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Communications to the Editor

A Multisubstrate Adduct Inhibitor of AICAR Transformylase

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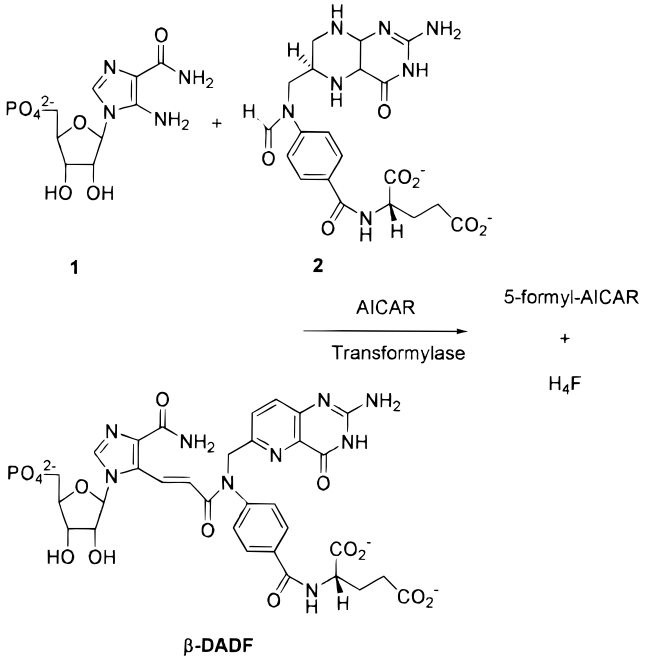
AICAR Tfase (5-aminoimidazole-4-carboxamide-ribonucleotide transformylase) catalyzes the transfer of the 10-formyl group of (6R,αS)-10-formyltetrahydrofolate (10-f-H_4F) to the 5-amino group of 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR) in the ninth step of de novo purine biosynthesis, Scheme 1. Virtually all organisms, with the exception of parasitic protozoa that scavenge purines from their environment, use this pathway to synthesize purines, which are essential for cell viability.[1] Rapidly dividing cancer cells, in particular, require a large amount of purines to sustain such growth, and consequently the de novo purine biosynthetic pathway is an attractive target for antineoplastic agents.[2] Moreover, AICAR Tfase requires a reduced folate cofactor, and some of the more successful chemotheraputic agents developed to date, such as methotrexate, are folate antimetabolites. To date there are no clinically useful inhibitors of AICAR Tfase nor is there an available crystal structure to assist in rational inhibitor design.

The principles of multisubstrate adduct inhibition offer several advantages over conventional inhibitor design.[3] The combination of two substrates into a single molecule increases the specificity of the inhibitor for the target enzyme since it is unlikely that other enzymes that use one of the components will recognize the multisubstrate adduct inhibitor (MAI). One can also expect an increase in the binding affinity due to an entropic advantage since the MAI possesses components

Scheme 1. Reaction Catalyzed by AICAR Tfase with Natural Substrates AICAR (**1**) and 10-f-H_4F (**2**); Also Shown Is the MAI for AICAR Tfase, β-DADF



of both individual substrates in a single molecule. Evidence from earlier kinetic studies in this laboratory indicate that the AICAR Tfase kinetic scheme follows a sequential mechanism with direct transfer of the formyl group, implying the formation of a ternary complex.[4,5] We have successfully used the MAI approach in preparing a picomolar inhibitor of glycinamide ribonucleotide transformylase[6,7] (GAR Tfase), an enzyme that catalyzes a similar formyl transfer from 10-f-H_4F. Here we report the synthesis and inhibitory properties of the first MAI for AICAR Tfase, β-DADF (Scheme 1).

