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Author(s)	Nakajima, Mado; Watanabe, Bunta; Han, Liyou; Shimizu, Bun-Ichi; Wada, Kei; Fukuyama, Keiichi; Suzuki, Hideyuki; Hiratake, Jun
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Glutathione-analogous peptidyl phosphorus esters as mechanism-based inhibitors of γ-glutamyl transpeptidase for probing cysteinyl-glycine binding site

Mado Nakajima[a, 1], Bunta Watanabe[a], Liyou Han[a], Bun-ichi Shimizu[a, 2], Kei Wada[b], Keiichi Fukuyama[c, 3], Hideyuki Suzuki[d], Jun Hiratake[a]*

[a] *Institute for Chemical Research, Kyoto University, Gokasho, Uji, Kyoto 611-0011, Japan*

[b] *Organization for Promotion of Tenure Track, University of Miyazaki, 5200 Kihara, Kiyotake, Miyazaki 889-1692, Japan*

[c] *Department of Biological Sciences, Graduate School of Science, Osaka University, Toyonaka, Osaka 560-0043, Japan*

[d] *Division of Applied Biology, Graduate School of Science and Technology, Kyoto Institute of Technology, Goshokaido-cho, Matsugasaki, Sakyo-ku, Kyoto 606-8585, Japan*

[1] Present address: *Shionogi Pharmaceutical Research Center, SHIONOGI & CO., LTD., 1-1, Futaba-cho 3-chome, Toyonaka, Osaka 561-0825, Japan*

[2] Present address: *Graduate School of Life Sciences, Toyo University, 1-1-1-Izumino. Itakura-machi, Oga-gun, Gumma 374-0193, Japan*

[3] Present address: *Department of Applied Chemistry, Graduate School of Engineering, Osaka University, Suita, Osaka 565-0871, Japan*

*Correspondence author
Phone: +81 774 38 3231

Fax: +81 774 38 3229
E-mail: hiratake@scl.kyoto-u.ac.jp

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Abstract

γ-Glutamyl transpeptidase (GGT) catalyzing the cleavage of γ-glutamyl bond of glutathione and its S-conjugates is involved in a number of physiological and pathological processes through glutathione homeostasis. Defining its Cys-Gly binding site is extremely important not only in defining the physiological function of GGT, but also in designing specific and effective inhibitors for pharmaceutical purposes. Here we report the synthesis and evaluation of a series of glutathione-analogous peptidyl phosphorus esters as mechanism-based inhibitors of human and *E. coli* GGTs to probe the structural and stereochemical preferences in the Cys-Gly binding site. Both enzymes were inhibited strongly and irreversibly by the peptidyl phosphorus esters with a good leaving group (phenoxide). Human GGT was highly selective for L-aliphatic amino acid such as L-2-aminobutyrate (L-Cys mimic) at the Cys binding site, whereas *E. coli* GGT significantly preferred L-Phe mimic at this site. The C-terminal Gly and a L-amino acid analogue at the Cys binding site were necessary for inhibition, suggesting that human GGT was highly selective for glutathione (γ-Glu-L-Cys-Gly), whereas *E. coli* GGT are not selective for glutathione, but still retained the dipeptide (L-AA-Gly) binding site. The diastereoisomers with respect to the chiral phosphorus were separated. Both GGTs were inactivated by only one of the stereoisomers with the same stereochemistry at phosphorus. The strict recognition of phosphorus stereochemistry gave insights into the stereochemical course of the catalyzed reaction. Ion-spray mass analysis of the inhibited *E. coli* GGT confirmed the formation of a 1:1 covalent adduct with the catalytic subunit (small subunit) with concomitant loss of phenoxide, leaving the peptidyl moiety that presumably occupies the Cys-Gly binding site. The peptidyl phosphonate inhibitors are highly useful as a ligand for X-ray structural analysis of GGT for defining hitherto unidentified Cys-Gly binding site to design specific inhibitors.

1. Introduction

γ-Glutamyl transpeptidase (GGT, EC 2.3.2.2) is a heterodimeric enzymes found widely in organisms ranging from bacteria, plants to mammals. GGT catalyzes the cleavage of the γ-glutamyl amide bond of glutathione (γ-L-glutamyl-L-cysteinylglycine), its *S*-conjugates, glutamine and other γ-glutamylamides to transfer the γ-glutamyl group to water (hydrolysis) or amino acids and peptides (transpeptidation) by the ping-pong mechanism via a γ-glutamyl-enzyme ester intermediate (Scheme 1A).[1-4] Mammalian GGT is bound to the external surface of cell membrane and is expressed in high concentrations in kidney tubules, biliary epithelium and brain capillaries.[2, 5] GGT plays a central role in glutathione homeostasis as the sole enzyme capable of cleaving γ-glutamyl bond of glutathione in the initial step of glutathione metabolism; GGT hydrolyzes the extracellular glutathione in concert with other dipeptidases to provide cells with cysteine, the rate-limiting substrate for intracellular *de novo* synthesis of glutathione.[4-8] GGT is also involved in the metabolism of xenobiotics by cleaving the γ-glutamyl bond of glutathione-*S*-conjugates as the initial step for their degradation and excretion.[2] Accordingly, the overexpression of GGT is often observed in human tumors, and its roles in tumor progression,[9, 10] growth acceleration[11] and the expression of such malignant phenotypes of cancer cells as drug resistance[11-13] and metastasis[14, 15] have been suggested repeatedly. GGT is also implicated in many physiological disorders such as neurodegenerative diseases,[16, 17] diabetes,[18] cardiovascular disease[19-21] and pulmonary disease.[22] Hence the serum GGT level has been used extensively as a diagnostic/prognostic marker of liver dysfunction, coronary heart diseases, Type 2 diabetes, stroke and atherosclerosis.[7, 21] While GGT is recognized widely as an integral component of cellular antioxidative defense system,[7, 8, 13] several lines of evidence have indicated that GGT promotes oxidative stress by producing Cys-Gly, a highly reactive thiol that generates reactive

oxygen species (ROS) via metal ion-catalyzed reduction of molecular oxygen.[23] Therefore, GGT can also be recognized as a pro-oxidant enzyme that potentially promotes oxidative stress.[24-27] The seemingly contradictory dual aspects of GGT, along with a complicated glutathione metabolism,[28] have made the physiological characterization of GGT rather complex. However, the implication of GGT in a number of pathological processes strongly suggests a causative role of this enzyme[7, 23, 25, 27] rather than a simple result of cellular adaptation to oxidative stress.[7, 8] Therefore, GGT is an attractive pharmaceutical target for cancer chemotherapy and a vast array of physiological disorders involving oxidative stress and glutathione metabolism.[13, 15, 22]

