Design, Synthesis, and Evaluation of the First Mechanism-Based Inhibitor of Glucosamine 6-Phosphate Synthase

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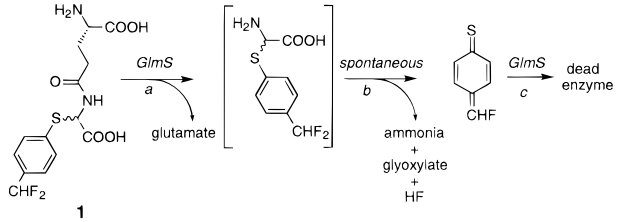
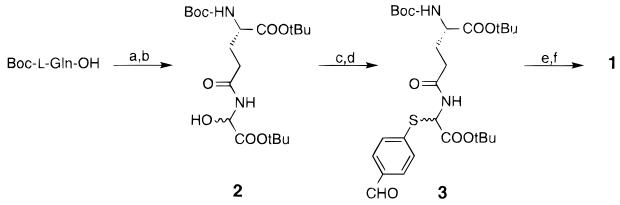
Glucosamine 6-phosphate synthase (GlmS, EC 2.6.1.16) catalyzes the transfer of NH_2 from the amide group of L-glutamine (glutamine dependent amidotransferase) to D-fructose 6-phosphate (6P).[1] The participation of the N-terminal cysteine residue in the catalysis and the structure of its glutamine binding domain[2] definitely established GlmS as a member of the N-terminal nucleophile(Ntn)-hydrolase superfamily.[3] The formation of glucosamine-6P catalyzed by GlmS is a key step in the biosynthesis of bacterial peptidoglycan and fungal chitin. Therefore, GlmS has been considered as an interesting therapeutic target. Glutamine site-directed inhibitors of GlmS actually display good *in vitro* antibacterial and antifungal activities when incorporated in oligopeptides.[4] Most of these inhibitors are affinity labels, *i.e.*, glutamine analogs incorporating one electrophilic function (halide,[4fg,5] α-keto epoxide,[6] α,β-unsaturated carbonyl derivatives[4]) that interact irreversibly with residue Cys^1 of GlmS. The need to find more specific inhibitors prompted us to design glutamine derivatives bearing a latent electrophilic function. As a first result of this novel approach, we present in this paper the first mechanism-based inhibitor, L-γ-glutamyl-2-[((*p*-difluoromethyl)phenyl)thio]glycine, referred to as compound **1**.

The good inhibitory properties of N^3-fumaroyl-2,3-diaminopropionate derivatives[7] had previously shown that the glutamine site of GlmS is large enough to accommodate a bulky group that would be linked to the N^5-amide nitrogen of substrate glutamine. During the course of our investigations in the chemistry of α-heteroatom-substituted glycines,[8] compound **1** was designed as a possible mechanism-based GlmS inhibitor. Enzyme-catalyzed hydrolysis of its peptide bond (Scheme 1, step *a*) should lead to the formation of glutamate and *N*-deacylated α-arylthioglycine which is known to be highly unstable.[9] Its decomposition (step *b*) would generate ammonia and glyoxylate on the one hand as well as HF and 4-thioquinone fluoromethide on the other hand. This latter powerful electrophile would then react with any active-site nucleophilic residue (step *c*), resulting in enzyme inactivation. Although previous approaches have been based on the generation of quinone methide or quinonimine methide[10,11] to achieve phosphatase[10d−f] or elastase[10c,11] inhibition, this is the first report of utilization of 2-heteroatom-substituted glycines in the inactivation of a glutamine-hydrolyzing enzyme.

Scheme 1. Putative Three-Step Mechanism of GlmS Inactivation by Compound **1**



Scheme 2. Synthesis of Compound **1**[a]



[a] Conditions: (a) 2-methylpropan-2-ol, DCC, DMAP (cat), dioxane, 78%; (b) *tert*-butyl glyoxylate, DMF, cyclohexane, reflux, 89%; (c) acetic anhydride, pyridine, 79%; (d) 4-mercaptobenzaldehyde, triethylamine, DMF, 95%; (e) DAST, dichloromethane, 39%; (f) 20% trifluoroacetic acid−dichloromethane, 61%.