β-DADF incorporates most structural features of the natural substrates with two exceptions. To reduce the complexity of the synthesis, the reduced pterin moiety of the natural cofactor, 10-f-H_4F, was replaced with an 8-deazafolate analogue. This aromatic compound is

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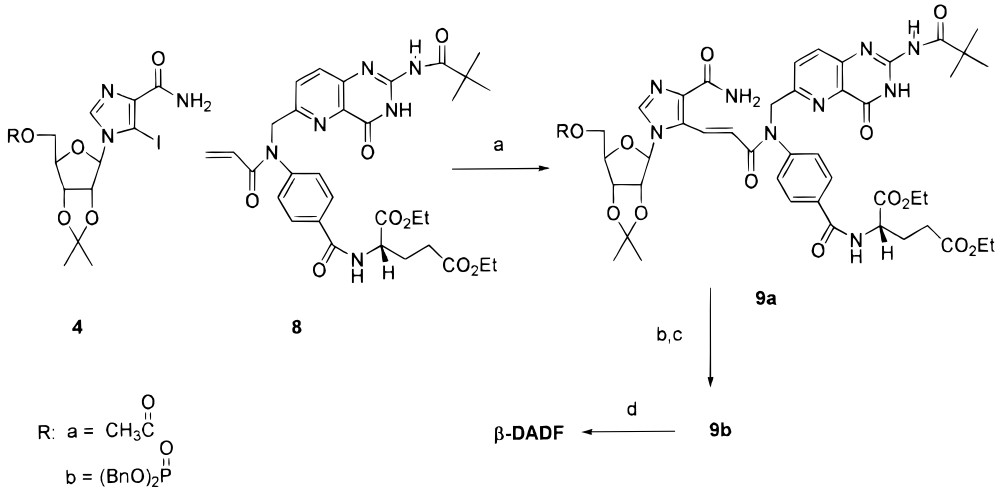
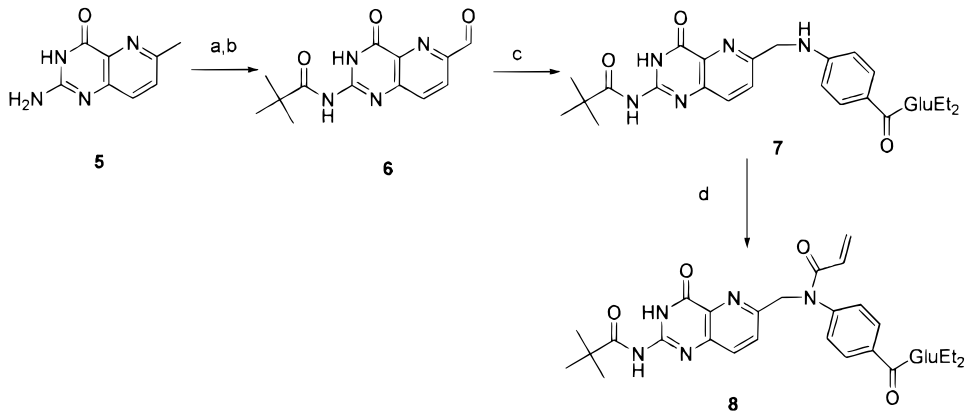
Scheme 2[a]



[a] Reagents and conditions: (a) 10 mol % $(PhCN)_2PdCl_2$, NEt_3, CH_3CN, 100 °C, 8 h; (b) NaOEt, EtOH, 25 °C, 1 h; (c) (i) dibenzyl diisopropylphosphoramidite, tetrazole, CH_2Cl_2, 25 °C, 1 h, (ii) MCPBA, −40 to 0 °C, 30 min; (d) (i) 50% TFA/H_2O, 25 °C, 3 h, (ii) 10 psi H_2, 10% Pd/C, CH_3OH, 25 °C, 2 h, (iii) 0.1 N NaOH, 25 °C, 4 days.

