# Irreversible Enzyme Inhibitors. CXXXIX.[1,2] *p*-(4,6-Diamino-1,2-dihydro-2,2-dimethyl-*s*-triazin-1-yl)phenylpropionylsulfanilyl 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

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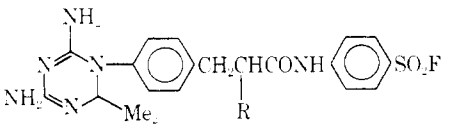
Thirteen candidate irreversible inhibitors of dihydrofolic reductase that have an alkyl, aryl, or aralkyl group on the propionamide bridge of the title compound (**1**) have been investigated with this enzyme from Walker 256 rat tumor, rat liver, three strains of L1210 mouse leukemia, and mouse liver. 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 $SO_2F$ group.

In an earlier study on substitution[5] 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. Compounds **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 inhibition[8] of tumor *vs.* liver enzyme could be achieved. The results are the subject of this paper.



1. R = H
2. R = $C_6H_5$
3. R = *o*-$C_6H_4CH_3$
4. R = *m*-$C_6H_4CH_3$
5. R = *p*-$C_6H_4CH_3$

**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 inhibitor[9] 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 inhibitor[9] 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 reported[6] 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) 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 CXXIX 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.

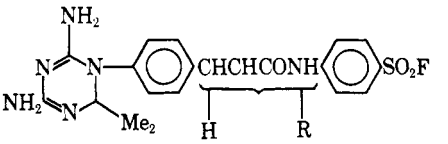
(9) A good irreversible inhibitor is defined as one that at an $I_{50} \simeq 6K_i$ concentration gives greater than 70% inactivation of an enzyme; an excellent irreversible inhibitor gives greater than 70% inactivation at $I_{50}/6 = K_i$; a fair irreversible inhibitor shows >70% inactivation at $30K_i \simeq 5I_{50}$. The dependence of the extent of irreversible inhibition on concentration of $SO_2F$-type irreversible inhibitors is due to the competition between enzyme inactivation and enzyme-catalyzed hydrolysis of the $SO_2F$ group within the enzyme–inhibitor complex;[10] the ratio of these two rates is sensitive to structural change.[3,5,11]

