Trifluoroacetyl-α-L-aspartyl-3,4-dinitroanilide (21). The 3,4-dinitroaniline has been obtained according to Nielsen et al.[17]

The title compound was prepared according to method B: yield 27%; TLC R_f (A) 0.47; mp 180–182 °C; $[\alpha]^{25}_D$ −58.5° (c 1, MeOH); NMR (Me_2SO-d_6) δ 11.25 (s, 1 H, NH Ar), 10.00 (m, 1 H, NH_α), 8.40–7.87 (m, 3 H, Ar), 4.83 (m, 1 H, CH), 2.90 (m, 2 H, CH_3). Anal. ($C_{12}H_9N_4O_3F_3$) C, H, N.

Trifluoroacetyl-α-L-aspartyl-2,3-dinitroanilide (22). The 2,3-dinitroaniline was prepared according to Nielsen et al.[17]

The title compound was prepared according to method B: yield 15%; TLC R_f (A) 0.37; mp 182–185 °C; $[\alpha]^{25}_D$ −88.8 °C (c 1, MeOH); NMR (Me_2SO-d_6) δ 10.03 (m, 2 H, NH_α, NH Ar), 8.15–7.90 (m, 3 H, Ar), 4.93 (m, 1 H, CH), 2.90 (m, 2 H, CH_2). Anal. ($C_{12}H_9N_4O_3F_3$) C, H, N.

Trifluoroacetyl-α-L-aspartyl-2-nitro-4-chloroanilide (23). This compound was prepared according to method B: yield 24%; TLC R_f (A) 0.39; mp 159–161 °C; $[\alpha]^{25}_D$ −95.9° (c 1, MeOH); NMR (Me_2SO-d_6) δ 10.67 (s, 1 H, NH Ar), 9.97 (m, 1 H, NH_α), 8.04–7.77 (m, 3 H, Ar), 4.85 (m, 1 H, CH), 2.83 (m, 2 H, CH_2). Anal. ($C_{12}H_9N_3O_6ClF_3$) C, H, N.

Trifluoroacetyl-α-L-aspartyl-2-chloro-4-nitroanilide (24). This compound was prepared according to method B: yield 23%; TLC R_f (A) 0.38; mp 162–164 °C; $[\alpha]^{25}_D$ −69.5° (c 1, MeOH); NMR (Me_2SO-d_6) δ 10.10 (m, 2 H, NH_α, NH Ar), 8.33–8.20 (m, 3 H, Ar), 5.03 (m, 1 H, CH), 2.85 (m, 2 H, CH_2). Anal. ($C_{12}H_9N_3O_6ClF_3$) C, H, N.

Trifluoroacetyl-α-L-aspartyl-3-methyl-4-nitroanilide (25). The 3-methyl-4-nitroaniline was prepared according to Wibaut.[18]

The title compound was prepared according to method B: yield 24%; TLC R_f (A) 0.44; mp 167–169 °C; $[\alpha]^{25}_D$ −55.9 (c 1, MeOH); NMR (Me_2SO-d_6) δ 10.71 (s, 1 H, NH Ar), 9.88 (d, 1 H, NH_α), 8.10–7.55 (m, 3 H, Ar), 4.83 (m, 1 H, CH), 2.85 (m, 2 H, CH_2), 2.53 (s, 3 H, CH_3). Anal. ($C_{13}H_{12}N_3O_6F_3$) C, H, N.

Acknowledgment. We thank Joseph Tsang and Judd Berman for helpful discussions. We thank the National Institute of Denatl Research for their support of this research through Grant DE 05476.

Registry No. **1**, 41696-59-7; **2**, 41566-94-3; **3**, 92398-66-8; **4**, 48193-99-3; **5**, 39219-30-2; **6**, 92398-67-9; **7**, 92398-68-0; **8**, 61980-46-9; **9**, 92398-69-1; **10**, 92398-70-4; **11**, 41567-05-9; **12**, 92398-71-5; **13**, 92398-72-6; **14**, 41567-06-0; **15**, 41567-00-4; **16**, 92398-73-7; **18**, 92398-74-8; **19**, 92398-75-9; **20**, 92398-76-0; **21**, 92398-77-1; **22**, 92398-78-2; **23**, 92398-79-3; **24**, 92398-80-6; **25**, 92398-81-7; *N*-(*tert*-butyloxycarbonyl)-L-aspartic acid β-*tert*-butyl ester, 1676-90-0; trifluoroacetic anhydride, 407-25-0; 4-nitro-*N*-methylaniline, 100-15-2; 3,4-dinitroaniline, 610-41-3; 2,3-dinitroaniline, 602-03-9; 3-methyl-4-nitroaniline, 611-05-2; *m*-chloroaniline, 108-42-9; *o*-chloroaniline, 95-51-2; *m*-cyanoaniline, 2237-30-1; *o*-cyanoaniline, 1885-29-6; *m*-nitroaniline, 99-09-2; *o*-nitroaniline, 88-74-4; *p*-toluidine, 106-49-0; *m*-toluidine, 108-44-1; *o*-toluidine, 95-53-4; 2-methyl-4-nitroaniline, 99-52-5; 2-methyl-3-nitroaniline, 603-83-8; 2-methyl-5-nitroaniline, 99-55-8; 4-chloro-2-nitroaniline, 89-63-4; 2-chloro-4-nitroaniline, 121-87-9.

