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Note

Synthesis and Characterization of Intermediate and Transition-State Analogue Inhibitors of γ-Glutamyl Peptide Ligases

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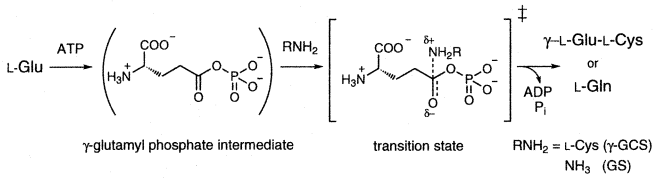
The phosphonodifluoromethyl ketone and phosphonofluoridate derivatives of L-glutamic acid were synthesized and characterized as analogues of the γ-glutamyl phosphate intermediate and the tetrahedral transition state, respectively, for the inhibition of γ-glutamylcysteine synthetase and glutamine synthetase. The former served as a poor inhibitor of both enzymes, but the latter inhibited glutamine synthetase with a K_i of 59 μM and partially inactivated the enzyme in an NH_3- and ATP-dependent manner.

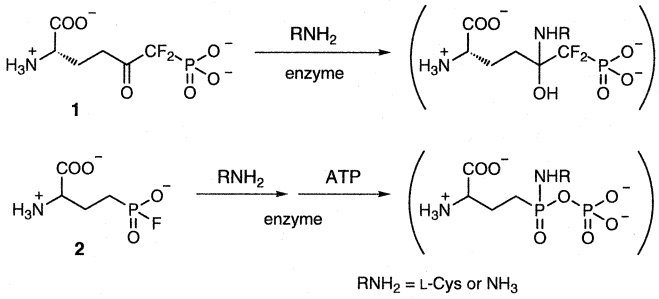
Key words: γ-glutamyl peptide ligase; intermediate analogue; transition-state analogue; phosphonodifluoromethyl ketone; phosphonofluoridate

γ-Glutamylcysteine synthetase (γ-GCS, EC 6.3.2.2) and glutamine synthetase (GS, EC 6.3.1.2) catalyze the ATP-dependent formation of a γ-glutamyl peptide bond to produce γ-L-glutamyl-L-cysteine and L-glutamine, respectively. γ-GCS is the rate-limiting enzyme in glutathione biosynthesis,[1] and GS is the key enzyme in nitrogen metabolism,[2] both of which are physiologically relevant. Hence, the inhibitors of these γ-glutamyl peptide ligases are important not only for use as therapeutic[3] and agrochemical agents[4] targeted toward the metabolism of the γ-glutamyl peptides, but also for use as mechanistic probes for the ATP-dependent peptide ligation.[5–7]

Considering that the reaction catalyzed by γ-GCS and GS is thought to proceed through the initial formation of a common γ-glutamyl phosphate intermediate followed by nucleophilic substitution by a substrate amine (L-Cys and NH_3 for γ-GCS and GS, respectively) (Scheme 1), we designed two compounds **1** and **2** as potential inhibitors. The phosphonodifluoromethyl ketone **1**, an analogue of the γ-glutamyl phosphate intermediate, was expected to undergo nucleophilic addition of the substrate amine to the electrophilic carbonyl to form a tetrahedral transition-state mimic in the enzyme active site (Scheme 2). The phosphonofluoridate **2** was chosen as a tetrahedral transition-state analogue in the hope that nucleophilic substitution by the substrate amine and phosphorylation by ATP would also afford a



Scheme 1. Proposed Reaction Mechanisms for *E. coli* γ-GCS and GS.



Scheme 2. Expected Inhibition Scheme.

transition-state mimic (Scheme 2). In this note, we report the synthesis and characterization of these two compounds, and their inhibitory activities toward *E. coli* γ-GCS and GS.

The synthesis of compound **1** is outlined in Scheme 3a. *N*-Trityl-L-glutamic acid dimethyl ester was treated with lithio(difluoromethyl)phosphonate[8] to give **3**. Deprotection by Me_3Si-I and alkaline hydrolysis, and subsequent cation-exchange chromatography (Dowex 50W × 8, H^+ form) afforded the product ketone **1**.[9] We also prepared the corresponding alcohol **5** by reducing the ketone **3** with $NaBH_4$ and then applying the same deprotection procedure.[10] Compound **1** was isolated in the keto form, which was confirmed by a combustion analysis.[9] In an aqueous solution, however, it existed as an equilibrium mixture of the following three components whose ratios (determined by ^{19}F-NMR) were dependent on the pH of the solution (Fig. 1): the desired keto form (b) and the hydrated form (a), which are predominant in acidic media, and the cyclic imine form