Scheme 3[a]



[a] Reagents and conditions: (a) pivalic anhydride, pyridine, reflux, 8 h; (b) selenium dioxide, *p*-dioxane, 100 °C, 3 h; (c) diethyl *N*-(*p*-aminobenzoyl)-L-glutamate, sodium triacetoxyborohydride, 1,2-dichloroethane, 25 °C, 4 h; (d) acryloyl chloride, NEt_3, CH_2Cl_2, 25 °C, 1 h.

more resistant to oxidation, and the stereochemicalrequirement of 10-f-H_4F at C-6 is absent in the 8-deazafolate analogue. This substitution is not expected to significantly reduce the binding affinity of β-DADF since 10-formyl-8-deazafolate is an alternative substrate for AICAR Tfase with a K_m of 102 μM compared to a K_m of 68 μM for 10-f-H_4F.[8] It has also been shown that 8-deazafolic acid inhibits *L. casei* AICAR Tfase with an IC_{50} of 6.4 μM.[9] The 5-exocyclic amino group of AICAR is also absent in β-DADF since we were not able to find a suitable electrophile that would react with this amine. The decreased nucleophilicity of the exocyclic amine is due to the electron-withdrawing 4-carboxamide, which reduces the pK_a of the 5-exocyclic amine to 2.4.[10]

Due to the lack of reactivity of the 5-amino group of AICAR, we decided to explore other methods for the coupling of AICAR and 10-f-H_4F moieties. Matsuda had reported palladium-catalyzed cross-coupling reactions of 5-iodoimidazole nucleosides with terminal alkynes[11] and methyl acrylate,[12] and this prompted us to try a similar approach. The synthesis of β-DADF was achieved via a convergent synthesis that centered on a palladium-catalyzed cross-coupling reaction of a suitably protected 5-iodoimidazole nucleoside (**4**) and a 10-acryloylfolate derivative **(8)** (Scheme 2). Initially, to minimize the number of transformations after the coupling, we had hoped to use a nucleoside derivative that had a protected 5′-phosphate already in place (**4b**), but this compound decomposed under the coupling conditions. Therefore, we prepared compound **4a**, which was readily accessible in three steps from commercially available AICAR. In the first step, acetonide formation was achieved with hydrogen chloride in ethanol−acetone according to the procedure of Robbins,[13] followed by acetylation of the 5′-OH with acetic anhydride andtriethylamine. The 5-iodo group was then introduced by diazotization with isoamyl nitrite in diiodomethane at 100 °C in a manner similar to that reported by Matsuda to prepare 5-iodo-1-(2,3,5-tri-*O*-acetyl-β-D-ribofuranosyl) imidazole-4-carboxamide.[11]

The synthesis of **8** is outlined in Scheme 3. Treatment of 6-methyl-8-deazapterin[14] with pivalic anhydride in pyridine afforded 2-pivaloyl-6-methyl-8-deazapterin. The 2-pivaloate group was important because it increased the solubility of the 8-deazapterin compounds and thus simplified purification. The 6-methyl group was oxidized with selenium dioxide in refluxing *p*-dioxane to the aldehyde **6** with overoxidation to the acid as a side reaction. The aldehyde was conveniently purified by chromatography on silica gel. Compound **6** readily

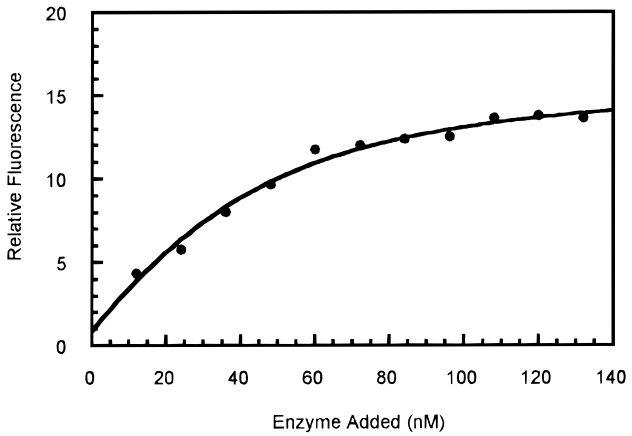


Figure 1. Fluorescence titration of 40 nM β-DADF with AICAR Tfase, excitation at 278 nm and emission at 415 nm. The line is the best fit of the data to eq 1; $K_D = 20$ nM.