Compound **1** was prepared according to Scheme 2. Condensation of suitably protected glutamine and *tert*-butyl glyoxylate[12] afforded the 2-hydroxyglycine **2** in 90% yield, as expected from earlier studies performed with simple amides.[13] After conversion of **2** into the acetoxy derivative, nucleophilic substitution with freshly prepared 4-mercaptobenzaldehyde[14] afforded *N*-(γ-glutamyl)-2-(arylthio)glycine **3** (76%). This aldehyde was converted with DAST[15] into the difluoro derivative and deprotected by trifluoroacetic acid to give compound **1** as an undefined mixture of epimers[16] in 12.4% overall yield from Boc-L-glutamine. Attempts to prepare the 2-(phenoxy)-glycine and the 2-(anilino)glycine analogs failed because of the instability of these products. Compound **1** which turned out to be perfectly stable under the conditions of enzyme assay even

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(1) Badet-Denisot, M.-A.; René, L.; Badet, B. *Bull. Soc. Chim. Fr.* **1993**, *130*, 249−255.

(2) Isupov, M. N.; Obmolova, G.; Butterworth, S.; Badet-Denisot, M.-A.; Badet, B.; Polikarpov, I.; Littlechild, J. A.; Teplyakov, A. *Structure* **1996**, *4*, 801−810.

(3) Brannigan, J. A.; Dodson, G.; Duggleby, H. J.; Moody, P. C. E.; Smith, J. L.; Tomchick, D. R.; Murzin, A. G. *Nature* **1995**, *278*, 416−419.

(4) (a) Kenig, M.; Abraham, E. P. *J. Gen. Microbiol.* **1976**, *94*, 37−45. (b) Chmara, H. *J. Gen. Microbiol.* **1985**, *131*, 265−271. (c) Milewski, S.; Chmara, H.; Andruszkiewicz, R.; Borowski, E.; Zaremba, M.; Borowski, J. *Drugs Exp. Clin. Res.* **1988**, *14*, 461−465. (d) Shoji, J.; Hindo, H.; Sakazaki, R.; Kato, T.; Hattori, T.; Matsumoto, K.; Tawara, K.; Kikuchi, J.; Terui, Y. *J. Antibiot.* **1989**, *42*, 869−874. (e) Andruszkiewicz, R.; Milewski, S.; Zeniawa, T.; Borowski, E. *J. Med. Chem.* **1990**, *33*, 132−135. (f) Andruszkiewicz, R.; Chmara, H.; Milewski, S.; Zeniawa T.; Borowski, E. *J. Med. Chem.* **1990**, *33*, 2755−2759. (g) Andruszkiewicz, R.; Chmara, H.; Zeniawa, T.; Borowski, E. *Eur. J. Med. Chem.* **1994**, *29*, 61−67.

(5) Auvin, S.; Cochet, O.; Kucharczyk, N.; Le Goffic, F.; Badet, B. *Bioorg. Chem.* **1991**, *19*, 143−151.

(6) (a) Chmara, H.; Zähner, H. *Biochim. Biophys. Acta* **1984**, *787*, 45−52. (b) Chmara, H.; Zähner, H.; Borowski, E. *J. Antibiot.* **1984**, *37*, 1038−1043. (c) Baldwin, J. E.; Adlington, R. M.; Mitchell, M. B. *J. Chem. Soc., Chem. Commun.* **1993**, 1332−1335. (d) Crossley, M. J.; Stamford, A. W. *Aust. J. Chem.* **1993**, *46*, 1443−1446.

(7) (a) Chmara, H.; Andruszkiewicz, R.; Borowski, E. *Biochim. Biophys. Acta* **1986**, *870*, 357−366. (b) Badet, B.; Vermoote, P.; Le Goffic, F. *Biochemistry* **1988**, *27*, 2282−2287. (c) Kucharczyk, N.; Denisot, M.-A.; Le Goffic, F.; Badet, B. *Biochemistry* **1990**, *29*, 3668−3676.