So far a number of GGT inhibitors have been reported,[4, 29] but acivicin, L-(α*S*,5*S*)-α-amino-3-chloro-4,5-dihydro-5-isoxazole, a classical glutamine antimetabolite antibiotic produced by *Streptomyces sviceus*,[30] has been used by far the most extensively for both *in vitro* and *in vivo* inhibition of GGT.[31-33] Acivicin, however, is not selective for GGT and inhibits many glutamine-dependent biosynthetic enzymes to show potent cyto- and neurotoxicity.[34, 35] The frequent use of acivicin as a GGT inhibitor, irrespective of its toxicity, is primarily due to the irreversible nature of its inhibition; acivicin reacts with the catalytic Thr residue of GGT to make a covalent adduct to inactivate the enzyme (Scheme 1B).[36, 37] Therefore the important criteria for GGT inhibitors of practical use both *in vitro* and *in vivo* are (1) irreversible inhibition with no regain of enzyme activity until new enzymes are *de novo* synthesized and (2) high selectivity for GGT without inhibiting glutamine-hydrolyzing biosynthetic enzymes. In view of these criteria, we reported a series of phosphonate esters as active-site directed and transition-state analogue inhibitors of GGT.[38, 39] The phosphonate esters react in a mechanism-based manner with the N-terminal Thr residue in the small subunit, the catalytic nucleophile of GGT,[40, 41] to inactivate the enzyme completely. Among those inhibitors,

compound **1** (a mixture of four stereoisomers, see Section 2.5 and Table 1) closely mimicking the transition state 1 (TS-1) for glutathione hydrolysis, exhibited an exceptionally higher inhibitory activity (> 180 times) toward human GGT than the phosphonate with a simple methoxy group in place of the peptidyl chain.[39] Interestingly, the *E. coli* enzyme did not show any extra sensitivity toward the phosphonate **1**. This observation suggested that the presence of the peptidyl chain significantly improved the affinity of compound **1** to the active site of human GGT, thereby facilitating the formation of the enzyme-inhibitor covalent complex that is closely analogous to the putative transition state TS-1 (Scheme 1A, C). We reasoned therefore that human GGT, but not the *E. coli* enzyme, held a defined Cys-Gly binding site that could provide an extra, or in some cases, a major binding energy to enhance the inhibitory activity of the covalent-type phosphonate inhibitors. The characterization of the Cys-Gly binding site of GGT is also important in reducing the toxicity of inhibitors by avoiding accidental inhibition of the glutamine-hydrolyzing enzymes; the inhibitors that could exploit the binding energy effectively from the Cys-Gly binding site, like compound **1**, should be bound tightly to the active site of GGT, but not to the active site of glutamine-hydrolyzing enzymes that are presumably devoid of the Cys-Gly binding site.

Several X-ray crystal structures of bacterial GGTs have been reported,[36, 42-45] but none of them were successful in identifying the Cys-Gly binding site. In contrast, the mammalian enzymes, whose natural substrate is glutathione and its *S*-conjugates, are expected to have a defined binding site for Cys-Gly. Very recently the X-ray crystal structure of human GGT was solved,[46] presenting the first tertiary structure of eukaryotic GGT. However, the Cys-Gly binding site is still undefined by the X-ray crystal structure of human GGT in complex with glutamate,

suggesting that the structural identification of Cys-Gly biding site is very difficult without a minutely designed ligand that could incorporate a Cys-Gly moiety.

Here we report the synthesis and evaluation of a series of glutathione-analogous peptidyl phosphorus esters **2a**-**g**, **3** and **5** that incorporated a Cys-Gly moiety in a transition-state analogous scaffold (Fig. 1). The extent of mechanism-based inhibition depending on the structural differences in the peptidyl moiety has gained insights into the structural and stereochemical preferences of the putative Cys-Gly binding site of human and *E. coli* GGTs. Human GGT was highly selective for a mimic of L-form of an aliphatic amino acid such as L-2-aminobutyrate (a L-Cys mimic) and L-norleucine (a L-Met mimic) at the Cys binding site, whereas *E. coli* GGT significantly preferred an aromatic amino acid (L-Phe) at this site. Both enzymes required the C-terminal Gly for effective inhibition, suggesting that *E. coli* GGT, as well as the human enzyme, still retained some characters for binding dipeptides such as L-amino acyl (L-AA)-Gly. The transition-state mimicry and the phosphorus stereochemistry of these inhibitors successfully allowed access to the stereochemical course of the nucleophilic attack of the catalytic Thr residue on the γ-carbonyl of glutathione in the catalyzed reaction.

2. Results and discussion

2.1. Analogue design

The synthesized phophonate diesters **2a**-**g** and **3** contain the peptidyl side chains that mimic L-2-aminobutyrylglycine (L-Abu-Gly, **2a**), glycylglycine (Gly-Gly, **2b**), L-β-chloroalanylglycine (L-β-ClAla-Gly, **2c**), L-β-iodoalanylglycine (L-β-IAla-Gly, **2d**), L-phenylalanylglycine (L-Phe-Gly, **2e**), L-norleucylglycine (L-Nle-Gly, **2f**), D-norleucylglycine (D-Nle-Gly, **2g**) and L-norleucine methyl ester (L-Nle-OMe, **3**) (Fig. 1). The L-Abu-Gly moiety of **2a** is a mimic of

L-Cys-Gly in glutathione with the assumption that a methyl is sterically analogous to SH. A halogen atom was introduced (chlorine and iodine for **2c** and **2d**, respectively) in the hope that the halogen atom also mimics the SH of the L-Cys residue. In view of L-Met as a preferred acceptor substrate of rat kidney GGT,[3, 47] the norleucyl series **2f** (L-Nle-Gly), **2g** (D-Nle-Gly) and **3** (L-Nle-OMe) were synthesized to mimic L-Met-Gly, D-Met-Gly and L-Met-OMe, respectively. The synthesis of a carboxy derivative of **3** was unsuccessful due to hydrolytic instability of the compound probably by intramolecular nucleophilic catalysis by the adjacent carboxy group.[48]

The phosphonate **4** carries a dipeptide mimic of L-Abu-Gly (same as **2a**), but with a poor leaving group (OMe) in place of phenoxy attached to the phosphorus. Compound **5** is a phosphinate version of **2b** with a Gly-Gly side chain (see Section 2.4).