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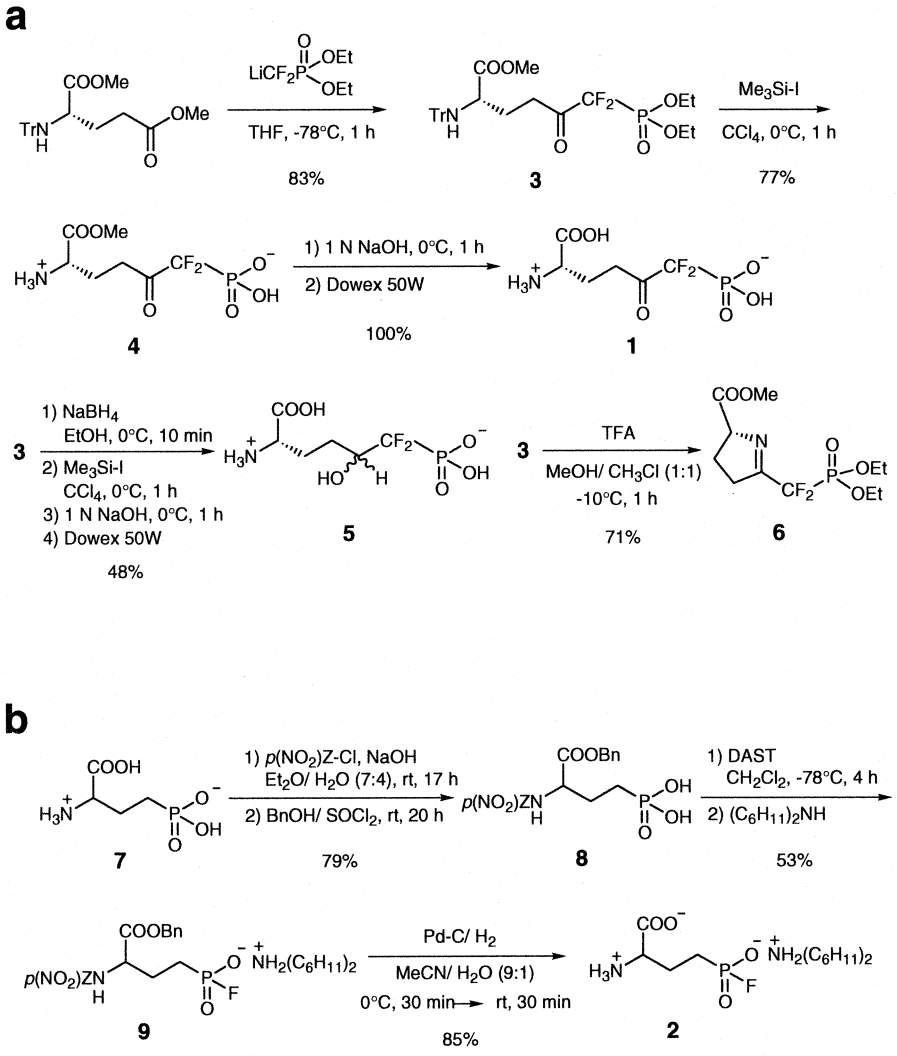
Abbreviations: γ-GCS, γ-L-glutamyl-L-cysteine synthetase; GS, L-glutamine synthetase; DAST, diethylaminosulfur trifluoride; AMP-PCP, 5′-adenylylmethylenediphosphonate

* This assignment was made by comparing the ^{13}C-NMR data with those of the following authentic compounds: the keto form (b) (δ_C=204.5, C=O) with the ketone **3** (δ_C=197.8, C=O), the hydrated form (a) [δ_C=97.2, $C(OH)_2$] with 3,3-difluoro-2-oxopropyl 4-methylphenyl sulfoxide in a hydrated form [δ_C=92.2, $C(OH)_2$],[11] and the cyclic imine form (c) (δ_C=178.4, C=N) with compound **6** (δ_C=172.5, C=N)[12] which was formed by the spontaneous cyclization upon deprotecting of the amino group of **3**.



Scheme 3. Synthetic Scheme.

