Available online at www.sciencedirect.com
SCIENCE DIRECT
Bioorganic & Medicinal Chemistry 14 (2006) 6043–6054
Bioorganic & Medicinal Chemistry

# γ-(Monophenyl)phosphono glutamate analogues as mechanism-based inhibitors of γ-glutamyl transpeptidase

Liyou Han,[a] Jun Hiratake,[a,*] Norihito Tachi,[a] Hideyuki Suzuki,[b] Hidehiko Kumagai[b,†] and Kanzo Sakata[a]

[a]*Institute for Chemical Research, Kyoto University, Uji, Kyoto 611-0011, Japan*
[b]*Graduate School of Biostudies, Kyoto University, Kitashirakawa, Sakyo-ku, Kyoto 606-8502, Japan*

Received 26 March 2006; revised 2 May 2006; accepted 3 May 2006
Available online 23 May 2006

**Abstract**—γ-Glutamyl transpeptidase (GGT, EC 2.3.2.2) catalyzes the hydrolysis and transpeptidation of extracellular glutathione and plays a central role in glutathione homeostasis. We report here the synthesis and evaluation of a series of hydrolytically stable γ-(monophenyl)phosphono glutamate analogues with varying electron-withdrawing para substituents on the leaving group phenols as mechanism-based and transition-state analogue inhibitors of *Escherichia coli* and human GGTs. The monophenyl phosphonates caused time-dependent and irreversible inhibition of both the *E. coli* and human enzymes probably by phosphonylating the catalytic Thr residue of the enzyme. The inactivation rate of *E. coli* GGT was highly dependent on the leaving group ability of phenols with electron-withdrawing groups substantially accelerating the rate (Brønsted $\beta_{lg} = -1.4$), whereas the inactivation of human GGT was rather slow and almost independent on the nature of the leaving group. The inhibition potency and profiles of the phosphonate analogues were compared to those of acivicin, a classical inhibitor of GGT, suggesting that the phosphonate-based glutamate analogues served as a promising candidate for potent and selective GGT inhibitors.

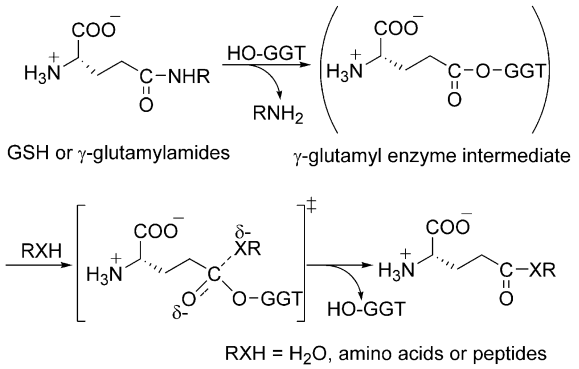

## 1. Introduction

γ-Glutamyl transpeptidase (GGT; EC 2.3.2.2) is a heterodimeric enzyme found widely among organisms from bacteria to mammals and catalyzes the first step in glutathione (GSH) metabolism.[1–4] In mammals, GGT is bound to the external surface of plasma membrane and is expressed in high concentrations in kidney tubules, biliary epithelium, and brain capillaries.[1,4,5] This enzyme plays critical roles in GSH homeostasis by breaking down extracellular GSH to provide cells with secondary source of cysteine, the rate-limiting substrate for intracellular GSH biosynthesis,[6–9] and in detoxification of electrophilic/oxidative xenobiotics through the metabolism of GSH conjugates to confer resistance against oxidative stress and anti-tumor drugs such as cisplatin.[10,11] The expression of GGT is often increased significantly in human tumors, and its roles in tumor progression[12] and the expression of malignant phenotypes of cancer cells such as drug resistance[10,11,13,14] and metastasis[15–17] have been repeatedly suggested.[18] GGT is also implicated in many physiological disorders such as neurodegenerative disease,[19,20] diabetes,[21,22] and cardiovascular disease[23,24] in relation to oxidative stress and GSH homeostasis.[25] Therefore, the important roles played by GGT in GSH-mediated detoxification and cellular response to oxidative stress strongly suggest that GGT is an attractive pharmaceutical target for cancer chemotherapy and a vast array of physiological disorders related to oxidative stress caused by reactive oxygen species.

GGT catalyzes the cleavage of the γ-glutamyl bond of GSH, its *S*-conjugates, and structurally diverse γ-glutamyl amides to transfer the γ-glutamyl group to water (hydrolysis) or to a variety of amino acids and peptides (transpeptidation) through a modified ping–pong mechanism involving a γ-glutamyl enzyme intermediate (Scheme 1).[26,27] Although a number of inhibitors have been reported to date, little compounds appear to serve as potent and specific inhibitors of GGT. (α*S*,5*S*)-α-Amino-3-chloro-4,5-dihydro-5-isoxazoleacetic acid

*Keywords*: γ-Glutamyl transpeptidase; γ-(Monophenyl)phosphono glutamate analogues; Mechanism-based inhibitor; Phosphonylation.
* Corresponding author. Tel.: +81 774 38 3231; fax: +81 774 38 3229; e-mail: hiratake@scl.kyoto.u-ac.jp
† Present address: Research Institute for Bioresources and Biotechnology, Ishikawa Prefectural University, Nonoichi-cho, Ishikawa 921-8836, Japan.

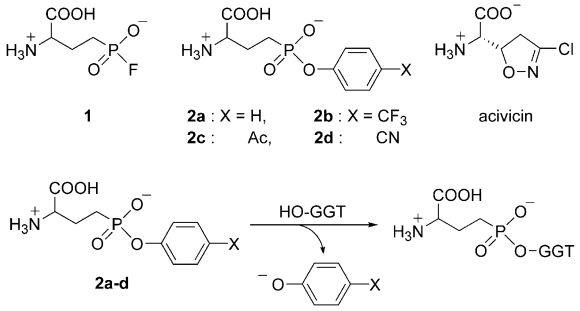
0968-0896/$ - see front matter © 2006 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmc.2006.05.008




**Scheme 1.** Proposed catalytic mechanism of GGT.

(acivicin, AT-125), a natural product derived from *Streptomyces sviceus*,[28] is a classical and most widely used irreversible inhibitor of GGT[29–31], but is a strong inhibitor of glutamine-dependent amidotransferases[32,33] and inactivates many physiologically important enzymes involved in purine and pyrimidine biosynthesis to exert potent cytotoxicity.[34,35] Other glutamine antagonists such as L-azaserine and 6-diazo-5-oxo-L-norleucine (DON) inhibit GGT,[36] but these compounds also serve as potent inhibitors of glutamine-dependent amidotransferases.[34] The reversible and weak inhibition of GGT by L-serine–borate complex[37] lead to the development of L-2-amino-4-boronobutanoic acid (γ-boroGlu), a γ-boronic acid analogue of glutamic acid, as a potent GGT inhibitor with an inhibition constant ($K_i$) of a nanomolar range.[38,39] This compound is believed to form a covalent bond with a hydroxy residue in the active site to mimic the transition-state of GGT catalysis, but the inhibition is reversible, and the inactivated enzyme regained activity rapidly.[38]

We previously reported that 2-amino-4-(fluorophosphono)butanoic acid (**1**), a γ-monofluorophosphono derivative of glutamic acid, served as an extremely effective mechanism-based affinity labeling agent that inactivated *Escherichia coli* GGT with a second-order rate constant for enzyme inactivation ($k_{on}$) of 48,000 $M^{-1}$ $s^{-1}$.[40] This compound was used successfully for the identification of the catalytic nucleophile of *E. coli* GGT as Thr-391,[40] the N-terminal residue of the small subunit, suggesting that GGT is a member of the N-terminal nucleophile hydrolase family.[41,42] Compound **1** is a promising lead for potent and selective inhibitors of GGT, because it reacts with the catalytic residue of GGT in a mechanism-based manner to form a stable monophosphonate bond with its catalytic Thr to inactivate the enzyme. However, the monofluorophosphonate **1** is highly reactive and is hydrolytically unstable in alkaline to neutral media.[40] Herein we report the synthesis and evaluation of a series of hydrolytically stable γ-(monophenyl)phosphono glutamate analogues **2a**–**d** with varying electron-withdrawing groups at the *para*-position of the leaving group phenols as mechanism-based inhibitors of *E. coli* and human GGTs (Scheme 2). The monophenyl phosphonates **2a**–**d** served as irreversible inhibitors that caused time-dependent inhibition of both the *E. coli* and human enzymes, but with significantly higher activity toward *E. coli* GGT. The dependence of the inactivation rates on the leaving group ability suggested significant differences in the inactivation transition state between the *E. coli* and human enzymes. The phosphonate-based glutamate analogues are compared with acivicin in terms of the potency and the mechanism of inactivation.

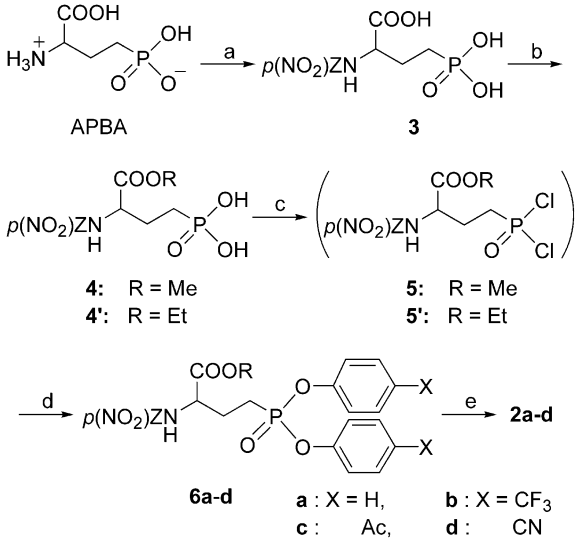



**Scheme 2.** Design of γ-(monophenyl)phosphono glutamate analogues **2a**–**d** as mechanism-based inhibitors of GGT.

## 2. Results and discussion

### 2.1. Synthesis and stability of monophenyl phosphonates *2a–d*

The monophenyl phosphonates **2a**–**d** were synthesized as shown in Scheme 3. The amino group of 2-amino-4-phosphonobutanoic acid (APBA) was protected with 4-nitrobenzyloxycarbonyl [*p*($NO_2$)Z] group, and the α-carboxy group was esterified selectively under acidic conditions to afford the methyl ester **4** or the ethyl ester **4′** with a free γ-phosphono group. The methyl ester **4**, however, was partially hydrolyzed (22%) to the acid **3**




**Scheme 3.** Synthesis of monophenyl phosphonates **2a**–**d**. Reagents: (a) *p*($NO_2$)ZCl, $NaHCO_3$; (b) $SOCl_2$, MeOH or EtOH, 75%; (c) $SOCl_2$, cat. DMF, 100%; (d) *p*-$XC_6H_4OH$, NaH, dry THF (X = H, $CF_3$, Ac and CN), 38–97%; (e) i—0.2 N NaOH; ii—$H_2$/5% Pd–C, MeOH or THF; **2a**: 59%, **2b**: 16%, **2c**: 80%, **2d**: 38%.

during purification on an ODS column (MeOH–$H_2O$), probably because the proximal acidic γ-phosphonyl group intramolecularly assisted the transesterification or hydrolysis of the α-carboxy group. The same transesterification was also observed with the *N*-protected monophenyl phosphonate derived from **6b** during chromatography on an ODS column (MeOH–$H_2O$) to give a small amount (ca. 10%) of the corresponding methyl ester (data not shown). This side reaction was completely prevented by using the ethyl ester **4′**.