2.2. Synthesis of phosphonate diesters 2a-g, 3 and 4

The synthesis of the peptidyl moieties is shown in Scheme 2. The preparation of the peptidyl phosphorus esters required a series of α-hydroxy acids with defined stereochemistry that mimic the L- or D-amino acid at the Cys moiety of glutathione. The chiral α-hydroxy acids **7a**, **e**-**g** were prepared from the corresponding L- or D-amino acids (**6a**, **e**-**g**, respectively) with retention of configuration by diazotization followed by hydrolysis.[49] The optical rotations of **7e**-**g** were in good agreement with the reported values.[49] The (*R*)-3-chloro-2-hydroxypropionic acid (**7c**) was prepared from commercial (*R*)-3-chloropropane-1,2-diol (**10**) by selective oxidation of the primary hydroxy with nitric acid.[50, 51] Each of the chiral α-hydroxycarboxylic acids **7a**, **c**, **e**-**g** and commercial grade glycolic acid (**7b**) was condensed with glycine benzyl ester monotosylate (**8**) by the conventional peptide coupling method to give the α-hydroxycarboxamides **9a**-**c**, **e**-**g**, respectively (Scheme 2). Iodine atom was introduced by treating the chloride **9c** with sodium

iodide in refluxing acetonitrile to give **9d** in 45% yield. Treatment of **7f** with thionyl chloride in methanol gave methyl (*S*)-2-hydroxyhexanoate (**11**) in 84% yield.

The peptidyl phosphonate diesters **2a-g**, **3** and **4** were synthesized as shown in Scheme 3. The hetero-diesters of phosphonic acid was prepared from the phosphonyl dichloride by stepwise reaction with phenol (a leaving group) and α-hydroxycarboxamides **9a-c**, **e-g** (a peptidyl side chain) according to our previously reported procedure.[39] Thus, the fully protected phosphonic acid **12** (racemic form)[39] was converted to the phosphonyl dichloride **13**. Selective substitution of a single chlorine atom of **13** was achieved by pre-mixing **13** and phenol, followed by the addition of Et_3N at low temperature (-65ºC) as a catalyst.[52] The resulting phosphonyl monochloro monophenyl ester was then allowed to react with the α-hydroxycarboxamides **9a-g** or the α-hydroxyester **11** to give the phosphonyl hetero-diesters **14a-g** and **15**, respectively. This procedure, however, usually accompanied the formation of the homo-diphenyl and homo-bis-peptidyl esters of **12** regardless of careful operations, and gave the desired hetero-diesters **14a-g** and **15** in moderate yields after careful chromatographic separation. The results under several reaction conditions indicated that the initial reaction with phenol should be conducted at sufficiently low temperature by initiating the reaction with NEt_3 for substitution of a single chlorine atom of **13**. However, the initial ratio of the intermediate monochloro monophenyl ester (δ_P 39.6) was not in agreement in several cases with the product ratio of the hetero-diesters, and the formation of the homo-bis-peptidyl esters was observed even in the reactions where complete consumption of the dichloride **13** (δ_P 49.2) was confirmed by ^{31}P NMR. This observation suggested that the intermediate monochloro monophenyl ester (δ_P 39.6) reacted with the second peptidyl alcohol by substitution with phenol, as well as with chlorine. It is noteworthy that the formation of homo-bis-peptidyl and diphenyl esters was not suppressed by

reversing the order of reaction of **13** with the α-hydroxycarboxamides and phenol (data not shown). Reaction of the dichloride **13** with methanol and **9a** by the same procedure afforded the peptidyl methyl phosphonate **16**. At this stage, the compounds **14a**-**g**, **15** and **16** were obtained as a mixture of equal amounts of four diastereoisomers (for **14a**, **c**-**g**, **15** and **16**) or four stereoisomers (two sets of enantiomers) (for **14b**) with respect to the stereogenic centers at the α-carbon of the glutamate moiety and the phosphorous atom. No attempts were successful in separating the diastereoisomers at this stage (see Section 2.2). Removal of the benzyl protecting groups by catalytic hydrogenation gave the desired phosphonates **2a**-**c**, **2e**-**g**, **3** and **4**. Interestingly, the iodine analogue **14d** did not react under the conventional hydrogenolysis conditions (H_2 gas /10% Pd-C). Hence the deprotection of **14d** was achieved under the nucleophilic C-O bond cleavage conditions using anisole/aluminum chloride to give **2d** in 22% yield.

2.3. Separation of diastereoisomers with respect to chiral phosphorus by MPLC

Starting with racemic 2-amino-4-phosphonobutanoic acid,[53, 54] the reaction with chiral α-hydroxycarboxamides **9a, c**-**g** and α-hydroxyester **11** gave the corresponding peptidyl phosphonate diesters **2a**, **c**-**g**, **3** and **4** as a mixture of four diastereoisomers with respect to the newly formed chiral phosphorus and the original chiral α-carbon of the glutamate moiety. In compound **2b**, the absence of a chiral carbon in the peptidyl chain gives a mixture of four stereoisomers (two sets of enantiomers) with respect to the same stereogenic centers.

