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STEREOCHEMISTRY OF DIHYDROFOLATE REDUCTASE INHIBITOR ANTITUMOR AGENTS: MOLECULAR STRUCTURE OF "BAKER'S ANTIFOL" (NSC 139105, TRIAZINATE) AND "INSOLUBLE BAKER'S ANTIFOL" (NSC 113423)

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SUMMARY. The crystal and molecular structures of 1-[3-chloro-4-(m-dimethylcarbamoylbenzyloxy)] phenyl-4,6-diamino-1,2-dihydro-2,2-dimethyl-s-triazine ethanesulfonate, (Baker's antifol), and 1-[4-(N-[3'-methyl-4'-fluorosulfonyl] phenyl) propanamide] phenyl-4,6-diamino-1,2-dihydro-2,2-dimethyl-s-triazine ethanesulfonate dihydrate (insoluble Baker's antifol) have been determined by X-ray crystallography. These compounds are, respectively, reversible and irreversible inhibitors of dihydrofolate reductase and show clinical promise for use in cancer chemotherapy. Both molecules adopt an extended conformation and are protonated at one of the triazine ring nitrogens.

INTRODUCTION

Tetrahydrofolic acid (FAH_4) and dihydrofolic acid (FAH_2), enzymatic reduction products of the B vitamin folic acid, are essential for purine and pyrimidine biosynthesis. In the reaction whereby deoxyuridylate is converted to thymidylate by thymidylate synthetase, FAH_4 is oxidized to FAH_2 and must then be re-reduced by dihydrofolate reductase (DHFR) if pyrimidine biosynthesis is to continue. Inhibition of either thymidylate synthetase or DHFR can lead to cellular deficiency of thymidylate and "thymine-less" cell death. If very selective inhibitors could be developed which could exploit evolutionary and species differences between enzymes of normal and tumor cells and bind only to tumor enzymes, these agents would be extremely valuable cancer chemotherapy drugs.

Much effort has been expended on obtaining possible selective DHFR inhibitors which could be used in cancer treatment, particularly by the late

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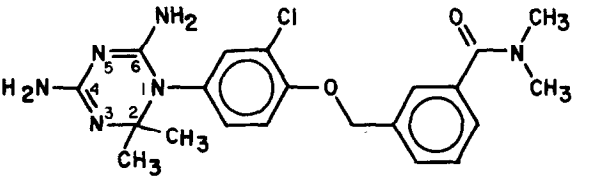
B. R. Baker in whose laboratory scores of compounds have been designed, synthesized and evaluated for *in vitro* and *in vivo* antitumor activity (1). The pioneering work of Baker and his co-workers has resulted in the development of a number of reversible and irreversible DHFR inhibitors which have shown activities against Walker 256 and Dunning leukemia ascites in the rat (2,3). Systematic design of new antifolate compounds has been hampered, however, by a lack of information on the three-dimensional stereochemistry of folic acid and of the inhibitors of DHFR. We have therefore undertaken a program of X-ray crystallographic analyses of folates and antifolates in order to supply conformational data which can aid in design and synthesis of new possible anticancer drugs.

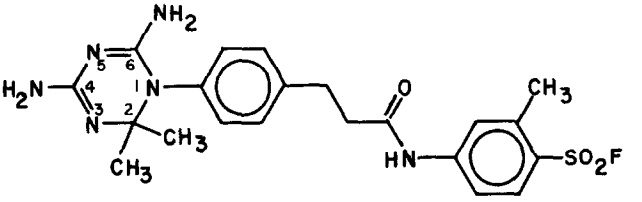
Overall the most active of Baker's compounds are 1-[3-chloro-4-(*m*-dimethylcarbamoylbenzyloxy)] phenyl-4,6-diamino-1,2-dihydro-2,2-dimethyl-*s*-triazine ethanesulfonate, (Baker's antifol, triazinate), and 1-[4-(N-[3'-methyl-4'-fluorosulfonyl] phenyl) propanamide] phenyl-4,6-diamino-1,2-dihydro-2,2-dimethyl-*s*-triazine ethanesulfonate dihydrate (insoluble Baker's antifol), which currently show promise in clinical trials as cancer chemotherapeutic drugs. It is these compounds whose three-dimensional crystal and molecular structures we now report. They are the first antifols, which have been used clinically in cancer chemotherapy to be crystallographically investigated. They are also the first antifolate compounds of a size comparable to folic acid to have their conformations determined, and thus may provide indirect evidence for the structures of folates and dihydrofolates.