The phosphonic acids **4** and **4′** were converted to the phosphonic diesters **6a–d** via the corresponding phosphono dichloridates **5** and **5′** followed by treatment with two equivalents of a series of *p*-substituted phenols and triethylamine.[43] Alkaline hydrolysis of **6a–d** followed by hydrogenolysis afforded the corresponding monophenyl phosphonates **2a–d**. The conditions for selective removal of the *p*($NO_2$)Z group are worth noting: the acetyl and the cyano groups attached to the phenyl ring are reduced under a standard condition for catalytic hydrogenolysis. In fact, the hydrogenolysis of the mono(4-acetylphenyl) phosphonate derived from **6c** in an acidic medium (MeOH/AcOH/$H_2O$) resulted in complete reduction of the acetyl group, along with the removal of the *p*($NO_2$)Z group. However, the use of a protic, but a neutral solvent system (MeOH–$H_2O$) combined with a less active catalyst (5% Pd–C) successfully cleaved the *p*($NO_2$)Z group without reducing the acetyl group. Since the cyano group is more susceptible to reduction than the acetyl group, the use of an aprotic and less polar solvent such as THF was required to prevent the reduction of the cyano group of **2d**. The use of less polar solvent, however, was problematic in product recovery, because the phosphonate monoesters **2b–d** were sparingly soluble in such solvents and were precipitated and deposited on the Pd–C as the reaction proceeded. Therefore, the products **2b–d** were recovered from the reaction mixture by extracting the Pd–C with hot water containing trifluoroacetic acid, followed by purification by crystallization or column chromatography (see Section 3).

The hydrolytic stability was examined with the least stable **2d** in $D_2O$ at 23 °C. No hydrolysis was observed in 12 h, suggesting that the monophenyl phosphonates **2a–d** were highly stable even with an electron-withdrawing group attached to the para position of the phenyl ring. The hydrolytic stability of **2a–d** was also examined at higher temperature (60 °C) at pH 5.5 for 12 h. Compound **2a** was very stable under these conditions, but the monophenyl phosphonates **2b–d** with an electron-withdrawing group were hydrolyzed partially to APBA according to the extent of leaving group ability of the phenols: 11%, 40%, and 65% hydrolysis was observed for **2b**, **2c**, and **2d**, respectively ($^{31}P$ NMR). Since the half-life ($t_{1/2}$) of the monofluorophosphonate **1** is 21.6 h (pH 5.5, 37 °C)[40] and 20 min (pH 7.5, 37 °C),[44] the hydrolytic stability of the monophenyl phosphonates **2a–d** was significantly improved by replacing the highly acidic leaving group (HF, p$K_a$ = 3.17) with less acidic substituted phenols.[45] However, less reactive electrophilic phosphonates may in turn lead to less potent inhibitory activities toward the enzyme. Therefore, the activities of **2a–d** were examined for inhibition of *E. coli* and human GGTs as model enzymes and were compared to those of monofluorophosphonate **1** and acivicin.

### 2.2. Inactivation of *E. coli* and human GGT by 2a–d

For evaluating the inhibitory activities of **2a–d**, we used the hydrolase, rather than the transpeptidase, activity of GGT, because the inhibitors **2a–d** have a *para*-substituted phenol moiety that may bind to the acceptor site of GGT.[46] Thus, a standard transpeptidase assay using a high concentration of an acceptor molecule (e.g., 10 mM Gly-Gly[47]) was thought to affect the binding of the inhibitors to underestimate the inhibitory activities, particularly when a continuous inhibition assay method is employed (see Section 3). Another reason is that we wanted to compare the inhibitory activities of **2a–d** with the activity of the enzyme, on the premise that the phosphonates **2a–d** serve as mechanism-based inhibitors that exploit the catalytic activity for enzyme inactivation. Transpeptidation is not a good measure of the intrinsic enzyme activity, because the apparent transpeptidase activity is highly dependent on the p$K_a$ of the amino group of the acceptor molecules that affects the effective concentration of the deprotonated form of the acceptor amino acid at a given pH.[48] Therefore, prior to the inhibition experiment, we measured the pH-dependence of the hydrolase activities of *E. coli* and human GGTs (Fig. 1). The pH-rate profiles indicated that the hydrolase activity of *E. coli* GGT was almost constant from pH 5.5 to 9.0,[49] but the activity of the human enzyme was highly dependent on pH: the apparent activity at pH 8 was about eight times higher than that at pH 5.5. The increase in the apparent activity at higher pH was probably due to the increase in the catalytic activity of the enzyme, because the activity was measured at sufficiently low substrate concentration (γ-Glu-AMC, 4.0 μM) to prevent autotranspeptidation,[47] and the observed convex upward curve is not likely to reflect

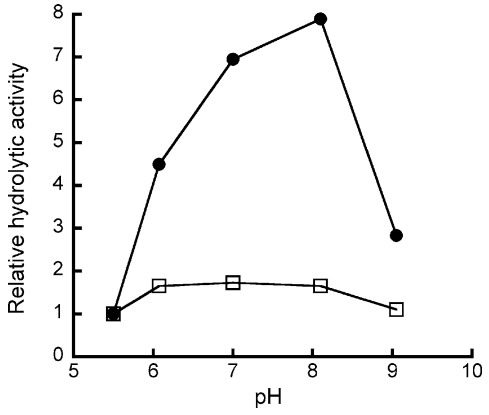



**Figure 1.** pH-rate profiles of the hydrolysis of human GGT (●) and *E. coli* GGT (□). The initial rates of hydrolysis were measured by using 0.2 and 4.0 μM γ-Glu-AMC as substrate for *E. coli* and human GGT, respectively, at 25 °C (see Section 3). The hydrolytic activities are shown relative to the rate of hydrolysis at pH 5.5 for each enzyme. The following buffer systems were used: pH 5.5, 100 mM succinate–NaOH; pH 6.1, 100 mM PIPES; pH 7.0, 100 mM MOPS; pH 8.1, 100 mM tricine–NaOH; pH 9.1, 100 mM $NaHCO_3$–$Na_2CO_3$ buffer.

the acid-base equilibrium of the amino group of the substrate.[48,50] We therefore measured the inhibitory activities of **2a**–**d** at pH 5.5 for *E. coli* GGT, and at pH 5.5 and 8.0 for human GGT, under the assay conditions for hydrolase activity.

Compounds **2b**–**d** inhibited both *E. coli* and human GGTs in a time-dependent manner as depicted by a series of typical progress curves that show the rate of enzyme inactivation increased with increasing concentration of the inhibitor (Fig. 2).

Compound **2a**, the least active monophenyl phosphonate, slowly inactivated *E. coli* GGT, but not the human enzyme either at pH 5.5 or 8.0. The time-dependent inhibition curves for **2b**–**d** were fit to the first-order rate Eq. (1) to determine the pseudo-first-order rate constant for enzyme inactivation ($k_{obsd}$) at each inhibitor concentration. The inhibition of *E. coli* GGT by **2a** was so slow that the values of $k_{obsd}$ were determined by measuring the remaining activity periodically by incubating the enzyme with varying concentrations of the inhibitor without substrate (discontinuous assay, see Section 3). A plot of $k_{obsd}$ against the inhibitor concentration was found to be linear for each inhibitor, and the second-order rate constant for enzyme inactivation ($k_{on}$) was calculated according to the Eq. (2) or (4). For comparison, the inactivation rates of *E. coli* and human GGTs by the monofluorophosphonate **1** and acivicin were also determined. The inhibition of human GGT by **1** was irreversible, because no regain of enzyme activity was observed after prolonged dialysis of the inactivated enzyme (data not shown). The inactivation of human GGT was probably caused by the phosphonylation of the catalytic nucleophile as confirmed with *E. coli* GGT inactivated by **1**.[40]

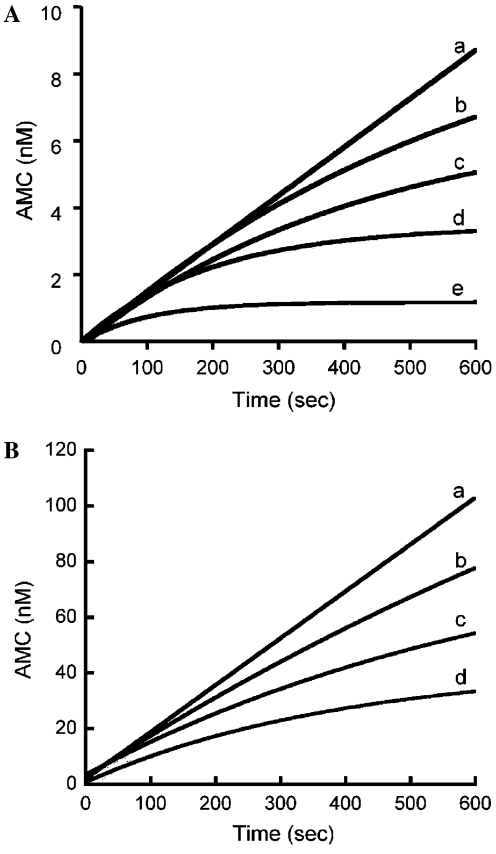



**Figure 2.** Typical time-dependent inhibition of GGT by the monophenyl phosphonate **2**. (A) Progress curves of *E. coli* GGT with 0.2 μM γ-Glu-AMC at pH 5.5 in the presence of varying concentrations of **2d**: (a) 0, (b) 7.08, (c) 14.2, (d) 35.4, (e) 70.8 μM. (B) Progress curves of human GGT with 4.0 μM γ-Glu-AMC at pH 8.0 in the presence of varying concentrations of **2c**: (a) 0, (b) 0.41, (c) 0.81, (d) 1.22 mM.