We found in the purification step that two sets of diastereoisomers were separated for compounds **2a**, **c**-**g**, **3** and **4** by reversed phase medium pressure liquid chromatography (MPLC) (Fig. 2). We collected each peak separately and designated peak A (slow-eluting) and peak B

(fast-eluting). In contrast, no chromatographic separation of diastereoisomers was observed with compound **2b**. This chromatographic behavior, in view of the distal stereogenic center at the α-carbon of the glutamate moiety, suggested that the separated diastereoisomers (peak A and B) of **2a**, **2c**-**g**, **3** and **4** were related to the difference in the stereochemistry at the chiral phosphorous (S_P* or R_P*) relative to the fixed stereogenic center at the adjacent peptidyl moiety. It seems that the stereogenic center (L_α or D_α) at the α-carbon of the glutamate moiety is not close enough to the chiral phosphorous and/or the peptidyl α-carbon to effect diastereoisomeric differences in the MPLC behavior. Hence each peak A and B is composed of a mixture of two diastereoisomers with respect to the distal chiral α-carbon (L_α or D_α) of the glutamate moiety. This view was supported by the spectroscopic data for peak A and B; each gave a single peak or very close two peaks (equal height) in ^{31}P NMR and an apparently single set of peaks in ^{1}H NMR. We therefore concluded that the separated diastereoisomeric pairs (peak A and B) of **2a**, **2c**-**g**, **3** and **4** were related to the chiral phosphorus and that the diastereoisomers belonging to peak A had the same stereochemistry at the chiral phosphorus (S_P*, see below), but it was opposite to that of the diastereoisomers in peak B (R_P*). Note that each peak A and B is still composed of equal amounts of two diastereoisomers with opposite stereochemistry (L_α or D_α) at the chiral α-carbon of the glutamyl moiety, but they behaved like enantiomers due to the distance of this chiral center. For the same reason, the diastereoisomers of compound **2b** behaved like enantiomers and were not separated by MPLC to give a mixture of four stereoisomers (two sets of enantiomers) with equal amounts.

For the phosphonate diesters **2a**, **c**-**g** and **3**, the slow-eluting peak A invariably showed high inhibitory activities, whereas the fast-eluting peak B exhibited little or no activity (see Sections 2.5 and 2.6). For this reason, we used the inhibitory activities of peak A (a mixture of two

diastereoisomers) in the following discussion. For compound **2b**, the inhibitory activity was evaluated for a mixture of four stereoisomers. The absolute configuration of the chiral phosphorus was not determined experimentally, but was assigned tentatively as S_P* and R_P* for the isomers in peak A and peak B, respectively, from our preliminary results on the X-ray crystal structural analysis of *E. coli* GGT in complex with **2a**-**A** (Supplemental Fig. S1, see Section 2.5 for details).

2.4. Synthesis of phosphinate ester 5

The phosphinate **5** was synthesized as shown in Scheme 4. The protected L-glutamic acid **17** was converted to the L-vinylglycine derivative **18** by the reported procedure.[55] Phosphorus atom was introduced by triethylborane-catalyzed radical addition of ammonium hypophosphite[56] to give the hydrophosphinic acid **19** in good yield. The crude product was treated as such with *N*,*O*-bis(trimethylsilyl)acetamide (BSA), and the resulting trivalent trimethylsiyl (TMS) phosphite was allowed to react by conjugate addition with *t*-butyl acrylate to give the (*S*)-phosphinic acid derivative **20** quantitatively. We initially tried to react with ethacrylamide **21** to synthesize directly the corresponding phosphinate derivative with a DL-Abu-Gly analogous peptidyl chain, but the reaction did not work with less reactive Michael acceptor ethacrylamide **21**. Hence we used the sequential three-step reaction involving the Michael addition to *tert*-butyl acrylate to give **20**, followed by deprotection of the *tert*-butyl ester and condensation with the glycine benzyl ester monotosylate (**8**) to give the full-length phosphinic acid **23** in a total yield of 50% from **18**. Treatment of **23** with oxalyl chloride followed by reaction with phenol gave the phosphinate ester **24** in 55% yield. Removal of the benzyl protecting groups by catalytic hydrogenolysis afforded the desired phosphinate ester **5** with a Gly-Gly mimic peptidyl chain.

Compound **5**, synthesized from L-vinylglycine derivative **18**, was composed of equal amounts of two diastereoisomers with respect to the chiral phosphorus, but, as seen for compound **2b**, the diastereoisomers of **5** behaved like enantiomers and were not separable by reversed phase MPLC due to the absence of a fixed stereogenic center at the α-carbon of the adjacent peptidyl moiety. Hence we used compound **5** for the following enzyme assays as a 1:1 mixture of two diastereoisomers with opposite stereochemistry at the chiral phosphorus. Compound **5** is the phosphinate version of compound **2b**, but it should be noted that compound **2b**, synthesized from racemic 2-amino-4-phosphonobutanoic acid, was a mixture of four stereoisomers (two sets of enantiomers) with respect to the stereogenic centers on the phosphorus (R_P* or S_P*) and the distal α-carbon (L_α or D_α) at the glutamyl moiety, whereas compound **5** was a mixture of two diastereoisomers (L_α, R_P*) and (L_α, S_P*). For clarity, the stereoisomeric composition and the configuration of each isomer for compounds **1**, **2a**-**g**, **3**, **4** and **5** are summarized in Table 1. The stereoisomeric composition of compound **1**[39] will be discussed in Section 2.5. The stereochemistry of peak A and B of compound **2g** is worth noting. This compound was synthesized from (*R*)-α-hydroxycarboxamide **9g**, a mimic of D-Nle-Gly, and its stereochemistry ($D_{\alpha'}$) at the α-carbon of the peptidyl chain adjacent to the phosphorus was opposite to that of compound **2f** ($L_{\alpha'}$) derived from (*S*)-α-hydroxycarboxamide **9f**. Therefore, the chromatographic behavior of the (R_P*, $D_{\alpha'}$) isomer of **2g** on MPLC should be similar to that of the (S_P*, $L_{\alpha'}$) isomer of **2f**, because, due to the distance of the remaining stereogenic center (L_α or D_α) at the α-carbon of the glutamate moiety, these two sets of diastereoisomers are pseudo-enantiomorphic and hence are expected to behave like enantiomers on MPLC. We therefore assigned the stereochemistry at the chiral phosphorus in peak A (slow-eluting) and peak B (fast-eluting) as R_P* and S_P*, respectively, for compound **2g**. Thus, the peak A of **2g** was composed of two

diastereoisomers (L_α, R_P*, $D_{\alpha'}$) and (D_α, R_P*, $D_{\alpha'}$), and peak B was a mixture of two diastereoisomers (L_α, S_P*, $D_{\alpha'}$) and (D_α, S_P*, $D_{\alpha'}$). The diastereoisomers contained in each peak A and B of **2g** were pseudo-enantiomorphic and were not separable by MPLC.