underwent reductive amination with diethyl *N*-(*p*-aminobenzoyl)-L-glutamate using sodium triacetoxyborohydride in 1,2-dichloroethane to give **7**. Treatment with acryloyl chloride and triethylamine in dichloromethane gave the desired 10-acryloylfolate **8**. The position of the acryloyl group was confirmed based on the ^{1}H NMR, since in compound **8** the 9-CH_2 is a singlet and the 10-NH resonance is absent, whereas in compound **7** the 9-CH_2 and 10-NH were coupled appearing as a doublet and triplet, respectively.

The palladium-catalyzed cross-coupling of **4a** and **8** to give **9a** was achieved using bis(benzonitrile)palladium chloride and triethylamine in acetonitrile at 100 °C in a sealed tube. Only one regioisomer was obtained, and the magnitude of the coupling constant, 15.8 Hz, indicates that the orientation of the double bond was trans. The 5′-acetate was then selectively removed with sodium ethoxide in ethanol, and phosphorylation of the 5′-OH was accomplished using a standard phosphoramidite procedure[15] to give **9b**. The acetonide was removed with 50% TFA/H_2O, followed by debenzylation with H_2 over 10% palladium on carbon. The ethyl and pivaloate esters were deprotected simultaneously with 0.1 N NaOH at 25 °C for 4 days to give β-DADF.[16] More concentrated NaOH solutions resulted in cleavage of the N-10 amide bond. The structure of β-DADF was confirmed by ^{1}H NMR, ^{31}P NMR, and mass spectral data.[17]

The thermodynamic dissociation constant, K_D, for the E-MAI complex was determined by following the enhancement in fluorescence at 415 nm (excitation at 278 nm) of β-DADF upon binding to AICAR Tfase (Figure 1). Aliquots of a concentrated AICAR Tfase solution were added to a 40 nM β-DADF solution, and measurements were taken after a 2-min incubation period.[18] The data were fit to an equation that describes the fluorescence in measurable quantities:[19]

$$F = F_o + \frac{(F_\infty - F_o)\{(E_T + I_T + K_D) - \sqrt{(E_T + I_T + K_D)^2 - 4E_T \cdot I_T}\}}{2I_T} \quad (1)$$

where I_T is the total inhibitor added, F_∞ is the fluorescence of the enzyme−inhibitor complex, F_o is the fluorescence of the inhibitor, F is the measured fluorescence, and E_T is the amount of enzyme added. The K_D was determined to be 20 nM, which shows that β-DADF binds tightly to AICAR Tfase, 10^3-fold more tightly than the individual substrates.[20] The inflection point in Figure 1 is close to the concentration of β-DADF indicating that the binding stoichiometry is 1:1. β-DADF was competitive with both substrates precluding a straightforward assessment of K_i; however an IC_{50} of 125 nM (10 nM AICAR Tfase, 25 μM AICAR, and 50 μM 10-f-H_4F) indicated that β-DADF was a potent inhibitor of AICAR Tfase.[21] β-DADF was also selective for AICAR Tfase; concentrations that completely inhibit AICAR Tfase have no effect on GAR Tfase, an enzyme that also uses 10-f-H_4F as a cofactor.[22]

To date, in all of the organisms in which AICAR Tfase has been characterized the enzyme exists as a bifunctional protein that contains both AICAR Tfase and inosine monophosphate cyclohydrolase (IMP cyclase) activity. IMP cyclase catalyzes the cyclization of 5-formyl-AICAR to IMP, and the question has arisen as to whether both of these activities reside in the same active site on the enzyme. Beardsley has prepared truncation mutants that have only AICAR Tfase activity suggesting that the two activities reside in different active sites.[23] Our results are in agreement: β-DADF concentrations which abolish AICAR Tfase activity have no effect on IMP cyclase activity, and therefore the two active sites are distinct.[24]