METHODS

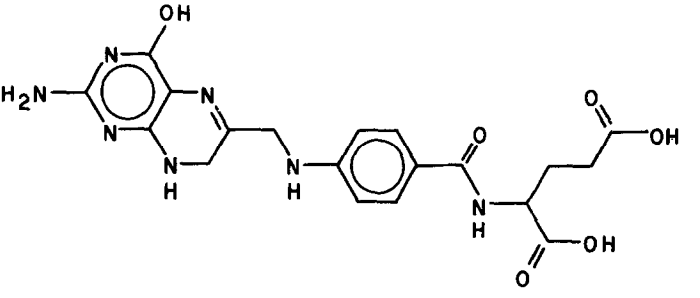
Colorless crystals of Baker's antifol (BAF) were obtained from slow evaporation of an ethanol-isopropanol-water solution. They are triclinic, space group $P\bar{1}$, with cell dimensions a = 11.522, b = 11.717, c = 11.474Å, α = 110.78°, β = 111.47°, γ = 87.85°. X-ray intensities were measured on an automated diffractomer using Mo$_{K\alpha}$ radiation. Of the 6151 reflections measured with interplanar spacings down to 0.78Å, 4985 had intensity significantly above background and were used in structure determination and refinement. The structure was solved by direct methods. Postions of 33 of the 36 non-hydrogen atoms were located in a three-dimensional E-map computed from the best set of phases generated by the program MULTAN. The remaining 3 atoms were located from a subsequent difference synthesis. After refinement of the atom postions



SOLUBLE BAKER'S ANTIFOL



INSOLUBLE BAKER'S ANTIFOL



DIHYDROFOLIC ACID

and thermal parameters by least-squares methods, a second difference fourier synthesis enabled all 31 hydrogen atoms to be located. Several additional cycles of least-squares refinement of all atom positions with anisotropic thermal parameters for the non-hydrogen atoms and isotropic ones for the hydrogens resulted in a final discrepancy index of R = 0.060.

Recrystallization of insoluble Baker's antifol (IBAF) from a hot water-dimethylformamide mixture yielded colorless triclinic crystals in space group $P\bar{1}$, with cell dimensions $\underline{a}$ = 12.929, $\underline{b}$ = 17.515, $\underline{c}$ = 6.572A, $\alpha = 99.08^{\circ}$, $\beta = 90.42^{\circ}$, $\gamma = 94.08^{\circ}$. Of the 5147 X-ray intensities measured on an automated diffractometer (minimum interplanar spacing of 0.84Å), 4345 had intensity significantly above background and were used in the structure determination. The structure was solved by direct methods. The locations of 32 of the 40 non-hydrogen atoms were obtained from an E-map computed from phases calculated by the program MULTAN. A subsequent difference synthesis located the remaining 8 atoms. Following several cycles of least-squares refinement, a second difference map was used to locate the 35 hydrogen atoms.

The least-squares refinement of all atom positions and anisotropic thermal parameters on the non-hydrogen atoms converged with a final discrepancy index, R = 0.052.

DISCUSSION

In their design of new antifolates Baker and his co-workers have had to adhere to key structural principles. Substituent groups which promote water solubility and thus make intravenous administration of the drug feasible have to be balanced with the need to maintain sufficient lipophilicity for good membrane permeability. Factors which influence active transport of drugs also have to be taken into account as it has been shown that compounds of comparable polarity can differ dramatically in their relative abilities to penetrate cell membranes (3). These properties depend not only on the drug molecule's functional groups, but also on molecular stereochemistry, that is, the manner in which the functional groups are distributed and oriented in three-dimensional space. Steric considerations indeed play a major role in antifolate drug design strategy: successful inhibitors may utilize one part of their molecular architecture to bind to DHFR outside the enzyme active site and another part to block the active site from reaction with the folate substrate. Differences in molecular stereochemistry will also determine differential antifolate binding characteristics to DHFR's of normal and tumor cells. Folic acid was isolated and characterized almost thirty years ago (4), but despite long term efforts in many laboratories, it has never been successfully crystallized for an X-ray crystallographic structure determination. Likewise, the conformation of methotrexate, the most widely used antifolate cancer chemotherapy agent, has not yet been crystallographically elucidated. However, since BAF and IBAF are both of comparable size to folic acid and methotrexate, and have demonstrated clinical anticancer potential as inhibitors of dihydrofolate reductase, the three-dimensional conformations of these two "non-classical" antifols will enable us to draw strong inferences on the three-dimensional stereochemistry of folic acid and the classical antifols.

Figures 1 and 2 are stereoscopic drawings showing the three-dimensional molecular structures of BAF and IBAF, respectively. A significant feature of both molecules is their linearly extended conformation. For BAF the relationship between the two substituted phenyl rings is described by the 176° phenyl-O-CH_2-phenyl dihedral angle. In IBAF this relationship is described by three dihedral angles, all close to 180°: a phenyl-CH_2-CH_2-C angle of -177°, a CH_2-CH_2-C-N angle of -174° and a CH_2-C-N-phenyl angle of -173°. In both molecules the triazine ring and the adjacent phenyl ring are nearly perpendicular. For BAF the C-N-C-C dihedral angle is 85°, the corresponding dihedral angle for IBAF is -87°. The two or four atom bridge between the phenyl groups is, in each case, in the plane of the central phenyl ring. In the case of IBAF the terminal phenyl ring is also in this plane, while for BAF the terminal phenyl is rotated so the dimethycarbamoyl group is on the same side of the molecular long axis as the chlorine atom. Protonation by the ethanesulfonic acid takes place at N_3 of the triazine ring for both molecules.