2.5. Inhibitory activities toward human GGT

The inhibitory activities of the phosphorus esters **2a**-**g**, **3**, **4** and **5** were evaluated against human and *E. coli* GGTs according to our previous method.[38, 39] Enzyme activity was measured using 7-(γ-L-glutamylamino)-4-methylcoumarin (γ-Glu-AMC)[57] as substrate, and release of 7-amino-4-methylcoumarin (AMC) was detected fluorimetrically and continuously as the progress of enzyme reactions. We employed a hydrolytic condition without using a γ-glutamyl acceptor substrate such as Gly-Gly,[3] because a high concentration of Gly-Gly may compete with the peptidyl moiety of the phosphorus esters **2a**-**g**, **3**, **4** and **5** for the enzyme Cys-Gly binding site, thereby complicating the inhibition kinetics. To suppress the possible auto-transpeptidation,[3, 4] the reaction was conducted under an acidic condition (pH 5.5), where the transpeptidation activity is relatively low,[58] and by using lower concentrations of substrate (4.0 and 0.2 μM of γ-Glu-AMC for human and *E. coli* GGT, respectively), which correspond to 1/3 and 1 K_m value for human and *E. coli* GGT, respectively.

The phosphonate diesters **2a**-**f** (peak A, except **2b**) with a good leaving group (phenoxide) served as potent and irreversible inhibitors of human GGT. Typical progress curves for the reaction catalyzed by human GGT in the presence of varying concentrations of compound **2a**-**A** are shown in Fig. 3A. The inhibitory activities were evaluated by calculating the second-order rate constant (k_{on}) for enzyme inactivation, assuming a bimolecular reaction of enzyme and inhibitor to form a covalent enzyme-inhibitor covalent complex (E-I) (see Section 2.7, Fig. 5).

Thus, the time-dependent inhibition curves were fit to the first-order rate equation (1) (see Section 4.3.2) to determine the observed pseudo-first-order rate constant for enzyme inactivation (k_{obs}) at each inhibitor concentration. A plot of k_{obs} against inhibitor concentration gave a straight line without saturation until the highest inhibitor concentration (data not shown), indicating that the reversible formation of EI complex was kinetically incompetent under the reaction conditions employed. Hence the k_{on} values, the second-order rate constants for enzyme inactivation, were calculated from the slope on the assumption that the inhibitor was competitive with the substrate [equation (2) in Section 4.3.2]. The results are summarized in Table 2. Among the inhibitors synthesized, the phosphonate **2a** exhibited the highest inhibitory activity (k_{on} = 145 $M^{-1}s^{-1}$) toward human GGT. This value, compared with the inhibitory activity of acivicin (k_{on} = 0.40 $M^{-1}s^{-1}$) measured under the same reaction conditions, indicated that **2a** was an extremely potent irreversible inhibitor of human GGT that inactivated the enzyme more than 360 times as quickly as acivicin. This observation was in accordance with our initial expectations that the L-Abu-Gly peptidyl chain in **2a** was highly analogous to the L-Cys-Gly moiety in glutathione to favor its binding to the active site of human GGT, to facilitate covalent bond formation with the catalytic Thr residue and to give the adduct that was a mimic of the putative transition state (TS-1) for the catalyzed reaction with glutathione (Scheme 1). A methyl as a good mimic of SH was also shown in the sulfoximine-based transition-state analogue inhibitors of γ-glutamylcysteine synthetase, the first and the rate-limiting enzyme in glutathione biosynthesis.[59] We previously reported that the parent compound **1**, a mixture of stereoisomers synthesized from racemic α-hydroxybutyrate, inactivated human GGT with a k_{on} value of 75 $M^{-1}s^{-1}$.[39] Assuming that the inhibition of human GGT is highly dependent on the stereochemistry at the chiral phosphorus, which will be discussed later, the inhibitory activity is then proportional to the stereoisomeric

composition. The observed activity of **1** (75 $M^{-1}s^{-1}$) was about a half that of **2a** that was a mixture of two diastereoisomers, suggesting that the previously synthesized **1** was a mixture of four stereoisomers (Table 1) rather than eight, probably due to the isolation of peak A solely during the chromatographic purification by MPLC. Therefore compound **1** was composed of (L$_{\alpha}$, S_P*, L$_{\alpha'}$), (L$_{\alpha}$, R_P*, D$_{\alpha'}$), (D$_{\alpha}$, S_P*, L$_{\alpha'}$) and (D$_{\alpha}$, R_P*, D$_{\alpha'}$) as shown in Table 1.

Removal of the ethyl group from the L-Abu-Gly peptidyl chain significantly decreased the inhibitory activity (**2b**). It should be noted that **2b** was racemic at both the chiral phosphorus and the α-carbon at the distal glutamate moiety, and was composed of equal amounts of four stereoisomers (Table 1). With an assumption that the only S_P* isomer is active, the observed k_{on} value of **2b** (a mixture of four) should be doubled when compared to that of **2a** (a mixture of two). The corrected value (89.0 $M^{-1}s^{-1}$) was still 61% of the k_{on} value of **2a**, indicating a significant contribution of the ethyl group to the enzyme inhibition. Interestingly, the introduction of a chloromethyl (**2c**) or iodomethyl group (**2d**) caused almost no difference in the inhibitory activity as compared to **2b**. We initially anticipated that a chlorine and iodine atoms might also work as a good mimic of SH, because the van der Waals volume of SH (14.8 cm^3 mol^{-1}) is similar to that of Cl (11.62 cm^3 mol^{-1}) and I (19.18 cm^3 mol^{-1}), as well as CH_3 (13.67 cm^3 mol^{-1}).[60] However, the introduction of a chloromethyl (**2c**) and iodomethyl (**2c**) had almost no impact on the activity of the inhibitors, whereas the introduction of an ethyl (**2a**) (CH_3 as SH mimic) increased the activity by 63% as compared to **2b**. This observation might be due to a little too small and large in size of chlorine and iodine atom, respectively, for a SH mimic, with a methyl group in between being a good compromise. In this sense, a bromine atom with a van der Waals volume of 14.40 cm^3 mol^{-1} [60] might be a reasonably good mimic of SH. Human GGT thus strictly recognized the size of the substituent attached to the β-carbon of the side chain of the Cys