The values of $k_{on}$ and the p$K_a$ of the leaving groups for **1**, **2a**–**d**, and acivicin are listed in Table 1. The monophenyl phosphonates **2a**–**d** were far more potent inhibitors of *E. coli* GGT than toward the human enzyme: the inactivation rates of *E. coli* GGT were at least two orders of magnitude larger than those of human GGT at pH 5.5. This also holds for the inhibition by acivicin, where human GGT was inactivated much more slowly than the *E. coli* enzyme. These results can be explained in part by the fact that *E. coli* GGT has a rather broad substrate specificity and is more specific for aromatic amino acids[49] as compared to the mammalian GGT.[26] Thus, the phosphonates **2a**–**d** with an aromatic moiety might be better accommodated by *E. coli* GGT than by the human enzyme to facilitate the enzyme inactivation.

Interestingly, the inhibition potency of the phosphonates **1** and **2a**–**d** toward *E. coli* GGT was highly dependent on the p$K_a$ of the leaving group, whereas the inactivation rate of human GGT was almost independent on the leaving group ability at either pH 5.5 or 8.0. As a result, the most reactive monofluorophosphonate **1** was by far the most potent inhibitor toward *E. coli* GGT, whereas the less reactive monophenyl phosphonates **2b**–**d** served as fairly good inhibitors of human GGT as compared to **1** and acivicin at pH 5.5. The difference became even smaller at pH 8, where the inhibitory activity of **2c** almost rivaled that of **1** and was more than three times higher than that of acivicin towards the human enzyme. Due to the large difference in the dependence of inhibitory activity on the leaving group ability, the selectivity of the phosphonates **2a**–**d** for *E. coli* GGT was significantly increased as the leaving group ability was increased: the phosphonate **2d**, for example, was ca. 2300 times more active toward *E. coli* GGT than toward the human enzyme at pH 5.5. These results indicate that there are significant differences between the *E. coli* and the human enzymes in the structure of the active site region and the catalytic mechanism.

It should be noted that the $k_{on}$ values for human GGT by the phosphonates **1** and **2a**–**d** increased by 6 to 19 times when the pH was raised from 5.5 to 8.0. The increase in the $k_{on}$ values corresponds approximately to the increase in the enzyme activity between pH 5.5 and 8.0 (Fig. 1), suggesting that the phosphonates **2b**–**d** served as mechanism-based inhibitors of GGT that reacted with the catalytic nucleophile by the assistance of the catalytic power of the enzyme. In contrast, the $k_{on}$ value of acivicin at pH 8.0 was only three times larger than that at pH 5.5, arguing against the correlation of the inhibition by acivicin with the catalytic mechanism

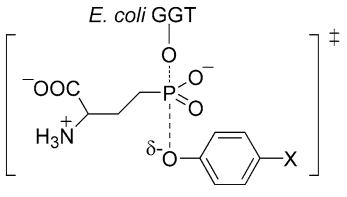
**Table 1.** Inhibitory activities of **1**, **2a–d** and acivicin toward *E. coli* and human GGT

| Inhibitor | Leaving group | p$K_a$ | $k_{on}$[a] ($M^{-1}$ $s^{-1}$) | | |
|---|---|---|---|---|---|
| | | | *E. coli* GGT (pH 5.5) | Human GGT (pH 5.5) | Human GGT (pH 8.0) |
| **2a** | $C_6H_5OH$ | 9.98 | 0.26 | NI[b] | NI[b] |
| **2b** | *p*-$CF_3$—$C_6H_4OH$ | 8.51 | 18 ± 1 | 0.031 ± 0.001 | 0.30 ± 0.1 |
| **2c** | *p*-Ac—$C_6H_4OH$ | 8.12 | 130 ± 20 | 0.34 ± 0.02 | 4.9 ± 0.3 |
| **2d** | *p*-CN—$C_6H_4OH$ | 7.95 | 270 ± 10 | 0.12 ± 0.01 | 2.3 ± 0.1 |
| **1** | HF | 3.17 | 49100 ± 800[c] | 0.82 ± 0.02 | 5.0 ± 0.3 |
| Acivicin | HCl | −6.18 | 4210 ± 10 | 0.40 ± 0.02 | 1.3 ± 0.1 |

[a] Second-order rate constant for enzyme inactivation.
[b] No inhibition.
[c] Ref. 40.

of GGT (see later). In fact, acivicin was shown to bind covalently to Ser406 of human GGT, but this residue is not essential for catalysis.[31] Acivicin is a potent inhibitor of *E. coli* GGT ($k_{on}$ = 4210 $s^{-1}$ $M^{-1}$, Table 1), but its inactivation rate was much smaller than expected from its leaving group ability ($Cl^-$), suggesting again that the mechanism of enzyme inactivation with acivicin was totally different from that with the phosphonates **1** and **2a–d**.

For quantitative analysis of the substituent effect on the inhibitory activity, Brønsted plots have been constructed for the inactivation of *E. coli* and human GGTs by the phosphonates **1** and **2a–d**. A large negative slope ($\beta_{lg}$ −1.4) with excellent linearity was obtained for the inactivation of *E. coli* GGT by the monophenyl phosphonates **2a–d** (Fig. 3A). Such a large leaving group dependence in phosphoryl transfer reactions[51] suggests that the reaction of the catalytic nucleophile of *E. coli* GGT with the electrophilic phosphorus proceeded via an anionic and dissociative transition state with substantial bond cleavage between the phosphorus and the leaving group as shown below. Interestingly, the plot of the monofluorophosphonate **1** was located well below the line, which is indicative of significantly lower activity of **1** than expected from its excellent leaving group ability of $F^-$. However, this can be attributed to the lower affinity of **1** rather than its reactivity, because the bimolecular reaction constant shown here ($k_{on}$) contains the kinetic parameter ($K_i$) with respect to the reversible binding step of the inhibitor before the formation of a covalent bond with the enzyme. Since *E. coli* GGT has a preference for aromatic amino acids as a γ-glutamyl acceptor,[49] the fluorine atom in **1** may be too small to be accommodated effectively in the acceptor site of the *E. coli* enzyme. If this is the case, then the excellent linearity obtained with **2a–d** cogently suggests that *E. coli* GGT accommodates these monophenyl phosphonates almost equally regardless of the substituent on the para position. This is also consistent with the broad substrate specificity of *E. coli* GGT.



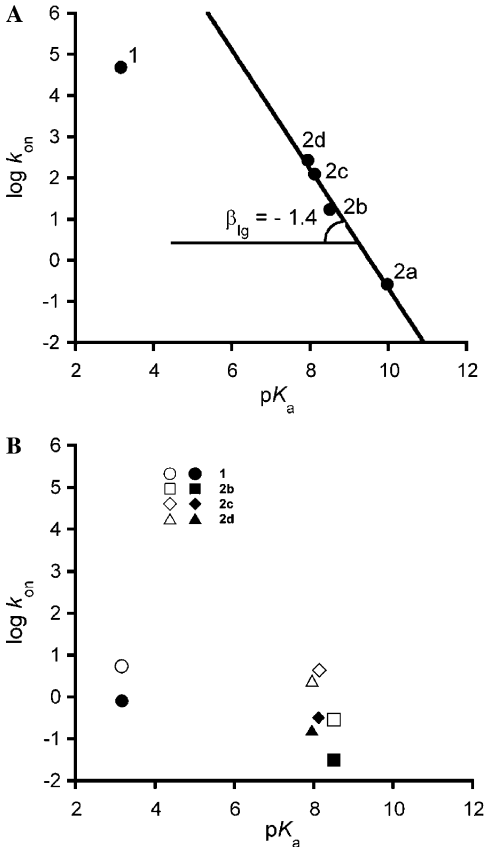
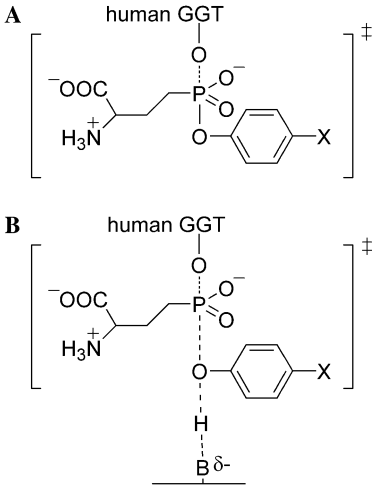




**Figure 3.** Brønsted plots relating the second-order rate constants for enzyme inactivation ($k_{on}$) by **1** and **2a–d** with the leaving group ability. (A) *E. coli* GGT at pH 5.5. (B) Human GGT at pH 5.5 (filled symbols) and pH 8.0 (open symbols).

In contrast, the Brønsted plot for the inactivation of human GGT gave a slope near zero with much poor linearity (Fig. 3B). A small value of $\beta_{lg}$ is consistent with a transition state that is primarily associative in character with little bond cleavage between the phosphorus and the leaving group as shown in the following figures (A), but is also consistent with general acid catalysis, where an acidic group in the active site provides a proton that offsets the development of negative charge on the leaving oxygen as the P–O bond breaks (B). Keillor et al. reported that the reaction catalyzed by rat kidney GGT proceeded via the general-acid-catalyzed acylation with an ammonium ion acting as a general acid, derived from the results of free energy relationships using a ser-

ies of *para*-substituted L-glutamyl γ-anilide as substrate.[52] Although the mechanism for the enzyme inactivation is different from that for the enzyme catalysis, the evidence for the general-acid-catalyzed acylation of GGT favors the participation of a general acid catalysis also in the phosphonylation of human GGT by the monophenyl phosphonates **2b–d**.