(8) Qasmi, D.; Rene, L.; Badet, B. *Tetrahedron Lett.* **1993**, *34*, 3861−3862.

(9) (a) Kingsbury, W. D.; Boehm, J. C.; Mehta, R. J.; Grappel, S. F.; Gilvarg, C. *J. Med. Chem.* **1984**, *27*, 1447−1451. (b) Kingsbury, W. D.; Boehm, J. C.; Perry, D.; Gilvarg, C. *Proc. Natl. Acad. Sci. U.S.A.* **1984**, *81*, 4573−4576. (c) Kraus, J.-L.; Attardo, G. *Eur. J. Med. Chem.* **1992**, *27*, 19−26.

(10) (a) Walsh, C. *Tetrahedron* **1982**, *38*, 871−909. (b) Abeles, R. H. *Chem. Eng. News* **1983**, Sept. 19, 48−56. (c) Wakselman, M. *Nouv. J. Chim.* **1983**, *7*, 439−447. (d) Myers, J. K.; Widlanski, T. S. *Science* **1993**, *262*, 1451−1453. (e) Born, T. L.; Myers, J. K.; Widlanski, T. S.; Rusnak, F. *J. Biol. Chem.* **1995**, *270*, 25651−25655. (f) Stowell, J. K.; Widlanski, T. S.; Kutateladze, T. G.; Raines, R. T. *J. Org. Chem.* **1995**, *60*, 6930−6936.

(11) Vergely, I.; Boggetto, N.; Okochi, V.; Golpayedani, S.; Reboud-Ravaux, M.; Kobaiter, R.; Joyeau, R.; Wakselman, M. *Eur. J. Med. Chem.* **1995**, *30*, 199−208.

(12) Carpino, L. A. *J. Org. Chem.* **1964**, *29*, 2820−2824.

(13) (a) Zoller, U.; Ben-Ishai, D. *Tetrahedron* **1975**, *31*, 863−866. (b) Schouteeten, A.; Christidis, Y.; Mattioda, G. *Bull. Soc. Chim. Fr.* **1978**, 248−254.

(14) Young, R. N.; Gauthier, J.-Y.; Coombs, W. *Tetrahedron Lett.* **1984**, *25*, 1753−1756.

(15) Diethylaminosulfur trifluoride. For a review, see: Hudlicky, M. *Org. React.* **1988**, *35*, 513−637.

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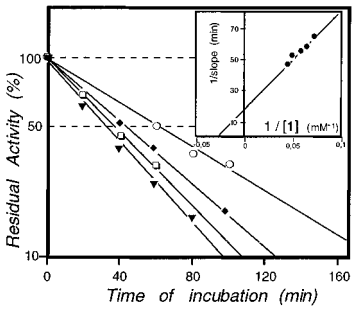

Figure 1. Time-dependent inactivation of GlmS. GlmS (13 μg) was incubated at 20 °C and pH 7.2 in 100 μL of 100 mM KPO_4 containing 10 mM fructose-6P and **1** at varying concentrations. At different time points, aliquots (5 μL) were withdrawn and the reaction was stopped by 200-fold dilution at 0 °C in 100 mM KPO_4 at pH 7.2 containing both saturating substrates (10 mM fructose-6P; 6 mM glutamine). Then residual activity is assayed at 37 °C by the described procedure.[7c] The enzyme remained 100% active during 120 min of incubation in the presence of fructose-6P and in the absence of **1**. Curves show time-dependent inhibition (semilog plot) with (○) 11.6, (◆) 14.5, (□) 17.4, and (▼) 22.6 mM. Inset: double reciprocal plot of similar inactivation experiments performed with **1** at 14.5, 16.0, 17.4, 20.3, and 22.6 mM. Above 25 mM, precipitation of **1** occurred. The data points represent the average of three determinations.

in the presence of 20 mM dithiothreitol was tested on *Escherichia coli* GlmS purified as described.[17]