moiety and that a SH group or its equivalent in size seems preferable at this site. This is highlighted by a significantly low activity of **2e** with a side chain mimicking L-Phe-Gly. This compound with a bulky phenyl group attached to the β-carbon was found to be an extremely weak inhibitor of human GGT (only 3% of compound **2a**). This result is consistent with the acceptor substrate specificity of rat kidney GGT, where L-Phe-Gly was a significantly poor dipeptide substrate.[3] It is noteworthy, however, that elongation of the side chain of **2a** by two methylene units did not severely affect the inhibitory activity; compound **2f** with a L-Nle-Gly-analogous side chain inhibited human GGT with a k_{on} value of 63.2 $M^{-1}s^{-1}$ that was 44% of that of **2a**. This result, combined with the moderate activities of **2c** and **2d**, and remarkably low activity of **2e**, indicated that the γ-position of the side chain that is usually occupied by the SH of Cys was strictly recognized by the enzyme, whereas the group beyond this position might be forced out of the active site, thereby escaping from the steric constraint and recognition by the enzyme. Furthermore, the stereochemistry at the α-carbon of the Cys moiety and the presence of the C-terminal Gly had a significant impact on the inhibition activity: the inversion of stereochemistry at the α-carbon of the Cys moiety (**2g**) or removal of the C-terminal Gly (**3**) resulted in a complete loss of inhibitory activity (Table 2). Thus, human GGT was inactivated strongly only by the phosphonate with a peptidyl side chain that is structurally and stereochemically equivalent to L-Cys-Gly. This is consistent with the observation that GGT accepts only L-amino acids or peptides as γ-glutamyl acceptor substrate[3] and is in good agreement with the putative substrate recognition and inhibition mechanisms proposed by us that human GGT strongly recognizes the negative charge at the C-terminal Gly of glutathione.[39]

We found that peak A of compounds **2a**, **c-f** was invariably active, whereas peak B was completely inactive towards human GGT. These results indicated that human GGT strictly

recognized the stereochemistry at phosphorus; the isomers A (tentatively assigned as S_P*) was exclusively accommodated and/or reacted with the active-site Thr residue, leading to enzyme inactivation. The assignment of the configuration at phosphorus was derived from our preliminary results on the X-ray crystallographic analysis of *E. coli* GGT in complex with **2a**-**A**, in which S_P configuration was observed at the chiral phosphorus that was covalently bound to the Oγ atom of Thr391 (Supplemental Fig. S1). Since both human and *E. coli* GGTs were inactivated by the same isomers (peak A), the stereochemical preference of both enzymes for the chiral phosphorus is the same (see Section 2.6). We therefore tentatively assigned the original stereochemistry at phosphorus of **2a**-**A** (peak A) as S_P* by assuming the in-line substitution at phosphorus with inversion of configuration [Note that the phosphorus stereochemical assignments for the phenyl phosphonate (before reaction) and the enzyme-inhibitor complex (after reaction) are the same (S_P*) due to the sequence rule for the ligands around the phosphorus, irrespective of the inversion of configuration].

Table 2 also highlights the strict stereochemical recognition by human GGT. Neither peak A nor peak B of the phosphonate **2g** was inhibitory toward human GGT, indicating that none of the four stereoisomers of **2g** (Table 1) was acceptable by the enzyme irrespective of the configuration at phosphorus. This was due to the D-configuration at the α-carbon of the Cys moiety of **2g**. Conversely, peak B of compound **2f** was totally inactive irrespective of the L-configuration at the α-carbon of the Cys moiety, because the isomers in peak B had the R_P* configuration at phosphorus. Therefore human GGT is highly selective for S_P*-phosphorus and L-α-carbon at the Cys moiety in the peptidyl chain, and does not accept any other stereoisomers.

Since the phosphonate inhibitors **2a**, **c**-**f** are a good mimic of the putative transition state (TS-1) for the catalyzed reaction of glutathione (Scheme 1), the stereochemistry at phosphorus is

closely related to the stereochemical outcome of the catalyzed reaction with glutathione. This is illustrated in Fig. 4. In the active site of human GGT, the isomer(s) **2f**-**A** with S_P* configuration at phosphorus is accommodated and reacted with the catalytic Thr residue (γOH) that approaches the phosphorus opposite to the phenoxy leaving group and then substitutes the phenoxy in line with inversion of configuration at phosphorus, thus making an enzyme-inhibitor covalent complex with S_P* configuration (panel A to B). In contrast, the isomer(s) **2f**-**B** with R_P* configuration at phosphorus is not reactive probably because of the unfavorable configuration for accommodation in the enzyme active site and/or for in-line substitution by the catalytic Thr residue. If this stereochemistry holds for the catalyzed reaction, then the nucleophilic attack of the Thr γOH occurs on the *Si*-face (above the plane) of the γ-carbonyl of glutathione to make a tetrahedral oxyanion with *S* configuration (panel C to D). This stereochemical course seems quite reasonable from a steric point of view: the catalytic Thr γO approaches the phosphorus (for **2a**-**A**) or the γ-carbonyl (for glutathione) from the less-hindered side that is opposite to the L-Cys side chain and makes a tetrahedral (S_P*)-adduct (for **2a**-**A**) or (*S*)-oxyanion (for glutathione) in which the O-Thr is located above the plane, while the side chain of the L-Cys moiety occupies below (Figure 4, B and D). Since the phosphorus stereochemistry of the active inhibitor is assigned tentatively as S_P*, we cannot rule out the possibility that all the stereochemistry shown in Fig. 4 would be reversed: the active-site Thr γOH attacks from the bottom side on the R_P* phosphorus of **2a**-**A** to make a (R_P*)-adduct, which corresponds to the attack of Thr γOH on the *Re*-face (below) of the γ-carbonyl of glutathione to make a (*R*)-tetrahedral intermediate. This mode of reaction, however, appears unfavorable from a stereochemical point of view, because Thr γOH attacks the phosphorus or the γ-carbonyl from the same side of the Cys side chain. The defined stereochemical assignments have to await the X-ray structural analysis of human GGT in

complex with **2a**-**A**.