### 2.3. Effect of 3-benzoylpropionic acid (3-BPA) on the inactivation of *E. coli* and human GGT by 1

The glutaminase activity of rat GGT was reported to be stimulated by maleate,[53,54] hippurate, and its analogues including 3-BPA,[55,56] but at the expense of the transpeptidase activity. The inactivation by acivicin or DON was also found to be accelerated by these compounds.[29,55,56] From these observations, Meister and Tate et al. concluded that maleate and the hippurate analogues were bound at the Cys-Gly binding site and that these acceptor site-directed binders modulated the enzyme activity by cooperative interactions between the γ-glutamyl donor and the acceptor binding domains of the GGT active site through conformational change.[56] It is interesting to test these hypotheses by using the fluorophosphonate **1** as a chemical probe, because the phosphonate **1** served as a mechanism-based inhibitor that reacted with the γ-glutamyl donor site, but does not have a large substituent that may bind to the acceptor site. We therefore measured the effect of 3-BPA on the inactivation of *E. coli* and human GGTs by **1** and acivicin (Fig. 4). Contrary to our initial expectations, the effect of 3-BPA was inhibitory, rather than accelerative, to the inactivation of both *E. coli* and human GGTs by **1** (Fig. 4A and C): the inactivation rate was decreased by 39% and 47% for *E. coli* and human GGT, respectively, in the presence of 40 mM 3-BPA. The same protective effect was observed in the inactivation of *E. coli* GGT by acivicin (Fig. 4B), where the addition of 3-BPA decreased the inactivation rate by 70%. However, the inactivation of human GGT by acivicin was significantly accelerated (ca. 30-fold) by the addition of 3-BPA (Fig. 4D). Of particular interest is that the acceleration of enzyme inactivation was observed solely for human GGT by acivicin. These results indicate some important facets regarding the differences between mammalian and bacterial GGTs and the properties of acivicin. First, the modulation of enzyme activity by acceptor site-directed molecules reported

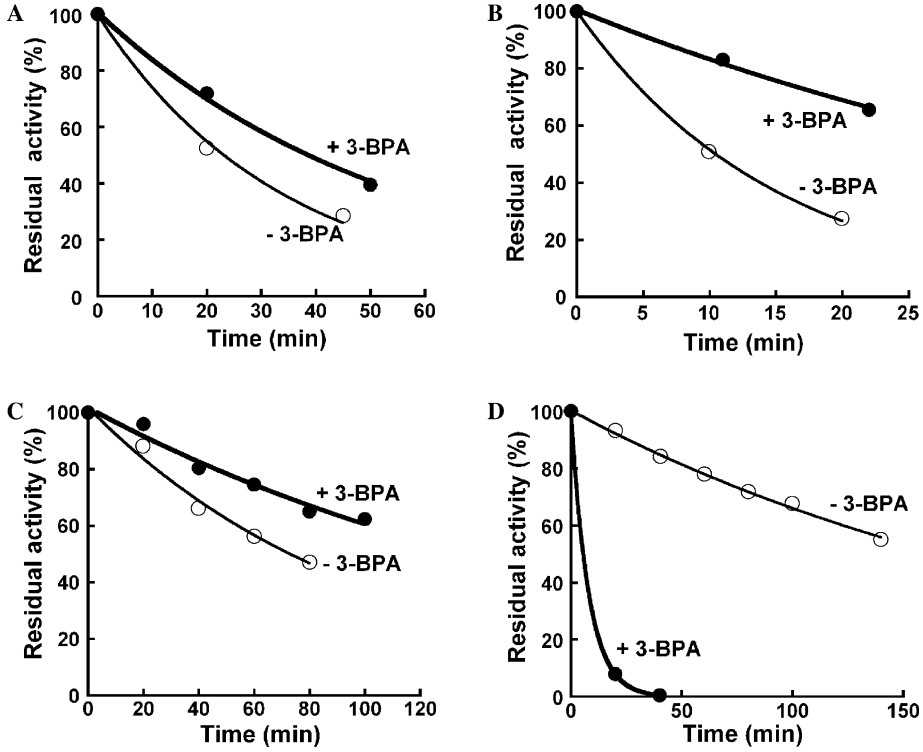



**Figure 4.** Effect of 3-benzoylpropionic acid (3-BPA, 40 mM) on the inactivation of *E. coli* GGT (A, B) and human GGT (C, D) by acivicin and monofluorophosphonate **1** in 100 mM succinate buffer (pH 5.5). Inhibitors: (A) 8.37 nM of **1**, (B) 0.17 μM acivicin, (C) 0.15 mM of **1**, (D) 0.20 mM acivicin.

with rat GGT[55,56] does not always hold for all the GGTs. At least, the *E. coli* enzyme does not appear to have such properties. This might be related to the differences in the physiological functions of bacterial and mammalian GGTs: the *E. coli* enzyme is a soluble protein located in the periplasmic space[49] and is probably responsible for utilization of exogenous γ-glutamyl peptides as an amino acid source.[57] In contrast, the mammalian GGT is anchored to the extracellular surface of the plasma membrane of cells with secretory or absorptive functions and is highly specific for glutathione and its analogues.[1] The in vivo role of hippurate was also implicated in the regulation of the activity of renal GGT.[55] Second, the protective effect of 3-BPA on the inactivation of human GGT by **1** (Fig. 4C) is indicative of binding of 3-BPA not only in the acceptor site, but also in the γ-glutamyl donor site. This notion is supported by the protective effect of 3-BPA on the inactivation of *E. coli* GGT either by compound **1** and acivicin. Finally, the inhibition of GGT by acivicin is a general observation, but its inactivation mechanism seems totally different from that by the mechanism-based inhibitors **1** and **2a**–**d** shown here. It is still unclear why 3-BPA significantly accelerated the inactivation of human GGT by acivicin, but it seems likely that the inhibition of GGT by acivicin is a rather fortuitous event that is not related to the enzyme catalysis, and its acceleration by 3-BPA may also be unrelated to the acceleration of the catalytic activity of the enzyme.

## 3. Experimental

### 3.1. Materials and methods

All chemicals were obtained commercially and used without further purification unless otherwise noted. 7-(γ-L-Glutamylamino)-4-methylcoumarin (γ-Glu-AMC) and (α*S*,5*S*)-α-amino-3-chloro-4,5-dihydro-5-isoxazoleacetic acid (acivicin) were purchased from Sigma. Racemic 2-amino-4-phosphonobutanoic acid (APBA) was synthesized by the reported procedure.[58,59] Racemic 2-amino-4-(fluorophosphono)butanoic acid (**1**) was synthesized previously.[40,44] Dry toluene and dry $CH_2Cl_2$ were prepared by distillation from $CaH_2$ and stored over molecular sieves 4 Å. Dry tetrahydrofuran (THF) was purchased from Kanto Kagaku (Tokyo, Japan). *E. coli* γ-glutamyltranspeptidase was purified from the periplasmic fraction of a recombinant strain of *E. coli* K-12 (SH642) by lysozyme treatment, ammonium sulfate precipitation, and chromatofocusing as described previously.[49] Human γ-glutamyltranspeptidase HC-GTP (T-72) was a generous gift from Asahi Kasei Corporation (Osaka, Japan). The latter enzyme preparation contained a large amount of bovine serum albumin (BSA) as enzyme stabilizer (GGT content <1%).

The protein concentration was determined by Bradford's method.[60] $^1H$, $^{13}C$, and $^{31}P$ NMR spectra were recorded on a JEOL JNM-AL 300 (300 MHz for $^1H$) or a JEOL JNM-AL 400 (400 MHz). Chemical shifts were recorded relative to the internal standard (tetramethylsilane for $^1H$ and $^{13}C$) or to the external standard [sodium 3-(trimethylsilyl)propanesulfonate for $^1H$ in $D_2O$ and 85% $H_3PO_4$ for $^{31}P$]. Infrared spectra were recorded on a Hitachi U-215 infrared spectrophotometer. Mass spectra were obtained with a JEOL JMS 700 spectrometer. Elemental analyses were performed on a Yanaco MT-5. Thin-layer chromatography was carried out using silica gel plates (Merck 5715, 0.25 mm). Compounds were purified by flash column chromatography on silica gel 60 (Merck 9385, 40–63 μm) or by medium-pressure reversed-phase column chromatography using a Yamazen YFLC (Yamazen Co., Osaka, Japan).

### 3.2. Synthesis

**3.2.1. 2-(*N*-4-Nitrobenzyloxycarbonylamino)-4-phosphonobutanoic acid (3).** 4-Nitrobenzyl chloroformate (12.1 g, 56.1 mmol) and $NaHCO_3$ (4.71 g, 56.1 mmol) were added portion wise over 2 h to a vigorously stirred solution of APBA (6.85 g, 37.4 mmol) and NaOH (4.48 g, 112 mmol) in a mixture of $H_2O$ (110 mL)–$Et_2O$ (40 mL) at 0 °C. The mixture was stirred vigorously at ambient temperature for 26 h. After completion of the reaction (TLC, BuOH/AcOH/$H_2O$ = 5:2:2, ninhydrin for consumption of APBA), the reaction mixture was washed with $Et_2O$ (3× 50 mL), acidified with 1 N HCl to pH 1, and was evaporated to dryness. Acetone (150 mL) was added to the residue, and the insoluble salt (NaCl) was removed by filtration. The residual oil was purified by medium-pressure reversed-phase column chromatography on a Daisogel SP-120-40/60-ODS-B (Daiso Co. Ltd., Osaka, Japan). The column was eluted with a linear gradient of MeOH/$H_2O$ (40–60%), and the fractions containing the product (TLC, BuOH/AcOH/$H_2O$ = 5:2:2, UV) were lyophilized to afford **3** as a highly hygroscopic solid (10.1 g, 75%): $^1H$ NMR (300 MHz, $D_2O$) $\delta_H$ 1.6–1.8 (m, 2H, $CH_2CH_2P$), 1.8–2.2 (2 × m, 2H, $CH_2CH_2P$), 4.2 (m, 1H, α-CH), 5.2 (s, 2H, benzyl), 7.6 (d, $J$ = 8.4 Hz, 2H) and 8.2 (d, $J$ = 8.3 Hz, 2H) ($C_6H_4NO_2$); $^{31}P$ NMR (121 MHz, $D_2O$) $\delta_p$ 27.71; Anal. Calcd for $C_{12}H_{15}N_2O_9P$: C, 39.79; H, 4.17; N, 7.73. Found: C, 39.62; H, 4.35; N, 7.63.