The conclusion that compound **1** behaved as a mechanism-based inhibitor was drawn from the following results. (i) When GlmS was incubated with **1** at concentrations 11−23 mM and saturating D-fructose-6P, a first-order loss of enzyme activity was observed (Figure 1) over a period of about 1.5 h.[18] This inactivation process displayed saturation kinetics, as shown from the positive *y*-intercept in the double reciprocal plot of the apparent inactivation rate *versus* inhibitor concentration (Figure 1, inset). Therefore, reversible enzyme/inhibitor complex formation is much faster than the inactivation step itself.[19] Values found for inactivation rate constant (k_{inact}) and irreversible inhibition constant (K_{irr}) are 0.054 ± 0.002 min^{-1} and 35.8 ± 0.1 mM, respectively. (ii) Extensive dialysis of inactivated enzyme did not result in any recovery of activity, demonstrating that the inhibition is an irreversible process. Furthermore, the ultraviolet spectrum (not shown) of this dialyzed dead enzyme was different from that of the native one as shown by appearence of a broad peak at $\lambda_{max} = 308$ nm. (iii) The presence of glutamate semialdehyde (0.27 mM), a potent glutamine site-directed reversible GlmS inhibitor ($K_i = 55$ μM),[20] totally protected the enzyme from inactivation. This demonstrates that inactivator **1** is directed against GlmS glutamine site. (iv) The inactivation resulted from inhibitor processing by the enzyme: glutamate dehydrogenase analysis of a GlmS (4.0 μM) solution inactivated to 66% by compound **1** (28 mM) revealed the formation of 21 ± 2 μM glutamate that is a 5.25-fold excess over the protein. Consistently, a simultaneous glyoxylic acid production was also detected by lactate dehydrogenase and NADH,[21] but the exact amount could not be quantified because of excessive background noise.[22] The 4 equiv (5.25-1) of 4-thioquinone methide released in the medium could add a water molecule (1,6-addition) to generate an unstable α-fluoro-α-(hydroxymethyl)phenyl thiol precursor of 4-mercaptobenzaldehyde. A new 214 nm absorbing compound was indeed detected in the HPLC profile of the inactivation mixture, but the minute quantity formed precluded its identification.

Although the four observations above meet the criteria required for suicide inhibition,[19] GlmS inactivation was prevented by nucleophiles such as dithiothreitol (5 mM), cysteine (10 mM) or tetra-*n*-butylammonium phenylthiolate (1.25 mM) which are not competitive inhibitors of the glutamine site. The possibility of enzyme reactivation by breaking an enzyme/inhibitor species adduct was discarded since extensive dialysis of inactivated enzyme in the presence of 5 mM dithiothreitol (5 mM) did not result in regain of activity. This protective effect may then suggest that the species responsible for inactivation was released into the solution (where it can be quenched by nucleophiles) before alkylating the enzyme.[19]

However, this assumption would be in contradiction with the absence of a lag time before the onset of inhibition and with the low partition ratio of about 7 ((5.25/0.66) − 1, see above) which was deduced from the quantitation of glutamate formed during inactivation. It is then possible that in the protection experiments the thiols enter the active site and compete with the active-site nucleophiles for the activated thioquinone methide. The design of a bulky thiol unable to enter the glutamine site of the native enzyme would certainly be of interest to elucidate this phenomenon. A similar behavior was actually observed in the inhibition of β-glucosidases by difluoromethyl arylglucosides.[23] Although the structure of native GlmS has not been determined yet, the active site has been consistently located at the bottom of a cleft widely exposed to solvent on the recently reported three-dimensional structure of the glutamine binding domain.[2]

The nature of the adduct between **1** and GlmS remains to be elucidated. The participation of cysteine1 in this adduct is highly unlikely since it is assumed to be still engaged in the γ-glutamyl thiol ester resulting from inhibitor processing by the catalytic thiol (Figure 1, step *a*).[1] The observed absorption at 308 nm, a wavelength higher than the typical value (∼260 nm) of phenylthiols or -thiolates[24] but lower than that reported for a doubly conjugated thioketone[25] (330 nm), might reveal further evolution of the methyl-substituted 4-fluoromethylphenyl thiol adduct resulting from nucleophilic 1,6-addition[26] on the thioquinone methide formed according to Scheme 1. Since electrospray mass spectroscopy failed to give reproducible results in the analysis of the inactive protein, further investigations are required to understand the molecular details of this inhibition.