In this model, we also proposed the mode of recognition of the Cys-Gly moiety; human GGT has a specific binding site for the Cys moiety to recognize strictly its stereochemistry (L_{α}') and the size of its side chain up until the sulfur atom probably by a narrow cavity around this part, but no recognition is inferred for any group beyond that position. This mode of recognition of the Cys residue is reasonable for GGT that accepts a variety of glutathione-*S*-conjugates as substrates. This is illustrated by the reaction of GGT with **2f**-**A** (Fig. 4, A and B) where the L-Nle residue is bound or recognized by the enzyme up until the γ-carbon. No stereoisomers with D-configuration at the Cys moiety are permitted for binding and reaction with the enzyme active site. In addition, the C-terminal carboxy group of the Gly moiety is strictly recognized by an electrostatic interaction with a positively charged active-site residue.[39] The active-site residue that recognizes the negative charge of the acceptor substrate in the transpeptidation reaction was recently reported to be Lys562.[61] Since the peptidyl chain of the present phosphonate inhibitors is equivalent to the acceptor substrate and the Cys-Gly moiety of glutathione, this residue is probably responsible for the recognition of the C-terminal negative charge in the present peptidyl phosphonate inhibitors, as well as that of the C-terminal Gly of the substrate glutathione.

A good leaving group such as phenoxy attached to the phosphorus was absolutely necessary for enzyme inactivation. The methyl ester **4** was totally inactive toward the enzyme, irrespective of the presence of S_P* phosphorus and L-Abu-Gly peptidyl chain. This is naturally understood by the inactivation mechanism; the electrophilic phosphorus with a good leaving group reacted with the active-site Thr γOH to phosphonylate and inactivate the enzyme (see Section 2.7 and Fig. 5). The structure of **4** is highly analogous to glutathione, but the enzyme activity was not affected appreciably even in the presence of a high concentration (1 mM) of compound **4** (Supplementary,

Fig. S2). This result indicated that, contrary to our initial expectations, the affinity of the phosphonate **4** with the enzyme active site was substantially low. This is probably due to the sp^3-hybridized tetrahedral phosphorus carrying a methoxy group that is substantially different in size and shape from the sp^2-hybridized γ-carboxamide of glutathione and from the tetrahedral oxyanion. If this is the case, the initial binding of **2a** is expected to be unfavorable, although the enzyme was inactivated by the phosphonate rather fast. In fact, the K_i value for a related phosphonate inhibitor was 0.17 mM.[39]

The phosphinate inhibitor **5**, a closely related analogue of the phosphonate **2b**, showed a substantially low activity (k_{on} = 1.4 $M^{-1}s^{-1}$) toward human GGT. The phosphinate **5** was a mixture of two diastereoisomers, while the phosphonate **2b** was four. Hence the net inhibitory activity of **5** was even worse and no more than 1.6% of that of **2b**. Despite its low activity, compound **5** caused a time-dependent inhibition of human GGT, giving a series of concave upward progress curves in the inhibition assay (Supplementary Fig. S3). This is indicative of covalent modification of the enzyme active site by the phosphinate **5**, but the reaction was substantially slow. The significantly lower activity of phosphinate **5** as compared to the phosphonate counterpart **2b** was totally unexpected from our initial expectations that the phosphinate ester **5** would be more reactive than the phosphonate ester **2b** due to the absence of the ground-state resonance stabilization by p_π-d_π conjugation with the oxygen and phosphorus.[62] We therefore speculated that, in the reaction of the active-site Thr γOH and the phosphorus inhibitors, the inductive (-I) effect by an electronegative oxygen had a larger impact on the reactivity than the p_π-d_π conjugation resonance effect. We reported previously that the reaction of GGT with phosphonate-diester based inhibitors proceeded via a dissociative transition state in which a substantial P-O bond cleavage is inferred.[39] In this transition state, the positively

charged phosphorus atom might be stabilized effectively by an electron-donating resonance effect of the oxygen to facilitate the reaction.

2.6. Inhibitory activities toward *E. coli* GGT

Under the same reaction conditions, the activities of **2a**-**f**, **3**, **4** and **5** (peak A, except **2b**) were evaluated for the inhibition of *E. coli* GGT. The peak A of the phosphonate diesters **2a**, **c**-**f** and **3** invariably caused time-dependent inhibition of *E. coli* GGT (Fig. 3B), whereas peak B was uniformly inactive.

In sharp contrast to human GGT, the *E. coli* enzyme was strongly inactivated by **2e** with a L-Phe-Gly analogous peptidyl chain that was more than 3.5 times as active as **2a**. This is consistent with the fact that aromatic L-amino acids such as L-Phe, L-His and L-DOPA serve as a good acceptor substrate of *E. coli* GGT in transpeptidation.[63] Inhibitors **2a**-**d** and **2f** inactivated *E. coli* GGT with k_{on} values ranging from 50.7 to 188, but no clear relationship was observed between their inhibition potency and the structure of the side chains at the Cys moiety. L-Met was reported to be nearly twice as good as L-Phe as an acceptor substrate,[63] but the inhibitor **2f** with a L-Nle-Gly mimetic peptidyl chain (k_{on} = 50.7 M^{-1} s^{-1}) was only13% as active as **2e**. A hydrogen bond involving a sulfur atom[64] might be playing a role in the recognition of Met as noted for capD, a GGT family enzyme from *Bacillus anthracis*.[61]