(17) A. T. Nielsen, R. T. Atkins, W. P. Norris, C. L. Coon, and M. E. Sitzmann, *J. Org. Chem.*, **45**, 2341 (1980).

(18) J. P. Wibaut, *Recl. Trav. Chim. Pays-Bas*, **32**, 287 (1913).

(19) We repeated the synthesis of the o-chloroanilide **4**, which Lapidus and Sweeney described as "not sweet", and found this compound to be slightly bitter.

A ^{1}H NMR Study of the Interactions and Conformations of Rationally Designed Brodimoprim Analogues in Complexes with *Lactobacillus casei* Dihydrofolate Reductase

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Physical Biochemistry Division, National Institute for Medical Research, Mill Hill, London NW7 1AA, England, and F. Hoffman-La Roche & Co., CH 4002, Basle, Switzerland, and Roche Products Ltd., Welwyn, England. *Received March 20, 1984*

A consideration of the detailed structural information available from X-ray crystallographic and NMR studies on complexes of dihydrofolate reductase with inhibitors has led to the design of trimethoprim analogues with improved binding properties. Computer graphic techniques have been used to predict which substituent groups were required at the 3′-O position of brodimoprim (2,4-diamino-5-(3,5-dimethoxy-4-bromobenzyl)pyrimidine) to make additional interactions with the enzyme. NMR spectroscopy provided a convenient method of assessing if the analogues were binding in the predicted manner. On the basis of this approach, the C4,C6-dicarboxylic acid analogue IX was designed to interact with Arg-57 and His-28 in the enzyme, and this analogue was found to bind 3 orders of magnitude more tightly than the parent brodimoprim.

The antibacterial drug trimethoprim (2,4-diamino-5-(3,4,5-trimethoxybenzyl)pyrimidine) acts by selectively inhibiting dihydrofolate reductase in bacterial cells. In the past, many trimethoprim analogues have been investigated in attempts to find inhibitors that are either more selective or more active against resistant strains. With the recent availability of detailed structural information on complexes of dihydrofolate reductase with inhibitors from both X-ray crystallography[1-4] and NMR spectroscopy,[5,6] we now have a framework for designing trimethoprim analogues with modified binding characteristics. These techniques also provide methods of monitoring the complexes formed with new inhibitors to assess whether or not they are binding in the predicted manner. Kuyper and co-workers[7] have recently used this structural approach to design a series of trimethoprim analogues with aliphatic ω-carboxylic acid substituents arranged to interact favorably with the con-

† National Institute for Medical Research.
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§ Roche Products Ltd.

(1) Matthews, D. A.; Alden, R. A.; Bolin, J. T.; Freer, S. T.; Hamlin, R.; Xuong, N.; Kraut, J.; Poe, M.; Williams, M.; Hoogsteen, K. *Science* **1977**, *197*, 452.
(2) Matthews, D. A.; Alden, R. A.; Bolin, J. T.; Filman, D. J.; Freer, S. T.; Hamlin, R.; Hol, W. G. J.; Kisliuk, R. L.; Pastore, E. J.; Plante, L. T.; Xuong, N.; Kraut, J. *J. Biol. Chem.* **1978**, *253*, 6946.
(3) Bolin, J. T.; Filman, D. J.; Matthews, D. A.; Hamlin, R. C.; Kraut, J. *J. Biol. Chem.* **1982**, *257*, 13650.
(4) Matthews, D. A.; Alden, R. A.; Freer, S. T.; Xuong, N.; Kraut, J. *J. Biol. Chem.* **1979**, *254*, 4144.
(5) Cayley, P. J.; Albrand, J. P.; Feeney, J.; Roberts, G. C. K.; Piper, E. A.; Burgen, A. S. V. *Biochemistry* **1979**, *18*, 3887.
(6) Birdsall, B.; Roberts, G. C. K.; Feeney, J.; Dann, J. G.; Burgen, A. S. V. *Biochemistry* **1983**, *22*, 5597.
(7) Kuyper, L. F.; Roth, B.; Baccanari, D. P.; Ferone, R.; Beddell, C. R.; Champness, J. N.; Stammers, D. K.; Dann, J. G.; Norrington, F. E. A.; Baker, D. J.; Goodford, P. J. *J. Med. Chem.* **1982**, *25*, 1120.