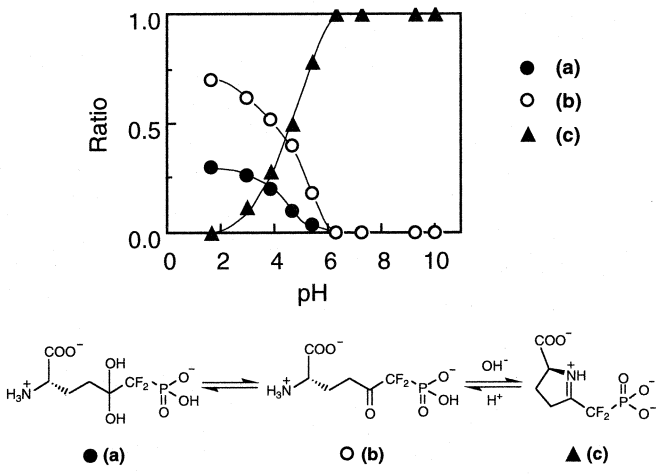


Fig. 1. Equilibrium Mixture of **1** in an Aqueous Solution.

[**1**] = 80.4 mM. pH was adjusted by adding K_2CO_3. The ratios of the hydrated form (a); ●, the keto form (b); ○, and the cyclic imine form (c); ▲ were calculated from the integral of the ^{19}F-NMR peak areas.

(c) which predominates under basic conditions.* Thus, the γ-carbonyl group of **1** was highly electrophilic as expected and the facile addition of nucleophiles such as water and an amine was actually found to take place.

The phosphonofluoridate **2** was synthesized as depicted in Scheme 3b. Commercially available amino acid **7** was derivatized as a *p*-nitrobenzyl carbamate and a benzyl ester to give **8**. The phosphonic acid **8** was effectively monofluorinated under mild conditions by using diethylaminosulfur trifluoride (DAST).[13] The monofluoridate **9** was purified by recrystallization as a dicyclohexylammonium salt. Hydrogenolysis gave the desired phosphonofluoridate **2** containing *ca.* 10% of the hydrolyzed byproduct **7**, and purification by silica gel column chromatography (Silica Gel 60N; Kanto Chemical; 21:5, $MeCN/H_2O$) afforded pure **2** as a white solid.[14] Compound **2** was stable under acidic conditions, and no hydrolysis was observed over a week at 4°C in an aqueous solution at pH 5. In alkaline to neutral media, however, **2** was susceptible to hydrolysis with, for example, a half-life of 20 min at 37°C in 1 M Tris-HCl (pH 7.5).

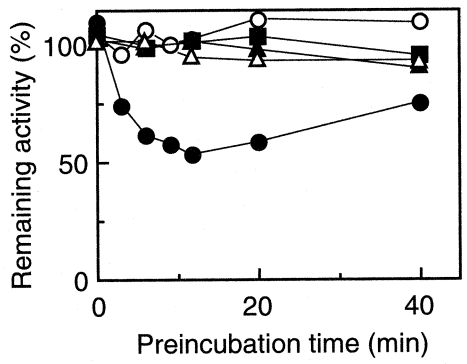


Fig. 2. Inactivation of GS by Compound **2**.

GS was incubated at 37°C with **2** (200 μM) in the presence of ATP (6 mM) and NH_4Cl (50 mM); ●, NH_4Cl alone; ▲, ATP alone; △, and AMP-PCP (6 mM) and NH_4Cl; ○ in 100 mM HEPES-KOH (pH 7.5). GS was preincubated with **7** (300 μM), ATP (6 mM) and NH_4Cl (50 mM); ■. The remaining activity was measured after a certain period of preincubation (abscissa) followed by a 100-fold dilution.

We first examined the inhibitory activity of **1**. Enzyme activity was measured by a pyruvate kinase-lactate dehydrogenase coupled enzyme assay.[15] Under physiological conditions (pH 7.5), at which compound **1** existed almost exclusively in the cyclic imine form (c), neither enzyme was inhibited with up to 6.1 mM of **1**, but moderate inhibition resulted in acidic media in which the hydrated form (a) and the keto form (b) existed as minor components. Thus, γ-GCS and GS were inhibited by 47% at pH 5.5 and 54% at pH 6.0, respectively, with 6.3 mM of **1**. The effective concentrations of the keto form (b) were 1 and 0.3 mM at pH 5.5 and 6.0, respectively, as calculated from the pH-ratio profile (Fig. 1). The keto form seemed to be inhibitory because the corresponding alcohol **5** failed to inhibit both enzymes up to 6.9 mM. Since moderate inhibition of γ-GCS and GS was observed, inactivation of the enzymes by **1** was next examined. The enzymes were incubated with compound **1** (6.3 mM) in the presence of ADP (1 mM)[16] and the corresponding substrate amine (150 mM of L-2-aminobutyrate and 50 mM of NH_4Cl for γ-GCS and GS, respectively) for 2 h at 37°C, and the remaining activity was measured after a 100-fold dilution. However, the remaining activities of γ-GCS and GS were 93 and 107%, respectively, relative to the control activity. Therefore, the inhibition scheme with respect to compound **1** did not seem to be operative for both enzymes.