It is likely that both ends of the folic acid antagonists reported here would bind to DHFR. The triazine end, being similar to folic acid, likely effects the enzyme inhibition by blocking the active site, while the dimethylbenzamide end of BAF and the phenylsulfonylfluoride end of IBAF probably interacts with the enzyme outside the active site. Hence, the distances, shown in Table I, between functional groups at the two ends of the antifolate will be a major factor in its DHFR inhibitory power.

Table I. Distances(Å) from Triazine Ring Center.

	BAF	IBAF
Terminal phenyl ring center	10.5	12.9
Carbonyl oxygen	11.9	9.9
Amide nitrogen	12.7	
Sulfonyl oxygens		16.3 - 16.6
Sulfonyl fluorine		16.5

This suggests a second binding site in DHFR 11-12Å from the active site, with a possible additional site at a distance of 16-17Å.

The linearly extended configuration as found for BAF and IBAF has also

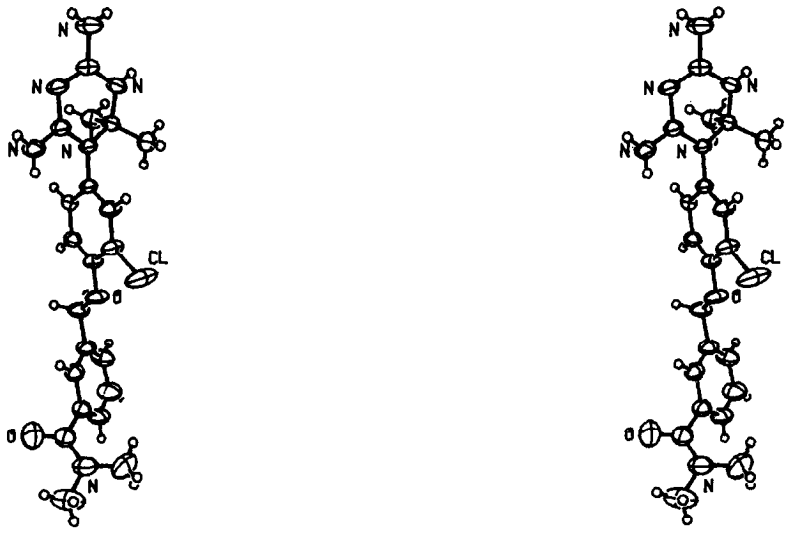


Figure 1. Stereoscopic drawing of Baker's Antifol (BAF).

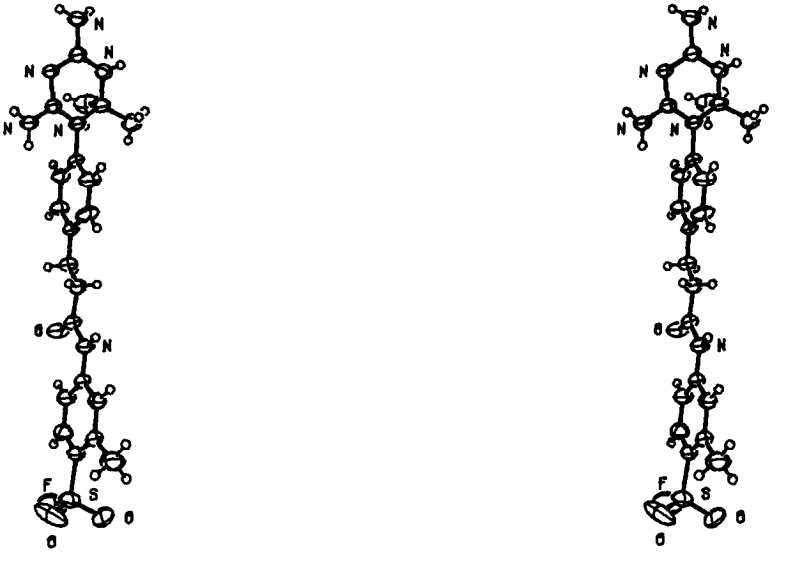


Figure 2. Stereoscopic drawing of Insoluble Baker's Antifol (IBAF).

been suggested for methotrexate bound to DHFR (5). Although not all individual atoms can be discerned in the enzyme-inhibitor complex, there are strong indications that methotrexate is linearly extended with the pyrimidine end in a hydrophobic pocket, the p-amino benzoyl moiety in a second hydrophobic pocket and the glutamic acid end bound at the enzyme surface.

These results suggest that other antifolates and folic acid itself likely adopt a similar extended conformation.

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