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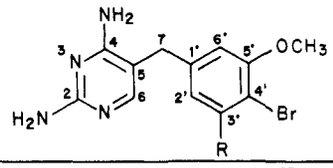
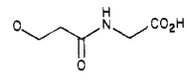
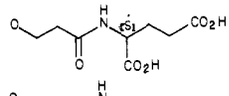
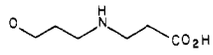
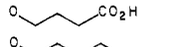
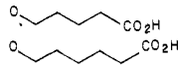
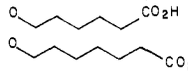
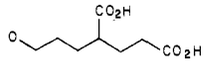
Table I. Inhibition Constants (K_i) and His-28 pK Values for Complexes of *L. casei* Dihydrofolate Reductase with Brodimoprim and Its Derivatives (I–IX)[8]



compd	R	K_i, nM	pK His-28
I	OCH_3	11.3	6.80
II		4.1	6.71
III		1.7	7.12
IV		5.3	6.71
V		0.9	
VI		0.2	6.83
VII		0.4	
VIII		0.6	6.80
IX		<0.01	7.80
methotrexate		0.004	7.80

served Arg-57 residue in dihydrofolate reductase isolated from *Escherichia coli* and have used crystallography to study their binding to the enzyme. Concurrently we have been exploring analogues of brodimorpim (I) that would be expected to make similar interactions with Arg-57 and additional interactions with other positively charged residues in *Lactobacillus casei* dihydrofolate reductase. We now describe these analogues and the use of high-resolution NMR spectroscopy to characterize the interactions and conformations of these analogues in their complexes with the enzyme.

Methods

Brodimoprim (I) and its analogues (II–IX) (see Table I) were synthesized by using procedures reported previously.[8,9]

Dihydrofolate reductase was isolated and purified from *L. casei* as described previously.[10] The enzyme used in the NMR experiments was lyophilized three times from ^2H_2O to exchange all the exchangeable protons for deuterons and was then examined as ~1 mM solutions in 50 mM potassium phosphate, 500 mM potassium chloride, ^2H_2O buffer containing 1 mM dioxane as an internal reference. Complexes of the brodimoprim analogues with the enzyme were formed by adding microliter volumes (containing slightly more than 1 molar equiv) of concentrated solutions of the ligands in ^2H_2O or Me_2SO-d_6 solution to the enzyme solution; the final Me_2SO-d_6 concentration was less than 2% v/v. Compound IX was a racemate, and 2 molar equiv were added to the enzyme in this case.

^1H NMR spectra were obtained at 25 °C with Bruker WH270 (270 MHz) and AM500 (500 MHz) spectrometers operating in the Fourier-transform mode. The pH dependence of the chemical shifts of the histidine C-2 proton resonances was determined and analyzed as described previously.[11,12] The pH measurements were made with a Radiometer Model 26 pH meter equipped with a glass electrode: the notation pH* denotes a pH meter reading uncorrected for deuterium isotope effects.

Transfer of saturation experiments, used to detect the resonances of protons in bound ligands, were carried out by using selective irradiations at 20-Hz intervals over the frequency range of interest as described previously.[5] The irradiation was applied for 1.0 s and then gated off before applying a 90° observing pulse. Saturation of a nucleus of the bound ligand on irradiation at its resonance frequency results in a decrease in intensity of the signal from the corresponding nucleus of the free ligand if the exchange rate of the latter is faster than its relaxation rate. Dihydrofolate reductase activity was determined by using a spectrophotometric assay by measuring the decrease in absorbance at 340 nm at 37 °C. A microcomputerized Kontron Uvicon 810 spectrophotometer was employed, and reaction rates were either traced on the recorder (usually for 5–10 min) or directly computed over the time intervals in which the reaction proceeded in a linear fashion, as judged by correlation coefficients close to 1.0. K_i values were determined according to Henderson[13] and as described in detail by Baccanari and Joyner[14] with the following modifications of the assay: the final volume of 2 mL contained 30 μM dihydrofolate, 55 μM NADPH, approximately 0.6 nM enzyme and inhibitor, if required. Enzyme was preincubated with inhibitor and NADPH and the reaction started by diluting aliquots of this mixture into the cuvette, containing buffer, NADPH, and dihydrofolate. Imidazole hydrochloride (100 mM), pH 7.0, containing 2 mM ascorbate and 2 mM EDTA was used as the buffer, and all reagents were dissolved in this buffer. The K_m of dihydrofolate used to calculate K_i was determined to be 2.2 μM under these conditions. The data were evaluated by using the equation $I_t/(1 - v_i/v_0) = K_i(1 + S/K_m)v_0/v_i + E_t$, where I_t is the total inhibitor concentration, v_0 is velocity of the uninhibited reaction, v_i is the velocity of inhibited reaction, S is the concentration of competing substrate, and E_t is the total enzyme concentration.[13]