Despite moderate efficiency, further use of glutamine derivatives releasing reactive leaving groups may constitute a general approach to the design of irreversible inhibitors of glutamine-hydrolyzing enzymes. The application of this concept to protease inhibition is currently under investigation.

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(16) It has never been possible to separate at any stage diastereomers on reverse-phase HPLC. Nonetheless, all compounds gave satisfactory analytical data. For compound **1**: ^{1}H NMR (300 MHz, DMSO-d_6) δ 2.02 (m, 2H, β-CH_2), 2.39 (m, 2H, γ-CH_2), 3.70 (t, $J = 6.4$ Hz, 1H, α-CH), 5.74 (d, $J = 8.8$ Hz, 1H, 2-CH), 7.09 (t, $J = 55$ Hz, 1H, CHF_2), 7.63 (m, 4H, CH_{arom}), 8.92 (d, $J = 8.8$ Hz, 1H, 2-NH); ^{13}C NMR (75 MHz, DMSO-d_6) δ 26.4 (β-CH_2), 30.9 (γ-CH_2), 52.3 (α-CH), 57.4 (2-CH), 114.6 (CHF_2 (t, $J_{CF} = 234$ Hz)), 126.0 (3′-CH and 5′-CH), 131.2 (2′-CH and 6′-CH), 132.4 (4′-C), 137.1 (1′-C), 168.9 (CONH), 170.5 and 170.9 (2 COOH); UV−vis (*c* 10^{-4} M, H_2O) λ_{max} 262 (3400) nm; MS (FAB$^+$, glycerol + HCl) *m/e* calcd for $C_{14}H_{16}F_2N_2S + H^+$ 363.0826, found 363.0808; 363 (100, MH^+); $[\alpha]_D^{20} + 3.4°$ (*c* 1.0, MeOH).

(17) Dutka-Malen, S.; Mazodier, P.; Badet, B. *Biochimie* **1988**, *70*, 287−290.

(18) In the absence of fructose-6P, inactivation occurred at a slower rate. GlmS incubation with 20.3 mM **1** under the conditions reported in Figure 1 resulted in a 28% loss in activity compared to 70% in the presence of fructose-6P.

(19) Silverman, R. B. *Mechanism-based enzyme inactivation*; CRC Press: Boca Raton, FL, 1988; Vol. 1.

(20) Bearne, S. L.; Wolfenden, R. *Biochemistry* **1995**, *34*, 11515−11520.

(21) Pineda, M.; Piedras, P.; Cárdenas, J. *Anal. Biochem.* **1994**, *222*, 450−455.

(22) The enzyme activity remained unaffected by glyoxylate concentrations within that range (20 μM). Under these conditions it exhibits a IC_{50} of 3 mM.

(23) Halazy, S.; Berges, V.; Ehrhard, A.; Danzin, C. *Bioorg. Chem.* **1990**, *18*, 330−344.

(24) (a) Jones, R. A.; Katritzky, A. R. *J. Chem. Soc.* **1958**, 3610−3613. (b) Albert, A.; Barlin, G. B. *J. Chem. Soc.* **1959**, 2384−2396.

(25) (a) Metzner, P.; Vialle, J. *Bull. Soc. Chim. Fr.* **1972**, 3138−3145. (b) Barton, D. H. R.; Choi, L. S. L.; Hesse, R. H.; Pechet, M. M.; Wilshire, C. *J. Chem. Soc., Perkin Trans. 1* **1979**, 1166−1175.

(26) Wagner, H.-U.; Gompper, R. Quinone methides. In *The chemistry of the quinonoid compounds*; Patai, S., Ed.; J. Wiley and Sons: New York, 1974; Chapter 18.