(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



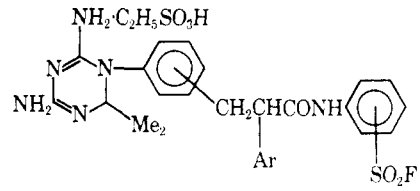
| No. | R | Enzyme source | Reversible[b] $I_{50}$,[d] $\mu M$ | Reversible[b] $K_i$,[e] $\mu M$ | Irreversible[c] Inhib, $\mu M$ | Irreversible[c] % EI[f] | Irreversible[c] Time, min | Irreversible[c] % inactvn |
|---|---|---|---|---|---|---|---|---|
| 1 | H | Walker 256[g] | 0.020 | 0.003 | 0.020 | 87 | 1, 3 | 50, 90[h] |
| | | Rat liver[g] | 0.006 | 0.001 | 0.020 | 95 | 8[i] | 50[h] |
| | | | | | 0.050 | 98 | <2, 60 | 70, 70[h] |
| | | L1210/FR8[g] | 0.080 | 0.01 | 0.070 | 84 | <2, 10 | 84, 84[h] |
| | | L1210/DF8[j] | 0.025 | 0.004 | 0.070 | 94 | 60 | 73[k] |
| | | L1210/0[j] | 0.012 | 0.002 | 0.070 | 98 | 60 | 53[k] |
| | | Mouse liver[g] | | | 0.070 | | 2, 60 | 38, 38[h] |
| 2 | $\alpha$-$C_6H_5$ | Walker 256[g] | 0.074 | 0.01 | 0.074 | 87 | 4, 9, 60 | 50, 71, 71[h] |
| | | Rat liver[g] | 0.18 | 0.03 | 0.074 | 84 | 2, 8, 30 | 50, 88, 94[h] |
| | | L1210/FR8 | 0.20 | 0.03 | 1.2 | 96 | <2, 60 | 65, 65[h] |
| | | | | | 0.16 | 86 | 60 | 27 |
| | | L1210/DF8 | 0.045 | 0.007 | 0.48 | 99 | 60 | 34[k] |
| | | L1210/0 | | | 0.48 | | 60 | 47 |
| 3 | $\alpha$-($o$-$C_6H_4CH_3$) | 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 | $\alpha$-($m$-$C_6H_4CH_3$) | L1210/DF8 | | | 0.42 | | 60 | 65[k] |
| | | L1210/0 | | | 0.42 | | 60 | 52[k] |
| | | Mouse liver | 0.20 | 0.03 | 0.42 | 92 | 60 | 20[k] |
| 5 | $\alpha$-($p$-$C_6H_4CH_3$) | L1210/FR8 | 0.63 | 0.1 | 3.1 | 97 | 60 | 13 |
| | | L1210/0 | | | 1.2 | | 60 | 0 |
| 6 | $\alpha$-Me | L1210/0 | 0.050[n] | | 0.10 | | 60 | 0 |
| | | L1210/DF8 | | | 0.10 | | 60 | 0 |
| 7 | $\alpha$-$(CH_2)_2C_6H_5$ | L1210/0 | 1.9[n] | | 3.8 | | 60 | 0 |
| | | L1210/DF8 | | | 3.8 | | 60 | 0 |
| 8 | $\beta$-Me | L1210/DF8[j] | | | 0.16 | | 60 | 40[k] |
| | | L1210/0[j] | 0.044 | 0.007 | 0.16 | 97 | 60 | 25[k] |
| | | Mouse liver[j] | 0.080 | 0.01 | 0.40 | 97 | 60 | 0[k] |
| | | Mouse spleen[j] | | | 0.16 | | 60 | 38[k] |
| 9 | N-Me | L1210/0 | 0.041[n] | | 0.082 | | 60 | 0 |
| 10 | $\alpha$-$C_6H_5$[l] | Walker 256 | 0.54 | 0.09 | 2.7 | 97 | 60 | 0 |
| | | L1210/FR8 | 0.48 | 0.06 | 2.4 | 97 | 60 | 0 |
| | | L1210/0 | | | 0.96 | | 60 | 0 |
| 11 | $\alpha$-$C_6H_5$[m] | Walker 256 | | | 0.65 | | 60 | 10[k] |
| | | L1210/DF8 | 0.13 | 0.02 | 0.65 | 97 | 60 | 0 |
| | | L1210/0 | | | 0.65 | | 60 | 10[k] |
| 12 | H[m] | Rat liver | 0.012 | 0.002 | 0.060 | 96 | 2, 20 | 72, 71 |
| | | L1210/0 | | | 0.10 | | 60 | 53[k] |
| | | L1210/DF8 | 0.018 | 0.003 | 0.10 | 98 | 60 | 49[k] |
| | | Mouse liver | | | 0.20 | | 60 | 26[k] |
| 13 | $\alpha$-($o$-$C_6H_4OCH_3$) | Walker 256 | | | 1.0 | | 60 | 83 |
| | | Rat liver | | | 1.0 | | 60 | 75 |
| | | L1210/DF8 | 0.47 | | 2.4 | 97 | 60 | 76[k] |
| | | L1210/0 | | | 2.4 | | 60 | 57[k] |
| 14 | $\alpha$-($m$-$C_6H_4OCH_3$) | Walker 256 | | | 0.70 | | 60 | 73 |
| | | Rat liver | | | 0.70 | | 60 | 57 |
| | | L1210/DF8 | 0.35 | | 1.8 | 97 | 60 | 71[k] |
| 15 | $\alpha$-($\alpha$-Naphthyl) | Walker 256 | | | 1.1 | | 60 | 78 |
| | | Rat liver | | | 1.1 | | 60 | 54 |
| | | L1210/DF8 | 0.57 | | 2.8 | | 60 | 61[k] |

[a] The technical assistance of Sharon Lafler, Diane Shea, and Carolyn Wade with these assays is acknowledged. [b] Assayed with 6 $\mu M$ dihydrofolate and 30 $\mu M$ 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 $\mu M$ TPNH as previously described.[7] [d] $I_{50}$ = concentration for 50% inhibition. [e] Estimated from $K_i = K_m[I_{50}]/[S]$ which is valid since $[S] = 6K_m = 6\ \mu M$ 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] $SO_2F$ connected *meta* to propionamide bridge. [n] $I_{50}$ on L1210/FR8 enzyme.[5]

inhibition of the mouse liver enzyme at a concentration of $5I_{50}$.