The early molecular modeling was carried out on a PDP11/40 host computer interfaced to a Megatek 7000 display processor and later molecular modelling on a VAX-11/780 host computer with an Evans Sutherland Color MPS and a Tektronix-4113/4662 color terminal system using software developed in the Roche research laboratories.

Results and Discussion

Design of Brodimoprim Analogues. Crystallographic data are available for the trimethoprim complex of *E. coli* dihydrofolate reductase[7] but not for that of the *L. casei* enzyme; it is therefore necessary to use structural information on related complexes as a basis for inhibitor design. We have used the atomic coordinates of the ternary complex of the *L. casei* enzyme with methotrexate and NADPH.[2,3] Figure 1a shows the methotrexate molecule and the two amino acid residues Arg-57 and His-28, which interact respectively with the α- and γ-carboxylates of the glutamate moiety of the inhibitor.

Both NMR measurements[5,15] and a comparison of the structures of the trimethoprim and methotrexate complexes of the *E. coli* enzyme[1,16] indicate that the 2,4-diaminopyrimidine ring of trimethoprim binds in the same site as the corresponding part of the 2,4-diaminopteridine ring of methotrexate. Using a combination of ring current shift calculations and transferred nuclear Overhauser effect measurements, we have previously determined the conformation of trimethoprim in its complexes with the *E. coli* and *L. casei* enzymes in solution[5,6] (this is essentially identical with the conformation found in the crystal

(8) Kompis, I.; Wick, A. *Helv. Chim. Acta* **1977**, *60*, 3025.
(9) Kompis, I.; Then, R. L. *Eur. J. Med. Chem.*, in press.
(10) Dann, J. G.; Ostler, G.; Bjur, R. A.; King, R. W.; Scudder, P.; Turner, P. C.; Roberts, G. C. K.; Burgen, A. S. V.; Harding, N. G. L. *Biochem. J.* **1976**, *157*, 559.
(11) Wyeth, P.; Gronenborn, A.; Birdsall, B.; Roberts, G. C. K.; Feeney, J.; Burgen, A. S. V. *Biochemistry* **1980**, *19*, 2608.
(12) Gronenborn, A.; Birdsall, B.; Hyde, E. I.; Roberts, G. C. K.; Feeney, J.; Burgen, A. S. V. *Biochemistry* **1981**, *20*, 1712.
(13) Henderson, P. J. F. *Biochem. J.* **1973**, *135*, 101.
(14) Baccanari, D. P.; Joyner, S. S. *Biochemistry* **1981**, *20*, 1710–1716.
(15) Roberts, G. C. K.; Feeney, J.; Burgen, A. S. V.; Daluge, S. *FEBS Lett.* **1981**, *131*, 85.
(16) Baker, D. J.; Beddell, C. R.; Champness, P. J.; Norrington, F. E. A.; Smith, D. R.; Stammers, D. K. *FEBS Lett.* **1981**, *126*, 49.