**3.2.2. Methyl 2-(*N*-4-nitrobenzyloxycarbonylamino)-4-phosphonobutanoate (4).** Thionyl chloride (3.22 g, 27.1 mmol) was added dropwise to MeOH (100 mL) at −5 °C, and the mixture was stirred for 15 min. Compound **3** (6.54 g, 18.1 mmol) was added to the solution, and the mixture was stirred at ambient temperature for 76 h. Volatiles were removed in vacuo, and the residual oil was purified by medium-pressure reversed-phase column chromatography (ODS, Daisogel) eluted with a linear gradient of MeOH/$H_2O$ (50–99%). The fractions containing the product (TLC, BuOH/AcOH/$H_2O$ = 5:2:2, UV) were collected and lyophilized to afford **4** as a highly hygroscopic amorphous solid (4.96 g, 73%, containing 22% of **3** derived from **4** by hydrolysis during chromatography). Analytical sample was obtained from the fractions containing pure **4**: $^1H$-NMR (300 MHz, acetone-$d_6$) $\delta_H$ 1.8 (m, 2H, $PCH_2CH_2$), 1.9–2.2 (2 × m, 2H, $PCH_2CH_2$), 3.7 (s, 3H, $CH_3O$), 4.3 (m, 1H, α-CH), 5.3 (s, 2H, benzyl), 7.08

(br d, $J$ = 8.1 Hz, 1H, NH), 7.6 (d, $J$ = 8.7 Hz, 2H) and 8.2 (d, $J$ = 8.7 Hz, 2H) ($C_6H_4NO_2$); $^{31}$P NMR (121 MHz, acetone-$d_6$) $\delta_p$ 31.44; HRMS (FAB, glycerol) calcd for $C_{13}H_{18}N_2O_9P$ ($MH^+$) 377.0750, found 377.0751; Anal. Calcd for $C_{13}H_{16}N_2O_9$ P·0.68 $H_2O$: C, 40.19; H, 4.76; N, 7.21. Found: C, 40.17; H, 4.77; N, 7.41.

**3.2.3. Ethyl 2-(*N*-4-Nitrobenzyloxycarbonylamino)-4-phosphonobutanoate (4′).** The ethyl ester **4′** was prepared from **3** and EtOH–HCl by the same procedure as described for the synthesis of the methyl ester **4**. The product was purified by medium-pressure reversed-phase column chromatography (ODS-S-50D, Yamazen Co.) eluted with a linear gradient of MeOH/$H_2O$ (50–65%) to give pure **4′** as a colorless amorphous solid (75%): $^1$H-NMR (300 MHz, acetone-$d_6$) $\delta_H$ 1.2 (t, $J$ = 7.1 Hz, 3H, $OCH_2CH_3$), 1.8 (m, 2H, $PCH_2CH_2$), 1.9–2.3 (2 × m, 2H, $PCH_2CH_2$), 4.2 (2 × q, $J$ = 7.1 Hz, 2H, $OCH_2CH_3$), 4.3 (m, 1H, α-CH), 5.3 (s, 2H, benzyl), 7.04 (br d, $J$ = 8.2 Hz, 1H, NH), 7.6 (d, $J$ = 8.8 Hz, 2H) and 8.2 (d, $J$ = 8.8 Hz, 2H) ($C_6H_4NO_2$), 9.14 [br s, 3H, COOH and P(O)(OH)$_2$]; $^{31}$P NMR (121 MHz, acetone-$d_6$) $\delta_p$ 32.67; Anal. Calcd for $C_{14}H_{19}N_2O_9P$: C, 43.09; H, 4.91; N, 7.18. Found: C, 42.99; H, 4.91; N, 7.17.

**3.2.4. Methyl 2-(*N*-4-nitrobenzyloxycarbonylamino)-4-(diphenylphosphono)butanoate (6a).** A solution of **4** (0.87 g, 2.31 mmol) in dry $CH_2Cl_2$ was gently refluxed in the presence of a catalytic amount of DMF under an argon atmosphere. To this solution was added oxalyl chloride (0.73 g, 5.75 mmol), and the mixture was refluxed for 2 h. During the addition, copious gas was evolved, and the heating was adjusted to keep gentle refluxing. The reaction mixture was evaporated in vacuo to give the corresponding phosphonodichloridate **5** as an yellow oil: $^1$H-NMR (300 MHz, $CDCl_3$) $\delta_H$ 2.2–2.8 (m, 4H, $PCH_2CH_2$), 3.8 (s, 3H, $CH_3O$), 4.5 (m, 1H, α-CH), 5.2 (m, 2H, benzyl), 5.8 (br, 1H, NH), 7.5 (d, $J$ = 8.7 Hz, 2H) and 8.2 (d, $J$ = 8.7 Hz, 2H) ($C_6H_4NO_2$); $^{31}$P NMR (121 MHz, $CDCl_3$) $\delta_p$ 53.15. A mixture of phenol (0.46 g, 4.91 mmol) and $NEt_3$ (0.70 g, 6.93 mmol) in dry $CH_2Cl_2$ (20 mL) was added dropwise over 10 min to a solution of **5** (2.31 mmol) in dry $CH_2Cl_2$ (10 mL) at 0 °C. After the addition was complete, the mixture was stirred at 0 °C for 1.5 h. The reaction mixture was concentrated, diluted with EtOAc (50 mL) and was washed successively with 1 N HCl (50 mL), $H_2O$ (50 mL), satd $NaHCO_3$ (50 mL) and sat. NaCl (50 mL). The organic layer was dried over anhydrous $Na_2SO_4$. Solvent was removed, and the residual oil was purified by flash column chromatography on silica gel 60 (EtOAc/hexane, 3:2) to give **6a** as a colorless oil (0.79 g, 65%): $^1$H-NMR (300 MHz, $CDCl_3$) $\delta_H$ 2.0–2.5 (m, 4H, $PCH_2CH_2$), 3.77 (s, 3H, $CH_3O$), 4.48 (m, 1H, α-CH), 5.18 and 5.22 (2 × d, $J$ = 15 Hz, 2H, benzyl), 5.8 (br d, $J$ = 7.8 Hz, 1H, NH), 7.1–7.4 (m, 10H, 2 × $C_6H_5$), 7.5 (d, $J$ = 8.7 Hz, 2H) and 8.2 (d, $J$ = 8.7 Hz, 2H) ($C_6H_4NO_2$); $^{31}$P NMR (121 MHz, $CDCl_3$) $\delta_p$ 28.69; IR (NaCl) $\nu_{max}$ 1715, 1590, 1510, 1490, 1340, 1260–1160 (br), 1060–1000 (br), 925, and 760 cm$^{-1}$; Anal. Calcd for $C_{25}H_{25}N_2O_9P$: C, 56.80; H, 4.77; N, 5.30. Found: C, 56.74; H, 4.77; N, 5.23.

**3.2.5. Ethyl 2-(*N*-4-nitrobenzyloxycarbonylamino)-4-[bis(4-trifluoromethylphenyl)phosphono]butanoate (6b).** The phosphonic acid **4′** (3.11 g, 7.97 mmol) was dissolved in thionyl chloride (5 mL) and a catalytic amount of DMF and was heated at 60 °C for 1 h. The resulting solution was evaporated to give the phosphonodichloridate **5′** as an yellow oil: $^1$H-NMR (300 MHz, $CDCl_3$) $\delta_H$ 1.3 (t, $J$ = 7.1 Hz, 3H, $OCH_2CH_3$), 2.1–2.8 (m, 4H, $PCH_2CH_2$), 4.3 (q, $J$ = 7.1 Hz, 2H, $OCH_2CH_3$), 4.5 (m, 1H, α-CH), 5.20 and 5.25 (2 × d, $J$ = 17.9 Hz, 2H, benzyl), 5.7 (br d, $J$ = 6.8 Hz, 1H, NH), 7.5 (d, $J$ = 8.6 Hz, 2H) and 8.2 (d, $J$ = 8.6 Hz, 2H ($C_6H_4NO_2$); $^{31}$P NMR (121 MHz, $CDCl_3$) $\delta_p$ 49.07. An oil suspension of 60% NaH (0.73 g, 18.3 mmol) was washed twice with hexane and was suspended in dry THF (15 mL) under an argon atmosphere. A solution of 4-trifluoromethylphenol (3.23 g, 19.9 mmol) in dry THF (15 mL) was added to this suspension at 0 °C under an argon atmosphere to give an yellowish emulsion. To this mixture, a solution of the phosphonodichloridate **5′** (7.97 mmol) in dry THF (15 mL) was added dropwise over 10 min at 0 °C. After the addition was complete, the mixture gave a clear yellowish solution. The mixture was stirred at 0 °C for 1 h, and at ambient temperature for further 1 h under an argon atmosphere. After the reaction was completed ($^{31}$P NMR), the reaction mixture was concentrated and diluted with EtOAc (100 mL), washed successively with 1 N HCl (100 mL), and sat. NaCl (100 mL). The organic layer was dried over anhydrous $Na_2SO_4$ and was evaporated to give **6b** as an yellowish oil (5.87 g): $^1$H-NMR (300 MHz, $CDCl_3$) $\delta_H$ 1.2 (t, $J$ = 6.6 Hz, 3H, $OCH_2CH_3$), 2.0–2.5 (m, 4H, $PCH_2CH_2$), 4.2 (m, $J$ = 6.8 Hz, 2H, $OCH_2CH_3$), 4.4 (m, 1H, α-CH), 5.08 and 5.17 (2 × d, $J$ = 13.0 Hz, 2H, benzyl), 5.9 (br d, $J$ = 6.7 Hz, 1H, NH), 7.2–7.5 (m, 10H, $CF_3C_6H_4$ and $C_6H_4NO_2$), 8.1 (d, $J$ = 7.7 Hz, 2H, $C_6H_4NO_2$); $^{31}$P NMR (121 MHz, $CDCl_3$) $\delta_p$ 25.51. Compound **6b** was used for the next reaction without further purification.

**3.2.6. Methyl 2-(*N*-4-nitrobenzyloxycarbonylamino)-4-[bis(4-acetylphenyl)phosphono]butanoate (6c).** The bis(4-acetylphenyl) phosphonate **6c** was synthesized from freshly prepared **5** (2.82 mmol) and 4-hydroxyacetophenone (7.0 mmol) according to the same procedure as described for the synthesis of **6b** and was purified by flash column chromatography on silica gel 60 (EtOAc/hexane, 4:1) to give **6c** as an yellow oil (1.47 g, 85%): $^1$H-NMR (300 MHz, $CDCl_3$) $\delta_H$ 2.1–2.5 (m, 4H, $PCH_2CH_2$), 2.6 (s, 6H, 2 × $CH_3CO$), 3.7 (s, 3H, $CO_2CH_3$), 4.5 (m, 1H, α-CH), 5.17 and 5.24 (2 × d, $J$ = 13.8 Hz, 2H, benzyl), 5.7 (d, $J$ = 7.8 Hz, 1H, NH), 7.2 (d, $J$ = 8.4 Hz, 4H) and 7.9 (d, $J$ = 8.4 Hz, 4H) (2 × $C_6H_4COCH_3$), 7.5 (d, $J$ = 8.4 Hz, 2H) and 8.2 (d, $J$ = 8.4 Hz, 2H) ($C_6H_4NO_2$); $^{31}$P NMR (121 MHz, $CDCl_3$) $\delta_p$ 25.04; HRMS (FAB, glycerol) calcd for $C_{29}H_{30}N_2O_{11}P$ ($MH^+$) 613.1588, found 613.1594; Anal. Calcd. for $C_{29}H_{29}N_2O_{11}P$: C, 56.87; H, 4.77; N, 4.57. Found: C, 56.60; H, 4.82; N, 4.38.