Phosphonofluoridate **2**, on the other hand, inhibited γ-GCS and GS with a K_i of 83 and 59 μM, respectively. Compound **2** was partially hydrolyzed to **7** in the assay mixture, and **7** was equally inhibitory (K_i=34 and 19 μM for γ-GCS and GS, respectively). We therefore examined if **2** (200 and 400 μM for γ-GCS and GS, respectively) would cause enzyme inactivation by incubating the enzymes in the presence of the substrate amine and ATP (1 and 6 mM for γ-GCS and GS, respectively) for 20 min. Although the remaining activity of γ-GCS was 102% (no inactivation), the residual activity of GS was only 59% relative to the control activity. This partial inactivation was time dependent and was unique to the phosphonofluoridate **2**, because the inactivation was not observed with the simple phosphonate **7** under the same conditions (Fig. 2). Furthermore, the lack of either NH_3 or ATP failed to inactivate the enzyme, and the replacement of ATP by AMP-PCP, a non-hydrolyzable ATP analogue, did not cause the enzyme inactivation (Fig. 2). This mode of GS inactivation by compound **2** is consistent with the initially expected inhibition scheme, although direct evidence for the formation of a transition-state mimic is not available at present.

Wedler *et al.* have proposed that the putative reaction intermediate, γ-glutamyl phosphate, is not a most recognized species in the course of the reaction catalyzed by *E. coli* GS in their study of an intermediate analogue, 4-(phosphonoacetyl)-L-α-aminobutyrate.[17] In the present study, we also found that the γ-glutamyl phosphate analogue **1** was a poor inhibitor of *E. coli* γ-GCS and GS, although it had a highly electrophilic carbonyl group. On the other hand, phosphonofluoridate **2** was found to be a better inhibitor of GS. Unlike compound **1**, compound **2** has a tetrahedral geometry on the γ-phosphorus atom from the outset. Indeed, all of the potent inhibitors of *E. coli* γ-GCS and GS reported so far are phoshinate- and sulfoximine-based compounds[5-7] having this geometry on the γ-atom. These results suggest that the tetrahedral geometry is essential for the inhibitor.

Acknowledgments

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9) Compound **1**: white crystal from acetone/H_2O, mp 141–143°C (dec), $[\alpha]_D^{26}$+11.58° (*c* 1.05, H_2O). IR (KBr) ν_{max} cm^{-1}: 3500–

2000, 1730, 1710, 1620, 1510, 1250, 1000. ^{1}H-NMR (400 MHz, D_2O) (mixture of keto and hydrated forms) δ_H: 2.2 (m, 2H, CH_2 CH_2CO), 3.1 (m, 2H, CH_2CH_2CO), 4.08 (t, J=6.7 Hz) and 4.13 (dd, J=5.7 and 6.9 Hz) (1H, α-H). ^{19}F-NMR (188 MHz, D_2 O) δ_F: −46.68 (d, $^2J_{F-P}$=90.9 Hz) and −44.14 (t, $^2J_{F-P}$=86.2 Hz). ^{31}P-NMR (81.0 MHz, D_2O) δ_P: 1.01 (t, $^2J_{P-F}$=85.9 Hz) and 3.70 (t, $^2J_{P-F}$=90.8 Hz). ^{13}C-NMR (99.5 MHz, D_2O) δ_C: 25.34 and 25.55 (s, CH_2CH_2CO), 32.3 and 35.8 (m, CH_2CH_2CO), 54.45 and 55.35 (s, $CHCO_2H$), 97.2 [m, $C(OH)_2$], 118.01 (dt, $^1J_{C-P}$=176, $^1J_{C-F}$=269 Hz) and 120.68 (dt, $^1J_{C-P}$=184, $^1J_{C-F}$ =268 Hz) (CF_2), 173.82 and 174.15 (s, CO_2H), 204.5 (m, C=O). *Anal.* Found: C, 27.28; H, 3.79; N, 5.31%. Calcd. for $C_6H_{10}F_2$ NO_6P: C, 27.00; H, 3.86; N, 5.36%.