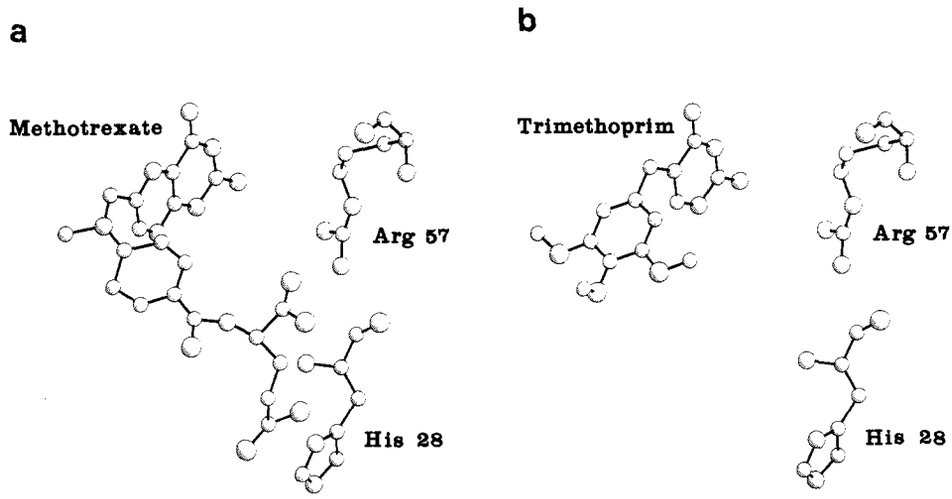


Figure 1. (a) Model of methotrexate in its bound conformation in the complex with *L. casei* dihydrofolate reductase and NADPH constructed from the crystal structure coordinates supplied by D. A. Matthews.[1] The His-28 and Arg-57 residues of the enzyme are also included. (b) Model of trimethoprim in its bound conformation in its complex with dihydrofolate reductase as deduced from NMR[5,6] and X-ray structural data.[1,2]

Table II. Conformations of the 2,4-Diamino-5-benzylpyrimidines

compd	θ_1,[a] deg	θ_2,[b] deg	compd	θ_1,[a] deg	θ_2,[b] deg
trimethoprim[5]	184	70	III	196	60
brodimoprim (I)	210	70	IX	205	53

[a] θ_1 = C4–(C5–C7)–C1′. [b] θ_2 = C5–(C7–C1′)–C6′.

structure of the *E. coli* enzyme–trimethoprim complex[7]). We have used this conformation in combination with the crystal structure coordinates[2,3] as the starting point for inhibitor design. Figure 1b shows trimethoprim in this conformation with its 2,4-diaminopyrimidine ring in the same position as the corresponding part of methotrexate in the methotrexate–NADPH–enzyme complex (see Figure 1a). It is clear that Arg-57 and His-28 cannot make any direct interactions with trimethoprim itself. Assuming that brodimoprim binds in the same conformation as trimethoprim, we have used model building and computer graphic techniques to investigate the types of substituent required to reach Arg-57 and His-28.

Docking of brodimoprim and its derivatives with the enzyme was performed in two steps. First, the 2,4-diaminopyrimidine ring was matched to the corresponding fragment of methotrexate in its bound conformation (cf. Figure 1b). The structure was then relaxed to minimize collisions with the enzyme and intramolecular strain. The resulting conformations of the benzylpyrimidine units for the various analogues (see Table II) came close to the conformation of trimethoprim bound to the *L. casei* enzyme deduced earlier on the basis of NMR studies.[5,6] From a consideration of this structure is became clear that in order to reach the basic residues Arg-57 and His-28 a chain with a terminal carboxylate group or groups should be attached at the 3′-position of brodimoprim (I). An ether bond was chosen as the link to the benzyl ring to maintain the similarity to brodimoprim. Molecular modelling experiments showed that the carboxylate group of VI, attached through a four-carbon chain to the 3′-O of brodimoprim, can reach the guanidinium group of Arg-57 without conformational strain within the ligand molecule and without steric conflicts with the surrounding protein. Similarly, a six-carbon link (VIII) readily permits an interaction between the carboxylate and His-28. The C4,C6-dicarboxylic acid (IX, Figure 2) fits well into the enzyme site, and the two carboxylates can interact simultaneously with Arg-57 and His-28. In the "optimal"

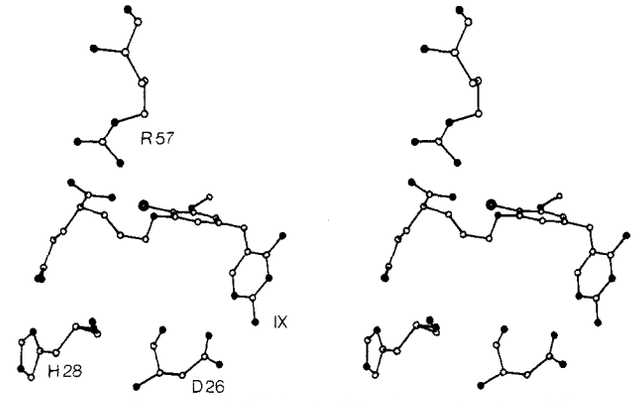


Figure 2. Compound IX docked in the active site of *L. casei* dihydrofolate reductase: the Asp-26, His-28, and Arg-57 residues are included.