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 $\beta$-methyl derivative (**8**) should be pursued, and (2) further $\alpha$-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 | $SO_2F$ position | Ar | % yield | Mp, °C dec | Formula[d] |
|---|---|---|---|---|---|---|
| 10 | *meta* | *para* | $C_6H_5$ | 32[b] | 235–236 | $C_{26}H_{27}FN_6O_3S \cdot C_2H_5SO_3H$ |
| 11 | *para* | *meta* | $C_6H_5$ | 47[c] | 215–216 | $C_{26}H_{27}FN_6O_3S \cdot C_2H_5SO_3H$ |
| 13 | *para* | *para* | *o*-$C_6H_4OMe$ | 60[b] | 208–209 | $C_{27}H_{29}FN_6O_4S \cdot C_2H_5SO_3H$ |
| 14 | *para* | *para* | *m*-$C_6H_4OMe$ | 43[b] | 230–231 | $C_{27}H_{29}FN_6O_4S \cdot C_2H_5SO_3H$ |
| 15 | *para* | *para* | α-Naphthyl | 45[c] | 229–231 | $C_{30}H_{29}FN_6O_3S \cdot C_2H_5SO_3H$ |

[a] Compounds were prepared by method B previously described;[5] each had an ir band at 1395–1405 $cm^{-1}$ characteristic of $SO_2F$. [b] Recrystallized from *i*-PrOH–$H_2O$. [c] Recrystallized from EtOH–$H_2O$. [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 inhibitor[9] of the rat liver enzyme and a fair to poor irreversible inhibitor of the dihydrofolic 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.

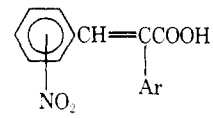
Three additional α-aryl derivatives (**13–15**) were then synthesized for evaluation. The *o*-anisyl derivative (**13**) was a fair irreversible inhibitor[9] of both the Walker 256 rat tumor and rat liver enzymes, but a fair to poor irreversible inhibitor of the L1210/DFS and L1210/0 enzymes; the *m*-anisyl (**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 Section[13]

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 of the routes described earlier.[5,8,12] The physical properties of **10–24** are given in Tables II–IV.

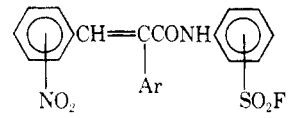
Table III
Physical Properties of



| No.[a] | $NO_2$ position | Ar | % yield | Mp, °C dec | Formula[d] |
|---|---|---|---|---|---|
| 16 | *meta* | $C_6H_5$ | 37 | 178–181[b] | |
| 17 | *para* | *o*-$C_6H_4OMe$ | 47 | 200–202 | $C_{16}H_{13}NO_5$ |
| 18 | *para* | *m*-$C_6H_4OMe$ | 35 | 220–222 | $C_{16}H_{13}NO_5$ |
| 19 | *para* | α-Naphthyl | 54 | >215[c] | $C_{19}H_{13}NO_4$ |

[a] Compounds were prepared by the method previously described for *p*-nitro-α-(*p*-tolyl)cinnamic acid[5] 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 | $SO_2F$ position | Ar | % yield | Mp, °C | Formula[d] |
|---|---|---|---|---|---|---|
| 20 | *meta* | *para* | $C_6H_5$ | 56[b] | 157–158 | $C_{21}H_{15}FN_2O_5S$ |
| 21 | *para* | *meta* | $C_6H_5$ | 58[b] | 141–142 | $C_{21}H_{15}FN_2O_5S$ |
| 22 | *para* | *para* | *o*-$C_6H_4OMe$ | 62[c] | 203–204 | $C_{22}H_{17}FN_2O_6S$ |
| 23 | *para* | *para* | *m*-$C_6H_4OMe$ | 66[b] | 204–205 dec | $C_{22}H_{17}FN_2O_6S$ |
| 24 | *para* | *para* | α-Naphthyl | 53[b] | 240–242 | $C_{25}H_{17}FN_2O_5S$ |

[a] All compounds were prepared by method A previously described.[5] [b] Recrystallized from MeOEtOH–$H_2O$. [c] Recrystallized from MeOEtOH. [d] All compounds gave correct analyses for C, H, N.

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

(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.