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References

(1) Warren, M. S.; Mattia, K. M.; Marolewski, A. E.; Benkovic, S. J. The transformylase enzymes of de novo purine biosynthesis. *Pure Appl. Chem.* **1996**, *68*, 2029−2036.
(2) Elion, G. B. The purine path to chemotherapy. *Science* **1989**, *244*, 41−47.
(3) Broom, A. D. Rational design of enzyme inhibitors: multisubstrate analogue inhibitors. *J. Med. Chem.* **1989**, *32*, 2−7.
(4) Mueller, W. T.; Benkovic, S. J. On the purification and mechanism of action of 5-aminoimidazole-4-carboxamide-ribonucleotide transformylase from chicken liver. *Biochemistry* **1981**, *20*, 344−350.
(5) Smith, G. K.; Mueller, W. T.; Slieker, L. J.; DeBrosse, C. W.; Benkovic, S. J. Direct transfer of one-carbon units in the transformylations of de novo purine biosynthesis. *Biochemistry* **1982**, *21*, 1270−1278.
(6) Inglese, J.; Blatchly, R. A.; Benkovic, S. J. A multisubstrate adduct inhibitor of a purine biosynthetic enzyme with a picomolar dissociation constant. *J. Med. Chem.* **1989**, *32*, 937−940.
(7) Inglese, J.; Benkovic, S. J. Multisubstrate adduct inhibitors of glycinamide ribonucleotide transformylase: synthetic and enzyme-assembled. *Tetrahedron* **1991**, *47*, 2351−2364.
(8) Smith, G. K.; Mueller, W. T.; Benkovic, P. A.; Benkovic, S. J. On the cofactor specificity of glycinamide ribonucleotide and 5-aminoimidazole-4-carboxamide ribonucleotide transformylase from chicken liver. *Biochemistry* **1981**, *20*, 1241−1245.
(9) Degraw, J. I.; Colwell, W. T.; Brown, V. H.; Sato, M.; Kisliuk, R. L.; Gaumont, Y.; Thorndike, J.; Sirotnak, F. M. Synthesis and biological activity of 8-deazahomofolic acid and its tetrahydro derivative. *J. Med. Chem.* **1988**, *31*, 150−153.
(10) Yamazaki, A.; Okutsu, M. Cyclization of 5-amino-1-β-D-ribofuranosylimidazole-4-carboxamide (AICA-riboside): a review. *J. Heterocycl. Chem.* **1978**, *15*, 353−358.
(11) Minakawa, N.; Takeda, T.; Sasaki, T.; Matsuda, A.; Ueda, T. Nucleosides and nucleotides. 96. Synthesis and antitumor activity of 5-ethynyl-1-β-D-ribofuranosylimidazole-4-carboxamide (EICAR) and its derivatives. *J. Med. Chem.* **1991**, *34*, 778−786.
(12) Minakawa, N.; Kojima, N.; Matsuda, A. Nucleosides and nucleotides. 138. Synthesis of 3-halo-3-deazainosines: conformational lock with the halogen at the position of the 3-deazainosine in *anti*-conformation. *Heterocycles* **1996**, *42*, 149−154.

(13) Srivastava, P. C.; Newman, A. R.; Matthews, T. R.; Robins, R. K. Synthesis of 5-amino-1-(5-deoxy-β-D-ribofuranosyl)imidazole-4-carboxamide and related 5′-deoxyimidazole ribonucleosides. *J. Med. Chem.* **1975**, *18*, 1237−1240.

(14) Kelley, J. L.; McLean, E. W. Synthesis and antimicrobial testing of 2-amino-6-hydroxymethyl-4-(3*H*)pyrido[3,2-*d*]pyrimidinone. *J. Heterocycl. Chem.* **1981**, *18*, 671−673.

(15) Yu, K.-L.; Fraser-Reid, B. A novel reagent for the synthesis of myo-inositol phosphates: N,N-diisopropyl dibenzyl phosphoramidite. *Tetrahedron Lett.* **1988**, *29*, 979−982.