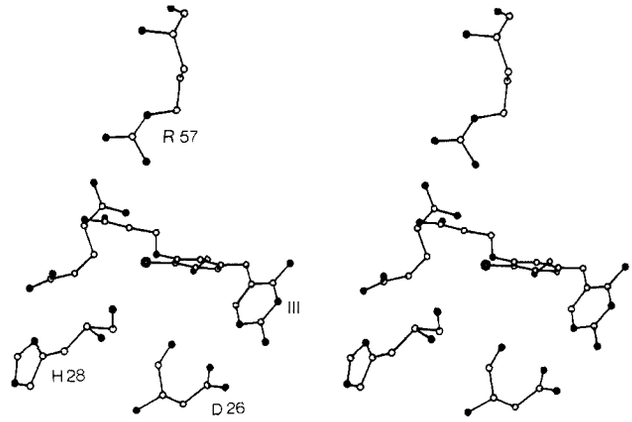


Figure 3. Compound III docked in the active site of *L. casei* dihydrofolate reductase.

binding conformation found in the modelling experiments, the α-carboxylate oxygens are 2.79 and 2.82 Å from the guanidinium nitrogens of Arg-57, and one γ-carboxylate oxygen is 2.62 Å from an imidazole nitrogen of His-28. The corresponding distances in the methotrexate complex are 2.60 and 3.03 Å (α-COO^-: Arg-57) and 2.62 Å (γ-COO^-: His-28). For the propionyl-L-glutamate derivative (III, Figure 3) satisfactory interatomic distances of 2.66 and 3.04 Å could be achieved for the α-carboxylate–Arg-57 interaction, but the γ-carboxylate could only approach to 3.07

Å from the imidazole of His-28, and even this required significant conformational strain (>10 kcal/mol). It is interesting that both in III and in methotrexate itself the two α-carboxylate oxygen–Arg-57 nitrogen distances are unequal, whereas in IX a "better"—more symmetrical—interaction can be achieved in the modelling experiments.

On the basis of the computer graphics studies, we synthesized the amino acid (II–IV) and aliphatic carboxylic acid (V–IX) analogues listed in Table I.

As noted above, design of these compounds was based on the assumption that brodimoprim has the same conformation as trimethoprim in its complex with the enzyme and further that the introduction of the side-chain substituents does not seriously perturb this bound conformation. We have used high-resolution ^{1}H NMR studies to characterize the bound conformations and also to monitor the interactions with His-28. Although His-28 is not conserved in the *E. coli* reductase, the enzymes from most other sources have a positively charged residue in a homologous position.[17] In complexes with the *L. casei* enzyme, NMR measurements can be conveniently used to detect changes in the pK value of His-28 that would accompany the predicted ion–ion electrostatic interactions.

Amino Acid Analogues II–IV. Brodimoprim and its analogues bind fairly tightly to the enzyme (K_i values < 10^{-8} M), and exchange between inhibitor molecules bound to the enzyme and free in solution is expected to be slow. Thus when a complex is examined in the presence of excess ligand, the bound and free forms of the ligands would be expected to give separate NMR spectra. We have shown previously[5,6] how the ^{1}H signals of bound trimethoprim can be detected and assigned by using transfer of saturation techniques to connect the bound and free species. We have now used this method to measure the ^{1}H chemical shifts of bound brodimoprim and some of its analogues. For some of the more tightly bound ligands, the rate of exchange of the ligand between bound and free states is too slow to allow detection of the bound signals using the transfer of saturation method. However, for brodimoprim and its Glu (III) and β-Ala (IV) analogues, the ^{1}H chemical shifts in the complex could be measured successfully, and the results for the H6 and H2′,6′ protons are summarized in Table III. The H6 proton has a very similar chemical shift in complexes of all three compounds, and this in turn is similar to the value measured previously in the trimethoprim–enzyme complex.[5] The H2′,6′ proton signal could not be detected in the brodimoprim complex; the signal is most probably very broad as a result of incomplete averaging of the H2′ and H6′ chemical shifts by ring flipping.[5] For complexes of the Glu (III) and β-Ala (IV) analogues, very high field shifts for the H2′,6′ protons were measured (−1.34 and −1.55 ppm); these are much larger than the corresponding value in trimethoprim. Using ring-current chemical shift calculations, we have shown previously that the shift of the H6 proton depends critically on the orientation of the benzyl ring with respect to the diaminopyrimidine ring: the close similarity between the H6 chemical shifts for trimethoprim, brodimoprim, and the Glu (III) and β-Ala (IV) analogues is strong evidence that the overall conformation is the same in all these complexes (within the ranges $\theta_1 = 180 \pm 30$; $\theta_2 = 90 \pm 34$). The observed H2′,6′ chemical shift (−0.61 ppm) in the trimethoprim–enzyme complex has been shown[5,6] to result from averaging of a large (−1.23 ppm) and small (+0.03 ppm) chemical shift by rapid flipping of the benzyl ring about the C7–C1′ bond. Assuming that a similar averaging takes place in the complexes formed by the Glu (III) and β-Ala (IV) analogues, we are unable to find a conformation for the analogues that will give calculated average H2′,6′ shifts as large as the observed chemical shifts (−1.34 and −1.55 ppm). However, if we make the reasonable assumption that the asymmetrically substituted benzyl ring of these analogues is no longer flipping in complexes with the enzyme, then the high-field signal would correspond to just one of the ortho protons. Indeed, in the conformation observed for trimethoprim bound to the enzyme, one of the ortho protons is calculated to have a chemical shift of −1.23 ppm.[5,6] The other ortho-proton resonance would go undetected if it was close to that of the free ligand as expected for this conformation (+0.03 ppm in trimethoprim). We conclude that brodimoprim and its Glu (III) and β-Ala (IV) analogues all have a conformation when bound to the enzyme similar to that of trimethoprim itself except that in the amino acid analogues the asymmetrically substituted benzyl ring is not flipping about the C7–C1′ bond. These results allow us to proceed with some confidence to a consideration of the binding properties of the various ligands.