**3.2.7. Ethyl 2-(*N*-4-nitrobenzyloxycarbonylamino)-4-[bis(4-cyanophenyl)phosphono]butanoate (6d).** The bis(4-cyanophenyl) phosphonate **6d** was synthesized from

freshly prepared **5′** (8.45 mmol) and 4-cyanophenol (21.2 mmol) according to the same procedure as described for the synthesis of **6b**. The crude product (5.97 g) was used in the next step without purification: $^1$H-NMR (300 MHz, $CDCl_3$) $\delta_H$ 1.3 (t, $J$ = 7.2 Hz, 3H, $OCH_2CH_3$), 2.0–2.5 (m, 4H, $PCH_2CH_2$), 4.2 (q, $J$ = 6.8 Hz, 2H, $OCH_2CH_3$), 4.5 (m, 1H, α-CH), 5.17 and 5.26 (2 × d, $J$ = 13.4 Hz, 2H, benzyl), 5.6 (br d, $J$ = 7.3 Hz, 1H, NH), 7.3 (d, $J$ = 9.1 Hz, 4H) and 7.7 (d, $J$ = 8.4 Hz, 4H) (2 × $C_6H_4CN$), 7.5 (d, $J$ = 8.4 Hz, 2H) and 8.2 (d, $J$ = 8.7 Hz, 2H) (2 × $C_6H_4NO_2$); $^{31}$P NMR (121 MHz, $CDCl_3$) $\delta_p$ 25.78.

**3.2.8. 2-Amino-4-(monophenylphosphono)butanoic acid (2a).** An aqueous solution (50 mL) of NaOH (0.40 g, 10.0 mmol) was added to a solution of **6a** (1.01 g, 1.91 mmol) in THF (20 mL). The mixture was stirred at ambient temperature for 25 h. After completion of the reaction (TLC, hexane/EtOAc, 1:4, UV), the reaction mixture was acidified (pH 2) with 2 N HCl and was evaporated to dryness. Acetone (100 mL) was added to the residue, and the resulting precipitate (NaCl) was removed by filtration through Celite. The filtrate was evaporated to dryness to give the N-protected monophenyl phosphonate as a deep red oil: $^1$H-NMR (300 MHz, acetone-$d_6$) $\delta_H$ 1.9–2.2 (m, 2H, $PCH_2CH_2$), 2.2–2.4 (m, 2H, $PCH_2$), 4.4 (m, 1H, α-CH), 5.3 (s, 2H, benzyl), 7.2–7.4 (m, 5H, $C_6H_5$), 7.7 (d, $J$ = 8.1 Hz, 2H) and 8.2 (d, $J$ = 8.7 Hz, 2H) ($C_6H_4NO_2$). The crude monophenyl phosphonate (1.91 mmol) was dissolved in MeOH/AcOH/$H_2O$ (8:2:1, 44 mL). Hydrogen gas was passed through the solution in the presence of 10% Pd–C (100 mg) for 1.5 h at ambient temperature. The Pd–C was removed by filtration through Celite, and the filtrate was evaporated. The residual oil was purified by medium-pressure reversed-phase column chromatography on an adsorption resin DIAION HP20SS (Mitsubishi Chemical Corp., Tokyo, Japan). The column was eluted with $H_2O$, and the fractions containing the product (TLC, BuOH/AcOH/$H_2O$, 5:2:2, ninhydrin) were collected and lyophilized to afford **2a** as a colorless solid (0.29 g, 59%): mp 173–175 °C; IR (KBr) $\nu_{max}$ 3600–2300 (br), 1700, 1590, 1485, 1160, 1040, 880, and 750 cm$^{-1}$; $^1$H-NMR (300 MHz, $D_2O$) $\delta_H$ 1.7–1.9 (m, 2H, $PCH_2CH_2$), 2.1-2.3 (m, 2H, $PCH_2CH_2$), 4.1 (t, $J$ = 6.0 Hz, 1H, α-CH), 7.1–7.2 and 7.3–7.4 (m, 5H, $C_6H_5$); $^{31}$P NMR (121 MHz, $D_2O$) $\delta_p$ 22.66; $^{13}$C NMR (75.4 MHz, $D_2O$) $\delta_C$ 25.1 (d, $^1J_{C\text{-}P}$ = 137.4 Hz, $PCH_2$), 27.0 ($PCH_2CH_2$), 55.9 (d, $^3J_{C\text{-}P}$ = 17.9 Hz, α-CH), 123.5, 127.1, 132.5 and 153.8 (d, $^2J_{C\text{-}P}$ = 7.4 Hz) ($C_6H_5$), 174.3 (C═O); HRMS (FAB, glycerol) calcd for $C_{10}H_{15}NO_5P$ ($MH^+$) 260.0689, found 260.0684; Anal. Calcd. for $C_{10}H_{14}NO_5P{\cdot}0.6\ H_2O$: C, 44.48; H, 5.67; N, 5.19. Found: C, 44.45; H, 5.51; N, 5.10.

**3.2.9. 2-Amino-4-[mono(4-trifluoromethylphenyl)phosphono]butanoic acid (2b).** The crude **6b** (7.97 mmol) was hydrolyzed by NaOH (1.93 g, 47.6 mmol) in a mixture of $H_2O$ (30 mL) and THF (45 mL) at ambient temperature for 36 h. After the reaction was complete (TLC, BuOH/AcOH/$H_2O$, 5:2:2, UV), the reaction mixture was worked up by the same procedure as described for the hydrolysis of **6a**. The crude product was purified by medium-pressure reversed-phase column chromatography (ODS-S-50B, Yamazen Co.). The column was eluted with a linear gradient of MeOH/$H_2O$ (50–90%), and the fractions containing the product (TLC, BuOH/AcOH/$H_2O$, 5:2:2, UV) were collected and evaporated to afford the N-protected mono(4-trifluoromethylphenyl) phosphonate as an yellowish solid (2.07 g, 51%): $^1$H-NMR (300 MHz, acetone-$d_6$) $\delta_H$ 2.0–2.4 (m, 4H, $PCH_2CH_2$), 4.4 (m, 1H, α-CH), 5.2 (2 × d, $J$ = 13.9 Hz, 2H, benzyl), 7.4 (d, $J$ = 8.3 Hz, 2H, $C_6H_4CF_3$), 7.6 (m, 4H, $C_6H_4CF_3$ and $C_6H_4NO_2$), 8.2 (d, $J$ = 8.7 Hz, 2H, $C_6H_4NO_2$); $^{31}$P NMR (121 MHz, acetone-$d_6$) $\delta_p$ 25.76; HRMS (FAB, glycerol) calcd for $C_{19}H_{19}F_3N_2O_9P$ ($MH^+$) 507.0781, found 507.0767. The mono(4-trifluoromethylphenyl) phosphonate (0.79 g, 1.56 mmol) was dissolved in THF (45 mL), and hydrogen gas was passed through the solution in the presence of 5% Pd–C (50 mg) at ambient temperature for 2 h. The reaction was monitored with TLC (BuOH/AcOH/$H_2O$, 5:2:2, ninhydrin). The precipitated **2b** and the Pd–C were collected by filtration, washed with THF, and extracted with hot $H_2O$ (30 mL). The extract was evaporated to give an yellow oil (200 mg). This residue was dissolved in trifluoroacetic acid (50 μL) and was diluted with $H_2O$ (1.9 mL). This solution was kept at ambient temperature for 3 days to give crystals. The crystals were collected by filtration and was washed with EtOH and $Et_2O$ successively to afford **2b** as light yellow crystals (68 mg, 16%): $^1$H-NMR (300 MHz, $D_2O$) $\delta_H$ 1.7–2.0 (m, 2H, $PCH_2CH_2$), 2.1–2.3 (m, 2H, $PCH_2$), 4.0 (m, 1H, α-CH), 7.3 (d, $J$ = 8.3 Hz, 2H) and 7.7 (d, $J$ = 8.3 Hz, 2H) ($C_6H_4CF_3$); $^{31}$P NMR (121 MHz, $D_2O$) $\delta_p$ 23.66; HRMS (FAB, glycerol) calcd for $C_{11}H_{14}F_3NO_5P$ ($MH^+$) 328.0562, found 328.0562; Anal. Calcd. for $C_{11}H_{13}F_3NO_5P$: C, 40.38; H, 4.00; N, 4.28. Found: C, 40.34; H, 4.06; N, 4.30.