10) Alcohol **5**: white solid. IR (KBr) ν_{max} cm^{-1}: 3700–2200, 1720, 1610, 1510, 1040. ^{1}H-NMR (200 MHz, D_2O) (mixture of two diastereomers) δ_H: 1.6–2.3 (m, 4H, CH_2CH_2CHOH), 3.9–4.1 (m, 2H, CHOH and α-H). ^{19}F-NMR (282 MHz, D_2O) δ_F: −50.63 (ddd, $^2J_{F-F}$=293 Hz, $^2J_{F-P}$=93.2 Hz, $^3J_{F-H}$=17.2 Hz) and −50.33 (ddd, $^2J_{F-F}$=293 Hz, $^2J_{F-P}$=89.8 Hz, $^3J_{F-H}$=17.2 Hz), −42.47 (ddd, $^2J_{F-F}$=293 Hz, $^2J_{F-P}$=93.2 Hz, $^3J_{F-H}$=8.6 Hz) and −42.32 (ddd, $^2J_{F-F}$=293 Hz, $^2J_{F-P}$=91.4 Hz, $^3J_{F-H}$=8.6 Hz). ^{31}P-NMR (121 MHz, D_2O) δ_P: 3.83 (t, $^2J_{P-F}$=92.3 Hz) and 3.87 (t, $^2J_{P-F}$=91.7 Hz). *Anal.* Found: C, 27.17; H, 4.60; N, 5.20%. Calcd. for $C_6H_{12}F_2NO_6P$: C, 27.39; H, 4.60; N, 5.32%.

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12) Cyclic imine **6**: colorless oil, $[\alpha]_D^{22}$+75.93° (*c* 1.08, EtOH). IR (KBr) ν_{max} cm^{-1}: 3350, 2950, 1740, 1650, 1270, 1200, 1020. ^{1}H-NMR (200 MHz, $CDCl_3$) δ_H: 1.38 and 1.39 (t, J=7.5 Hz, 6H, $PO_2CH_2CH_3$), 2.3 (m, 2H, CH_2CH_2C=N), 2.9 (m, 2H, CH_2C H_2C=N), 3.76 (s, 3H, CO_2CH_3), 4.3 (m, 4H, $PO_2CH_2CH_3$), 4.92 (m, 1H, α-H). ^{19}F-NMR (282 MHz, $CDCl_3$) δ_F: −36.92 (d, $^2J_{F-P}$=97.7 Hz). ^{31}P-NMR (81.0 MHz, $CDCl_3$) δ_P: 4.31 (t, $^2J_{P-F}$=99.8 Hz). ^{13}C-NMR (99.5 MHz, $CDCl_3$) δ_C: 16.32 and 16.34 (d, $^3J_{C-P}$=5.8 Hz, $PO_2CH_2CH_3$), 25.85 (s, CH_2CH_2 C=N), 34.80 (d, $^3J_{C-F}$=1.6 Hz, CH_2CH_2C=N), 52.22 (s, $CHCO_2CH_3$), 65.16 (d, $^2J_{C-P}$=6.6 Hz) and 65.28 (d, $^2J_{C-P}$=6.7 Hz) ($PO_2CH_2CH_3$), 74.85 (s, CO_2CH_3), 114.68 (dt, $^1J_{C-P}$=208 Hz, $^1J_{C-F}$=261 Hz, CF_2), 171.29 (s, CO_2CH_3), 172.52 (dt, $^2J_{C-P}$ =14.2 Hz, $^2J_{C-F}$=25.3 Hz, C=N). *Anal.* Found: C, 41.93; H, 6.09; N, 4.38%. Calcd. for $C_{11}H_{18}F_2NO_5P$: C, 42.22; H, 5.79; N, 4.47%.

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14) Compound **2**: white solid, mp 152–155°C (dec). IR (KBr) ν_{max} cm^{-1}: 3700–2300, 1620, 1220. ^{1}H-NMR (200 MHz, D_2O) δ_H: 1.0–2.2 (m, 24H, CH_2CH_2P and cyclohexyl), 3.2 (m, 2H, cyclohexyl), 3.78 (t, J=6.0 Hz, 1H, α-H). ^{19}F-NMR (282 MHz, D_2O) δ_F: 12.59 (d, $^1J_{F-P}$=980 Hz). ^{31}P-NMR (81.0 MHz, D_2O) δ_P: 26.98 (d, $^1J_{P-F}$=982 Hz). HRMS (FAB, glycerol) m/z (MH^+): calcd. for $C_{16}H_{33}FN_2O_4P$, 367.2162; found, 367.2173.

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