The inhibition constants (K_i) for brodimoprim and its amino acid analogues II–IV are given in Table I. These values were necessarily determined in the presence of the coenzyme NADPH and reflect the binding of the inhibitor to the coenzyme–enzyme complex. The measured K_i values indicate only modest increases in binding over the parent compound brodimoprim. Thus the Gly analogue II, which is expected to interact with Arg-57, shows only a 3-fold increase in binding while the Glu analogue III, which is expected to reach both Arg-57 and His-28, binds less than 1 order of magnitude more tightly than does brodimoprim. The relatively modest increases in binding seen for the amino acid analogues suggests that optimal electrostatic interactions with Arg-57 and His-28 are not being made. Information on the proposed interactions with the latter residue can be obtained by measuring the pK values of histidine residues in the complexes.

The seven histidine residues of *L. casei* dihydrofolate reductase have been extensively studied by NMR[11,12,18] and the assignments for some of their C2 proton resonances are known unequivocally. One of these, His-28, shows significant changes in its pK value when it interacts directly with carboxylate groups: for example, in the complex of methotrexate with the enzyme, the glutamate γ-carboxylate of methotrexate interacts directly with His-28 and increases its pK by ~1 pH unit[1,18] (estimated to contribute ~6 kJ mol^{-1} to the binding energy).[19] Similar effects are seen in complexes with folate or (*p*-aminobenzoyl)-L-glutamate.[18]

We have measured the ^{1}H NMR spectra of the complexes of the enzyme with brodimoprim and compounds II–IV as a function of pH* over the range 5.0–8.0 and estimated the pK values of the histidine residues from the titration curves of the C2 proton chemical shifts as a function of pH* using nonlinear regression methods.[11,12] The pK values for most of the histidines did not vary significantly in the different complexes, but those for

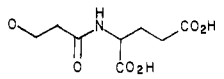
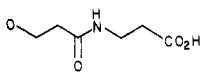
(17) Volz, K. W.; Matthews, D. A.; Alden, R. A.; Freer, S. T.; Hansch, C.; Kaufman, B. T.; Kraut, J. *J. Biol. Chem.* **1982**, *257*, 2528.

(18) Birdsall, B.; Griffiths, D. V.; Roberts, G. C. K.; Feeney, J.; Burgen, A. S. V. *Proc. R. Soc. London, Ser. B* **1977**, *196*, 251.

(19) Antonjuk, D. J.; Birdsall, B.; Cheung, H. T. A.; Clore, G. M.; Feeney, J.; Gronenborn, A.; Roberts, G. C. K.; Tran, T. Q. *Br. J. Pharmacol.* **1984**, *81*, 309.

(20) Emsley, J. W.; Feeney, J.; Sutcliffe, L. H. "High Resolution Nuclear Magnetic Resonance Spectroscopy"; Pergamon Press: Oxford, 1966; Vol. 2.

Table III. ^{1}H NMR chemical shifts of H6 and H2′,6′ in Complexes of Brodimoprim Analogues with *L. casei* Dihydrofolate Reductase

compd	proton	free, ppm	bound, ppm	shifts from models,[a] ppm
brodimoprim (I)	H6	3.80	2.74	−1.12
	H2′,6′	2.91		
	H6	3.79	2.74	−1.12
	H2′,6′	2.90	1.37	−1.34
	H6	3.79	2.74	−1.12
	H2′,6′	2.90	1.16	−1.55
trimethoprim	H6	3.62	2.76	−1.10
	H2′,6′	2.90	2.10	−0.61
2,4-diamino-5-methylpyrimidine[b] (X)	H6	3.86		
1-methyl-3,4-dimethoxy-4-bromobenzene[c] (XI)	H2′,H6′	2.71		

[a] Shifts measured relative to those in model compounds X and XI, which have no contributions from conformation-dependent "internal" ring current shifts. [b] Hurlbert, S., personal communication. [c] Estimated from substituent shift effects; see p 1140 of ref 20.

