ H
14	<i>para</i>	<i>para</i>	<i>m</i> -C ₆ H ₄ OMe	43 ^b	230-231	C ₂₇ H ₂₉ FN ₆ O ₄ S · C ₂ H ₅ SO ₃ H
15	<i>para</i>	<i>para</i>	α -Naphthyl	45 ^c	229-231	C ₃₀ H ₂₉ FN ₆ O ₃ S · C ₂ H ₅ SO ₃ H

^a Compounds were prepared by method B previously described;⁵ each had an ir band at 1395-1405 cm⁻¹ characteristic of SO₂F. ^b Recrystallized from *i*-PrOH-H₂O. ^c Recrystallized from EtOH-H₂O. ^d All compounds gave correct analyses for C, H, F.

reversible inhibition of tumor enzymes could be achieved; such a study is reported below.

When the propionamide side chain of **2** was moved to the *meta* position of the 1-phenyl group as in **10**, irreversible inhibition of the enzyme from Walker 256, L1210/FR8, or L1210/0 was lost. When the sulfonyl fluoride group of **1** was moved to the *meta* position, the resultant **12** was a fair irreversible inhibitor of the enzyme from Walker 256;¹² **12** has now been observed to be a fair irreversible inhibitor⁹ of the rat liver enzyme and a fair to poor irreversible inhibitor of the dihydrofolate reductase from L1210/0, L1210/DFS, and mouse liver, being least effective on the latter enzyme. Insertion of the α -phenyl group (**11**) on **12** eradicated the irreversible inhibition of the L1210 and Walker 256 enzymes.

Three additional α -aryl derivatives (**13-15**) were then synthesized for evaluation. The *o*-anisyl derivative (**13**) was a fair irreversible inhibitor⁹ of both the Walker 256 rat tumor and rat liver enzymes, but a fair to poor irreversible inhibitor of the L1210/DFS and L1210/0 enzymes; the *m*-anisyl (**14**) and α -naphthyl (**15**) derivatives gave similar results with the two rat enzymes and the L1210 enzyme.

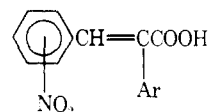
From this study it can be concluded that the α -aryl derivatives of **1** are not good candidates for animal studies; irreversible inhibition is poor to fair and relatively nonselective and secondly, this type of substitution is detrimental to reversible inhibition. Whether or not more effective compounds derived from the β -methyl derivative (**8**) can be achieved remains to be determined.

Experimental Section¹³

The synthesis of a number of the compounds in Table I have been previously described as indicated. The remaining compounds (**10**, **11**, **13-15**) were synthesized by proper modification

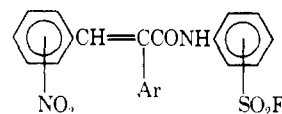
(12) B. R. Baker and G. J. Lourens, *J. Med. Chem.*, **11**, 39 (1968), paper CXII of this series.

of the routes described earlier.^{5,8,12} The physical properties of **10-24** are given in Tables II-IV.

 TABLE III
 PHYSICAL PROPERTIES OF


No. ^a	NO ₂ position	Ar	% yield	Mp, °C dec	Formula ^d
16	<i>meta</i>	C ₆ H ₅	37	178-181 ^b	
17	<i>para</i>	<i>o</i> -C ₆ H ₄ OMe	47	200-202	C ₁₅ H ₁₃ NO ₃
18	<i>para</i>	<i>m</i> -C ₆ H ₄ OMe	35	220-222	C ₁₅ H ₁₃ NO ₃
19	<i>para</i>	α -Naphthyl	54	>215 ^c	C ₁₉ H ₁₃ NO ₃

^a Compounds were prepared by the method previously described for *p*-nitro- α -(*p*-tolyl)cinnamic acid⁵ and recrystallized from EtOH. ^b T. R. Lewis, M. G. Pratt, E. D. Homiller, B. F. Tullar, and S. Archer, *J. Am. Chem. Soc.*, **71**, 3749 (1949), have recorded mp 183-185°. ^c Gradually decomposes over this temperature. ^d Compounds **17-19** gave correct analyses for C, H, N.

 TABLE IV
 PHYSICAL PROPERTIES OF


No. ^a	NO ₂ position	SO ₂ F position	Ar	% yield	Mp, °C	Formula ^d
20	<i>meta</i>	<i>para</i>	C ₆ H ₅	56 ^b	157-158	C ₂₅ H ₁₆ FN ₂ O ₅ S
21	<i>para</i>	<i>meta</i>	C ₆ H ₅	58 ^b	141-142	C ₂₅ H ₁₆ FN ₂ O ₅ S
22	<i>para</i>	<i>para</i>	<i>o</i> -C ₆ H ₄ OMe	62 ^c	203-204	C ₂₂ H ₁₇ FN ₂ O ₅ S
23	<i>para</i>	<i>para</i>	<i>m</i> -C ₆ H ₄ OMe	66 ^b	204-205 dec	C ₂₂ H ₁₇ FN ₂ O ₅ S
24	<i>para</i>	<i>para</i>	α -Naphthyl	53 ^b	240-242	C ₂₅ H ₁₇ FN ₂ O ₅ S

^a All compounds were prepared by method A previously described.⁵ ^b Recrystallized from MeOEtOH-H₂O. ^c Recrystallized from MeOEtOH. ^d All compounds gave correct analyses for C, H, N.

(13) Melting points were taken in capillary tubes on a Mel-Temp block and are uncorrected. Each analytical sample had ir and uv spectra compatible with their assigned structure; each gave combustion values for C, H, and N or F within 0.4% of theoretical.