**3.2.10. 2-Amino-4-[mono(4-acetylphenyl)phosphono]butanoic acid (2c).** Compound **6c** (1.47 g, 2.40 mmol) was hydrolyzed by NaOH (0.65 g, 15.6 mmol) in a mixture of $H_2O$ (75 mL) and THF (45 mL) at ambient temperature for 15 h. After the reaction was complete (TLC, hexane/EtOAc, 1:4, UV), the reaction mixture was worked up by the same procedure as described for the hydrolysis of **6a** to give the crude N-protected mono(4-acetylphenyl) phosphonate as an yellow oil (1.52 g): $^1$H-NMR (300 MHz, acetone-$d_6$) $\delta_H$ 2.0–2.4 (m, 4H, $PCH_2CH_2$), 2.6 (s, 3H, $CH_3CO$), 4.4 (m, 1H, α-CH), 5.3 (s, 2H, benzyl), 7.3 (d, $J$ = 8.9 Hz, 2H, $C_6H_4Ac$) and 7.7 (d, $J$ = 8.9 Hz, 2H, $C_6H_4NO_2$), 8.0 (d, $J$ = 8.5 Hz, 2H, $C_6H_4Ac$), 8.2 (d, $J$ = 8.7 Hz, 2H, $C_6H_4NO_2$). The crude mono(4-acetylphenyl) phosphonate (2.41 mmol) was dissolved in MeOH/$H_2O$ (4:1, 25 mL), and hydrogen gas was passed through the solution in the presence of 5% Pd–C (100 mg) at ambient temperature for 5.5 h. The reaction was monitored with TLC (BuOH/AcOH/$H_2O$, 5:2:2, ninhydrin). The precipitated **2c** and the Pd–C were collected by filtration, washed with THF, and extracted with hot 10% TFA in water (5 mL). The extract was evaporated to give crude **2c** as a deep red oil (0.67 g). The oil was washed with EtOH and dissolved in 33% HBr/AcOH (4 mL). The volatile was removed in vacuo to give the HBr salt

as a deep red oil. The oil was dissolved in EtOH (5 mL) and filtered through Celite. To this filtrate was added propylene oxide (2.5 mL), and the mixture was kept at 4 °C overnight. The precipitate was filtered and washed with EtOH and $Et_2O$, successively, to afford **2c** as light yellow powder (0.58 g, 80%): $^1$H-NMR (300 MHz, $D_2O$) $\delta_H$ 1.7–2.0 (m, 2H, $PCH_2CH_2$), 2.1-2.3 (m, 2H, $PCH_2$), 2.6 (s, 3H, $CH_3CO$), 4.0 (t, $J$ = 5.8 Hz, 1H, α-CH), 7.2 (d, $J$ = 8.1 Hz, 2H) and 8.0 (d, $J$ = 8.4 Hz, 2H) ($C_6H_4Ac$); $^{31}$P NMR (121 MHz, $D_2O$) $\delta_p$ 23.44; HRMS (FAB, glycerol) calcd for $C_{12}H_{17}NO_6P$ ($MH^+$) 302.0794, found 302.0800; Anal. Calcd for $C_{12}H_{16}NO_6P{\cdot}0.12\ H_2O$: C, 47.50; H, 5.39; N, 4.62. Found: C, 47.51; H, 5.39; N, 4.52.

**3.2.11. 2-Amino-4-[mono(4-cyanophenyl)phosphono]butanoic acid (2d).** Crude **6d** (8.45 mmol) was hydrolyzed by NaOH (0.81 g, 20.3 mmol) in a mixture of $H_2O$ (50 mL) and THF (40 mL) at ambient temperature for 24 h. After the reaction was complete (TLC, BuOH/AcOH/$H_2O$, 5:2:2, UV), the reaction mixture was worked up by the same procedure as described for the hydrolysis of **6a**. The crude product was purified by medium-pressure reversed-phase column chromatography (ODS-S-50B, Yamazen Co.). The column was eluted with a linear gradient of $CH_3CN/H_2O$ (20–50%), and the fractions containing the product (TLC, BuOH/AcOH/$H_2O$, 5:2:2, UV) were collected and evaporated to afford the N-protected mono(4-cyanophenyl) phosphonate as an yellow solid (1.08 g, 56%): $^1$H-NMR (300 MHz, acetone-$d_6$) $\delta_H$ 2.1–2.4 (m, 4H, $PCH_2CH_2$), 4.4 (m, 1H, α-CH), 5.3 (s, 2H, benzyl), 7.0 (br s, 1H, NH), 7.4 (d, $J$ = 8.6 Hz, 2H, $C_6H_4CN$), 7.6 (d, $J$ = 8.9 Hz, 2H, $C_6H_4NO_2$), 7.8 (d, $J$ = 8.8 Hz, 2H, $C_6H_4CN$), 8.2 (d, $J$ = 8.7 Hz, 2H, $C_6H_4NO_2$); $^{31}$P NMR (121 MHz, acetone-$d_6$) $\delta_p$ 25.76; HRMS (FAB, glycerol) calcd for $C_{19}H_{19}N_3O_9P$ ($MH^+$) 464.0860, found 464.0856; Anal. Calcd for $C_{19}H_{18}N_3O_9P0.3\ H_2O$: C, 48.69; H, 4.00; N, 8.96. Found: C, 48.67; H, 3.99; N, 8.99. Hydrogen gas was passed through a mixture of the mono(4-cyanophenyl) phosphonate (0.73 g, 1.58 mmol) and 5% Pd–C (200 mg) in THF (30 mL) at ambient temperature for 4 h. The reaction was monitored with TLC (BuOH/AcOH/$H_2O$, 5:2:2, ninhydrin). The precipitated**2d** and the Pd–C were collected by filtration, washed with THF, and was extracted with TFA (2 mL). The extract was evaporated to give crude **2d** as a deep red oil (442 mg). The oil was dissolved in 33% HBr/AcOH (10 mL) and was evaporated to give the hydrogen bromide salt as an oil. The oil was dissolved in EtOH (30 mL) and filtered through Celite. To this filtrate was added propylene oxide (10 mL), and diethyl ether (10 mL) was added to promote the precipitation of the product. The mixture was kept at 4 °C for 12 h to give **2d** as yellow powder (170 mg, 38%): $^1$H-NMR (300 MHz, $D_2O$) $\delta_H$ 1.7–2.0 (m, 2H, $PCH_2CH_2$), 2.1–2.3 (m, 2H, $PCH_2$), 4.0 (t, $J$ = 6.0 Hz, 1H, α-CH), 7.3 (d, $J$ = 8.6 Hz, 2H) and 7.8 (d, $J$ = 8.7 Hz, 2H) ($C_6H_4CN$); $^{31}$P NMR (121 MHz, $D_2O$) $\delta_p$ 23.67; HRMS (FAB, glycerol) calcd for $C_{11}H_{14}N_2O_5P$ ($MH^+$) 285.0641, found 285.0647; Anal. Calcd for $C_{11}H_{13}N_2O_5P$: C, 46.49; H, 4.61; N, 9.86. Found: C, 46.28; H, 5.25; N, 9.43.

### 3.3. Hydrolytic stability of 2a–d

The hydrolysis of **2d** was monitored by $^{31}$P NMR (121 MHz) by incubating 29.3 mM of **2d** in $D_2O$ at 23°. The ratio of **2d** and the hydrolyzed product, APBA, was calculated from the integration of the $^{31}$P NMR peak areas ($\delta_P$ 23.67 for **2d**, 24.32 for APBA).

### 3.4. Enzyme assay

The hydrolytic activity of *E. coli* GGT was measured fluorimetrically by using 0.2 μM γ-Glu-AMC as substrate[61] at pH 5.5 at 25 °C. An aliquot (10 μL) of an enzyme stock solution was added to 100 mM succinate–NaOH buffer (pH 5.5) in a total volume of 1 mL containing 100 μL γ-Glu-AMC stock solution (2 μM in water) at 25 °C. The release of 7-amino-4-methylcoumarin (AMC) was monitored continuously with a Hitachi F-2000 spectrophotometer (350 nm excitation, 440 nm emission). AMC concentrations were calculated from a standard calibration curve of fluorescence intensity ($F$) versus AMC concentration ($C$): $\Delta F/\Delta C = 0.11\ nM^{-1}$. The fluorescence intensity was proportional to the concentration of AMC up to 2.0 μM. The Michaelis constant ($K_m$) for γ-Glu-AMC was determined as 0.2 μM under these conditions. Protein was determined by the Bio-Rad protein assay dye, using bovine serum albumin as standard.

The hydrolytic activity of human GGT was measured under the same conditions, except that the final substrate concentration was 4.0 μM in 100 mM succinate–NaOH buffer (pH 5.5) or 100 mM tricine–HCl (pH 8.0). The $K_m$ values for γ-Glu-AMC were determined as 12.6 μM (pH 5.5) and 4.0 μM (pH 8.0), respectively, under these conditions.

### 3.5. Time-dependent inhibition assay

The inhibition of GGT was measured under pseudo-first order rate conditions for both continuous and discontinuous inhibition assay methods. In the continuous inhibition assay (for **1**, **2b–d** with *E. coli* and human GGT), the enzyme was added to a preincubated mixture of varying concentrations of the inhibitor and the substrate (0.2 or 4.0 μM γ-Glu-AMC for *E. coli* and human GGT, respectively) in 100 mM sodium succinate buffer (pH 5.5) or 100 mM tricine–HCl (pH 8.0) at 25 °C. Time-dependent inhibition of the enzyme was measured by monitoring the product release (AMC) continuously for 10 min. Pseudo-first-order rate constants for enzyme inactivation ($k_{obsd}$) were determined by fitting each curve to a first-order rate equation:[62]

$$[P] = [P]_\infty[1 - \exp(-k_{obsd}t)] \quad (1)$$

where [P] and $[P]_\infty$ are the concentrations of product formed at time $t$ and at the time approaching infinity, respectively. Since a plot of $k_{obsd}$ versus inhibitor concentration ([I]) exhibited no saturation until the highest possible inhibitor concentration, the second-order rate

constant for enzyme inactivation ($k_{on}$) was determined according to the Eq. (2) derived from the kinetic mechanism described below:

$$\begin{array}{l} E + S \overset{K_m}{\rightleftharpoons} ES \rightarrow E + P \\ \downarrow k_{on}[I] \\ E\text{-}I \end{array}$$

$$k_{obsd} = k_{on}[I]/(1 + [S]/K_m) \quad (2)$$

where S is γ-Glu-AMC; [S] and $K_m$ are 0.2 and 0.2 μM (*E. coli* GGT), and 4.0 and 12.6 μM (human GGT). Nonlinear regression analyses of kinetic data were performed using the KaleidaGraph program (Synergy Software).

In the discontinuous inhibition assay (for **2a** with *E. coli* and human GGT), the enzyme (final concd of 0.5 μg/mL of crude human GGT) was incubated at 25 °C in 100 mM succinate–NaOH (pH 5.5) or 100 mM tricine–HCl (pH 8.0) containing varying concentrations of inhibitor. An aliquot (10 μL) of the solution was withdrawn at various times and was assayed for remaining activity. The values for $k_{obsd}$ were obtained by fitting the remaining activities to the following equation:

$$a_t/a_0 = \exp(-k_{obsd}t) \quad (3)$$

where $a_t$ is the remaining activity at time $t$, and $a_0$ is the initial activity ($t = 0$) before inactivation. Nonlinear regression analyses of kinetic data were also performed using the KaleidaGraph program. The values for $k_{on}$ were calculated according to the following equation:

$$k_{obsd} = k_{on}[I] \quad (4)$$

### 3.6. Effect of 3-benzoylpropionic acid (3-BPA) on the inhibition of GGT

In a discontinuous assay, the enzyme was incubated at 25 °C in a solution of 100 mM succinate–NaOH (pH 5.5) or 100 mM tricine–HCl (pH 8.0) containing the inhibitor and 3-BPA (40 mM). An aliquot (20 μL) was withdrawn at various times and was assayed for remaining activity.