(16) β-DADF was purified by reversed-phase HPLC on a Whatman Partisil 10 ODS-3 9.4-mm × 50-cm column with a linear gradient of 0−50% acetonitrile in 0.1% TFA/H_2O over 30 min.

(17) ^{1}H NMR (D_2O): δ 8.34 (s, 1 H, imidazole H-2); 7.96 (d, 1 H, vinyl H, J = 15.9 Hz); 7.86 (d, 2 H, benzoyl H, J = 8.6 Hz); 7.60 (q, 2 H, folate H-7,8); 7.45 (d, 2 H, benzoyl H, J = 8.0 Hz); 6.72 (d, 1 H, vinyl H, J = 15.8 Hz); 5.63 (d, 1 H, H-1′, J = 7.0 Hz); 5.27 (m, 2 H, folate 9-CH_2); 4.58 (m, 1 H, H-2′); 4.33 (m, 2 H, H-3′,4′); 4.07 (m, 1 H, glutamic acid α-H); 3.89 (m, 2 H, 5′-CH_2); 2.32 (t, 2 H, glutamic acid γ-H's, J = 7.7 Hz); 2.23−1.98 (m, 2 H, glutamic acid β-H's). ^{31}P NMR (D_2O): δ 4.4 (s, 5′-PO_4). HRMS: calcd for $C_{32}H_{34}N_9O_{15}P$, MH^+ 816.1990; found, 816.2000.

(18) Fluorescence titration assays were carried out at 25 °C, pH 7.5 in 33 mM HEPES buffer and 25 mM KCl. Enzyme was purified from *E. coli* BL21(DE3) containing a plasmid that codes a His-tagged human *pur*H cDNA. The concentration of β-DADF was determined by measuring the concentration of inorganic phosphate (Chen, P. S.; Toribara, T. Y.; Warner, H. Microdetermination of phosphorus. *Anal. Chem.* **1956**, *28*, 1756−1758) in a solution of β-DADF that was hydrolyzed in 6 N HCl at 100 °C in a sealed tube for 12 h.

(19) Taira, K.; Benkovic, S. J. Evaluation of the importance of hydrophobic interactions in drug binding to dihydrofolate reductase. *J. Med. Chem.* **1988**, *31*, 129−137.

(20) K_m for AICAR is 17 μM, and K_m for 10-f-H_4F is 60 μM.[23]

(21) Inhibition assays were performed by following the production of H_4F at 298 nm in 33 mM Tris buffer containing 25 mM KCl at pH 7.5, 25 °C. Although β-DADF is a potent inhibitor of AICAR Tfase, its therapeutic potential may be limited due to its anionic nature, which most likely prohibits transport across biological membranes.

(22) *E. coli* GAR Tfase (5 nM) was assayed by following the production of 5,8-dideazafolate at 295 nm in 50 mM Tris buffer, pH 7.5, 25 °C, containing 40 μM 10-f-5,8-dideazafolate and 140 μM β-GAR. The presence of 10 μM β-DADF had no effect on the production of 5,8-dideazafolate, a concentration that completely inhibited 10 nM AICAR Tfase (50 μM 10-f-H_4F and 25 μM AICAR).

(23) Rayl, E. A.; Moroson, B. A.; Beardsley, G. P. The human *pur*H gene product, 5-aminoimidazole-4-carboxamide ribonucleotide formyltransferase/IMP cyclohydrolase cloning, sequencing, expression, purification, kinetic analysis, and domain mapping. *J. Biol. Chem.* **1996**, *271*, 2225−2233.

(24) IMP cyclase (10 nM) was assayed by following the conversion of 5-formyl-AICAR to IMP at 248 nm in 33 mM Tris buffer containing 25 mM KCl, pH 7.5, 25 °C. Concentrations of 10 μM β-DADF had no effect on the rate of IMP production.

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