His-28 showed substantial variations. The pK values of His-28 in the complexes with compounds I–IV are given in Table I. Brodimoprim itself produced a small (0.13 unit) increase in pK, presumably due to small changes in protein conformation on ligand binding which alter the environment of His-28. The only one of these compounds that produced a substantial change was the Glu analogue III (0.32 unit increase in pK), which is substantially less than the change observed in complexes of the enzyme with folate and methotrexate analogues (~1 unit increase in pK). The smaller increase in pK in the complex with the Glu analogue III clearly shows that although a γ-CO_2^-–His-28 interaction is present it is much weaker than in methotrexate. These results agree with the molecular modelling conclusions presented earlier. Although these analogues are binding in approximately the manner predicted, their modest increase in binding suggested that less rigid molecules might allow the interactions to be optimized (at the cost of some entropic disadvantage).

Aliphatic Carboxylate Acid Analogues V–IX. In an attempt to find more tightly binding compounds, we therefore examined the aliphatic carboxylate analogues V–IX. Because of the flexibility of the chains it is more difficult to predict from computer graphics the exact chain length to provide optimal binding, and for this reason several aliphatic carboxylate substituents of varying chain length were examined. The K_i values (see Table I) for these compounds indicate that all the analogues are binding more tightly than the amino acid analogues. The tightest binding among the monocarboxylic acids was found when the methylene chain length had four atoms (compound VI): this compound binds 50 times more tightly than brodimoprim and shows no perturbation of His-28 (see pK values in Table I). It thus seems likely that it is in fact interacting with Arg-57 as predicted from the computer graphic studies. On this basis, the six-carbon monocarboxylic acid analogue VIII should have a sufficiently long chain extension to allow interaction with His-28. Somewhat surprisingly, the pK of His-28 in the complex of VIII with the enzyme was completely unperturbed even though the analogue binds fairly tightly. Although the C6 chain is sufficiently long and flexible to reach His-28, it appears that the carboxylate group prefers to bind elsewhere (almost certainly to Arg-57). This could arise simply from entropic considerations if, when VIII is bound to the enzyme with its diaminopyrimidine ring located in the appropriate site, its flexible side chain has many more conformations that result in the ω-CO_2^- group being in the vicinity of Arg-57 rather than in that of His-28. Alternatively it could be that His-28 can only make the appropriate interaction with a carboxylate group when the Arg-57 has already made a similar interaction. If either of these explanations is correct, then a C4,C6-dicarboxylic acid analogue (IX) should make effective interactions with both Arg-57 and His-28. Gratifyingly, this analogue was found to bind 3 orders of magnitude more tightly to the enzyme than brodimoprim (see K_i value in Table I), and furthermore, the pK of His-28 is increased by 1 unit compared with the enzyme–brodimoprim complex. Clearly the γ-CO_2^- group is now binding optimally to His-28. The C4,C6-dicarboxylic acid analogue IX has a K_i value (<0.01 nM) approaching that of methotrexate (0.004 nM) in spite of the fact that it is a racemate. The interactions of IX with Arg-57 and His-28 appear to contribute more than a factor of 1000 to binding (corresponding to a change in binding energy of >4 kcal/mol ($\Delta G_{app} = -RT \ln (1/K_i)$). However, the corresponding interactions of methotrexate (estimated by comparing the binding of methotrexate and its α,γ-diamide[19]) contribute only ca. 2.6 kcal/mol. This difference may be related to the suggestion from the molecular modelling studies that IX is able to make a more symmetrical and perhaps more favorable interaction with Arg-57 than is methotrexate (although the apparent lack of symmetry in the methotrexate interaction could simply result from small inaccuracies (0.1–0.3 Å) in the crystal structure data[17]). The marked increase in binding produced by the addition of the carboxylate substituents is not accompanied by any decrease in the selectivity of the inhibitors for the bacterial as compared to the mammalian enzyme.[9]

In this work we have shown that computer graphic techniques based on structural information from crystallography and NMR can be applied effectively to the design of inhibitors with much improved binding properties. In particular, it is clear that high-resolution NMR techniques provide a rapid, convenient, and effective method for assessing the assumptions involved in such an approach and testing its predictions.

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