### 3.7. Regain of activity of inactivated human GGT

The enzyme was incubated with 1 mM of **1** in 100 mM succinate–NaOH (pH 5.5) at 25 °C for 20 min to inactivate the enzyme completely. The excess inhibitor was removed by rapid gel filtration at 4 °C through a Bio-Spin column (Bio-Rad) equilibrated with 100 mM 3,3-dimethylglutaric acid–NaOH buffer (pH 6.0). The inactivated enzyme was dialyzed against 100 mM 3,3-dimethylglutaric acid–NaOH buffer (pH 6.0) at 4 °C for 1 week. The regain of hydrolytic activity was measured using γ-Glu-AMC as substrate. The control experiment was conducted under the same conditions without using the inhibitor.

## Acknowledgments

We thank Asahi Kasei Corporation for a generous gift of human γ-glutamyl transpeptidase HC-GTP (T-72). This study was supported in part by a Grant-in-Aid for Scientific Research from Japan for the Promotion of Science for J.H. (contract No. 16310152).

## References and notes

1. Taniguchi, N.; Ikeda, Y. *Adv. Enzymol. Rel. Areas Mol. Biol.* **1998**, *72*, 239.
2. Hiratake, J.; Suzuki, H.; Kumagai, H. In *Handbook of Proteolytic Enzymes*; Barrett, A. J., Rawlings, N. D., Woessner, J. F., Eds., 2nd ed.; Elsevier: Amsterdam, 2004; pp 2090–2094.
3. Zhang, H.; Forman, H. J.; Choi, J. *Methods Enzymol.* **2005**, *401*, 468.
4. Whitfield, J. B. *Crit. Rev. Clin. Lab. Sci.* **2001**, *38*, 263.
5. Hanigan, M. H.; Frierson, H. F. J., Jr. *Histochem. Cytochem.* **1996**, *44*, 1101.
6. Hanigan, M. H.; Ricketts, W. A. *Biochemistry* **1993**, *32*, 6302.
7. Vincent, B. R.; Mousset, S.; Jacquemin-Sablon, A. *Eur. J. Biochem.* **1999**, *262*, 873.
8. Ruoso, P.; Hedley, D. W. *Cancer Chemother. Pharmacol.* **2004**, *54*, 49.
9. Anderson, M. E.; Meister, A. *Proc. Natl. Acad. Sci. U.S.A.* **1983**, *80*, 707.
10. Godwin, A. K.; Meister, A.; O'Dwyer, P. J.; Huang, C.-S.; Hamilton, T. C.; Anderson, M. E. *Proc. Natl. Acad. Sci. U.S.A.* **1992**, *89*, 3070.
11. Hanigan, M. R.; Gallagher, B. C.; Townsend, D. M.; Gabarra, V. *Carcinogenesis* **1999**, *20*, 553.
12. Hanigan, M. H. *Carcinogenesis* **1995**, *16*, 181.
13. Lewis, A. D.; Hayes, J. D.; Wolf, C. R. *Carcinogenesis* **1988**, *9*, 1283.
14. Hanigan, M. H.; Pitot, H. C. *Carcinogenesis* **1985**, *6*, 165.
15. Benlloch, M.; Ortega, A.; Ferrer, P.; Segarra, R.; Obrador, E.; Asensi, M.; Carretero, J.; Estrela, J. M. *J. Biol. Chem.* **2005**, *280*, 6950.
16. Ortega, A. L.; Carretero, J.; Obrador, E.; Gambini, J.; Asensi, M.; Rodilla, V.; Estrela, J. M. *J. Biol. Chem.* **2003**, *278*, 13888.
17. Obrador, E.; Carretero, J.; Ortega, A.; Medina, I.; Rodilla, V.; Pellicer, J. A.; Estrela, J. M. *Hepatology* **2002**, *35*, 74.
18. Pompella, A.; De Tata, V.; Paolicchi, A.; Zunino, F. *Biochem. Pharmacol.* **2006**, *71*, 231, and references cited therein.
19. Owen, A. D.; Schapira, A. H.; Jenner, P.; Marsden, C. D. *Ann. N.Y. Acad. Sci.* **1996**, *786*, 217.
20. Sian, J.; Dexter, D. T.; Lees, A. J.; Daniel, S.; Jenner, P.; Marsden, S. D. *Ann. Neurol.* **1994**, *36*, 356.
21. Lee, D.-H.; Ha, M.-H.; Kim, J.-H.; Christiani, D. C.; Gross, M. D.; Steffes, M.; Blomhoff, R.; Jacobs, D. R. *Diabetologia* **2003**, *46*, 359.
22. Meisinger, C.; Löwel, H.; Heier, M.; Schneider, A.; Thorand, B. *J. Internal Med.* **2005**, *258*, 527.
23. Paolicchi, A.; Minotti, G.; Tonarelli, P.; De Cesare, D.; Mezzetti, A.; Dominici, S.; Comporti, M.; Pompella, A. *J. Invest. Med.* **1999**, *47*, 151.
24. Ruttmann, E.; Brant, L. J.; Concin, H.; Diem, G.; Rap, K.; Ulmer, H. *Circulation* **2005**, *112*, 2130.
25. Townsend, D. M.; Tew, K. D.; Tapiero, H. *Biomed. Pharmacother.* **2003**, *57*, 145.
26. Allison, R. D. *Methods Enzymol.* **1985**, *113*, 419.

27. Keillor, J. W.; Ménard, A.; Castonguay, R.; Lherbet, C.; Rivard, C. *J. Phys. Org. Chem.* **2004**, *17*, 529.
28. Hanka, L. J.; Diez, A. *Antimicrob. Agents Chemother.* **1973**, *3*, 425.
29. Gardell, S. J.; Tate, S. S. *FEBS Lett.* **1980**, *122*, 171.
30. Stole, E.; Seddon, A. P.; Wellner, D.; Meister, A. *Proc. Natl. Acad. Sci. U.S.A.* **1990**, *87*, 1706.
31. Smith, T. K.; Ikeda, Y.; Fujii, J.; Taniguchi, N.; Meister, A. *Proc. Natl. Acad. Sci. U.S.A.* **1995**, *92*, 2360.
32. Jayaram, H. N.; Cooney, D. A.; RRyan, J. A.; Neil, G. L.; Dion, R. L.; Bono, V. H. *Cancer Chemother. Rep.* **1975**, *59*, 481.
33. Zalkin, H. *Adv. Enzymol. Rel. Areas Mol. Biol.* **1998**, *72*, 87.
34. Earhart, R. H.; Neil, G. L. *Adv. Enzyme Regul.* **1985**, *24*, 179.
35. Chittur, S. V.; Klem, T. J.; Shafer, C. M.; Jo Davisson, V. *Biochemistry* **2001**, *40*, 876.
36. Tate, S. S.; Meister, A. *Proc. Natl. Acad. Sci. U.S.A.* **1977**, *74*, 931.
37. Tate, S. S.; Meister, A. *Proc. Natl. Acad. Sci. U.S.A.* **1978**, *75*, 4806.
38. Stein, R. L.; DeCicco, C.; Nelson, D.; Thomas, B. *Biochemistry* **2001**, *40*, 5804.
39. London, R. E.; Gabel, S. A. *Arch. Biochem. Biophys.* **2001**, *385*, 250.
40. Inoue, M.; Hiratake, J.; Suzuki, H.; Kumagai, H.; Sakata, K. *Biochemistry* **2000**, *39*, 7764.
41. Brannigan, J. A.; Dodson, G.; Duggleby, H. J.; Moody, P. C. E.; Smith, J. L.; Tomchick, D. R.; Murzin, A. G. *Nature* **1995**, *378*, 416.
42. Suzuki, H.; Kumagai, H. *Biochemistry* **2002**, *277*, 43536.
43. Hirschmann, R.; Yager, K. M.; Taylor, C. M.; Witherington, J.; Sprengeler, P. A.; Phillips, B. W.; Moore, W.; Smith, A. B., III *J. Am. Chem. Soc.* **1997**, *119*, 8177.
44. Inoue, M.; Hiratake, J.; Sakata, K. *Biosci. Biotechnol. Biochem.* **1999**, *63*, 2248.
45. Bartlett, P. A.; Lamden, L. A. *Bioorg. Chem.* **1986**, *14*, 356.
46. Thompson, G. A.; Meister, A. *J. Biol. Chem.* **1977**, *252*, 6792.
47. Tate, S. S.; Meister, A. *Methods Enzymol.* **1985**, *113*, 400.
48. Cook, N. D.; Peters, T. J. *Biochim. Biophys. Acta* **1985**, *832*, 142.
49. Suzuki, H.; Kumagai, H.; Tochikura, T. *J. Bacteriol.* **1986**, *168*, 1325.
50. McIntyre, T. M.; Curthoys, N. P. *J. Biol. Chem.* **1979**, *254*, 6499.
51. Hengge, A. C.; Onyido, I. *Curr. Org. Chem.* **2005**, *9*, 61.
52. Ménard, A.; Castonguay, R.; Lherbet, C.; Rivard, C.; Roupioz, Y.; Keillor, J. W. *Biochemistry* **2001**, *40*, 12678.
53. Tate, S. S.; Meister, A. *Proc. Natl. Acad. Sci. U.S.A.* **1974**, *71*, 3329.
54. Thompson, G. A.; Meister, A. *J. Biol. Chem.* **1979**, *254*, 2956.
55. Thompson, G. A.; Meister, A. *J. Biol. Chem.* **1980**, *255*, 2109.
56. Gardell, S. J.; Tate, S. S. *J. Biol. Chem.* **1983**, *258*, 6198.
57. Suzuki, H.; Hashimoto, W.; Kumagai, H. *J. Bacteriol.* **1993**, *175*, 6038.
58. Kosolapoff, G. M. *J. Am. Chem. Soc.* **1948**, *70*, 1971.
59. Chambers, J. R.; Isbell, A. F. *J. Org. Chem.* **1964**, *29*, 832.
60. Bradford, M. M. *Anal. Biochem.* **1976**, *72*, 248.
61. Smith, G. D.; Ding, J. L.; Peters, T. J. *Anal. Biochem.* **1979**, *100*, 136–139.
62. Stein, R. L.; Strimpler, A. M.; Edwards, P. D.; Lewis, J. J.; Mauger, R. C.; Schwartz, J. A.; Stein, M. M.; Trainor, D. A.; Wildonger, R. A.; Zottola, M. A. *Biochemistry* **1987**, *26*, 2682.