In contrast to human GGT, the *E. coli* enzyme was inactivated slowly by **2g** with a D-Nle-Gly peptidyl chain, suggesting that the *E. coli* enzyme was not highly selective for the L-configuration at the α-carbon of the Cys-Gly peptidyl chain. With respect to the chiral phosphorus, the *E. coli* enzyme was not highly selective, either; *E. coli* GGT was inactivated slowly by **2f**-**B** and **2g**-**A** both with R_P* phosphorus, whereas these compounds were totally

inactive toward human GGT. It appears therefore that *E. coli* GGT does not particularly prefer L-Cys or its equivalent at the Cys-Gly binding site, and that the enzyme does not strictly recognize the stereochemistry at chiral phosphorus and the α-carbon of the Cys moiety. The properties of *E. coli* GGT suggest that the natural and preferred substrates of *E. coli* GGT are not glutathione or its *S*-conjugates, but other exogenous γ-glutamyl amides that can be processed by this enzyme as a nutrition source.[65] According to the substrate preference of the *E. coli* enzyme, such γ-glutamyl peptides presumably contain an aromatic moiety (L-Phe, Tyr, Trp and so on) or its equivalent at the peptide moiety. The identification of those γ-glutamyl peptides is intriguing in terms of natural occurrence of novel γ-glutamyl compounds.

The relatively non-selective nature of the *E. coli* enzyme is supported by the inhibitory activity of **3** that is devoid of the C-terminal Gly moiety. This compound was totally inactive toward human GGT, but an appreciable activity (k_{on} = 8.8 M^{-1} s^{-1}) was observed with *E. coli* GGT. Compound **3**, however, was substantially less active (ca. 17%) than **2f**, implying that the C-terminal Gly still contributed noticeably to the affinity of the inhibitor in the active site. This result is indicative of a putative Cys-Gly binding site still conserved in *E. coli* GGT. Several X-ray crystal structural analyses have been reported for bacterial GGTs,[42-45, 66] but none of them was successful in identifying the Cys-Gly binding site. In the crystal structures of *E. coli* GGT, the detection of intact glutathione failed even after the soaking of the enzyme crystals into a glutathione solution for a short period followed by rapid freezing.[42] An attempt to detect the structure of intact glutathione in complex with *Helicobactor pylori* GGT was made by using a catalytically inactive mutant enzyme (Thr380Ala), but the Cys-Gly portion of the enzyme-bound glutathione was invisible by X-ray structural analysis due to considerable mobility beyond the γ-glutamyl group.[44] These results suggested that no defined Cys-Gly binding site was present in

bacterial GGTs. However, the properties of *E. coli* GGT as probed by the present phosphonate inhibitors cogently suggested the presence of a putative or remaining characters of the binding site for the Cys-Gly moiety that accommodates the amino acyl (AA)-Gly moiety of γ-glutamylamide substrates or an acceptor substrate AA-Gly. In this regard, we could exploit the differences in the structural and stereochemical preferences of enzymes in the Cys-Gly binding site for generating selective inhibitors; a phosphonate diester, for example, with a D-AA-Gly or L-AA side chain that incorporates an appropriate aromatic substituent at the AA moiety would be selective for *E. coli* GGT without inhibiting the human enzyme.

We previously reported the inhibitory activities of a series of simple phosphonate diesters with a substituted phenol as a leaving group.[39] The *E. coli* enzyme was uniformly two orders of magnitude more sensitive to those inhibitors than human GGT. It is worth noting that in the present phosphonate inhibitors **2a**-**f** with a peptidyl side chain, the inhibitory activities (k_{on} values) toward *E. coli* GGT were not so much different from those toward the human enzyme (Table 2). These observations can be explained by the difference in the extent of recognition of the Cys-Gly moiety by the enzymes; human GGT is highly selective for the Cys-Gly moiety of inhibitors and is strongly inhibited only by the inhibitors with an appropriate peptidyl chain or its equivalent that fits the defined Cys-Gly binding site, whereas the *E. coli* enzyme is relatively unselective for the Cys-Gly moiety and is inhibited generally by simple phosphonates without the Cys-Gly peptidyl moiety. Hence the phosphonate diesters without an appropriate Cys-Gly mimic peptidyl chain are rather weak inhibitors of human GGT, but can be highly inhibitory toward the *E. coli* enzyme. The present peptidyl phosphonates **2a**-**f** have the Cys-Gly mimicking peptidyl chain and hence are potent inhibitors of human GGT as well as the *E. coli* enzyme, if other requirements such as stereochemistry at the phosphorus and the side chain of the peptidyl

moiety are met. This view is consistent with the extremely weak inhibition of human GGT by acivicin (k_{on} = 0.4 M^{-1} s^{-1}) that lacks the Cys-Gly moiety, whereas the *E. coli* enzyme is highly sensitive to this compound (k_{on} = 4200 M^{-1} s^{-1}) (Table 2).[39] In this regard, the recently reported inhibitor OU749 and its analogues by Hanigan *et al.* bound to the acceptor substrate binding site can be an attractive option for selective inhibition of mammalian GGTs,[67-69] but their reversible nature with micro-molar K_i values might be insufficient for complete elimination of GGT activities for a certain period of time *in vitro* and *in vivo* as was observed with acivicin.

The phosphonate **4** with a poor leaving group and the phosphinate **5** did not inhibit *E. coli* GGT at all. Since the phosphinate **5** was weakly inhibitory toward human GGT, it stands out as the sole compound that inhibited human GGT selectively among the phosphorus ester-based inhibitors. The structure of **5** might be a good template for designing novel inhibitors selective for human GGT.

2.7. Mass spectroscopic analysis of *E. coli* GGT in complex with inhibitor

The structural defining of the Cys-Gly binding site of human GGT is one of the most challenging, but the most rewarding subjects in the studies on GGT. Directing towards this goal, the peptidyl phosphonate inhibitors **2a-f** was designed also for use as a ligand for X-ray structural analysis of human GGT in view of the enzyme-inhibitor adduct that mimics the putative transition state (TS-1) for glutathione degradation (Scheme 1). For this purpose, the Cys-Gly mimicking peptidyl chain has to remain bound to the phosphorus after the reaction with the enzyme active site. This was confirmed by an ion-spray mass spectroscopy of *E. coli* GGT inactivated by compound **2a-A**. We used *E. coli* GGT rather than the human enzyme, because highly and heterologously glycosylated human GGT would make the mass analysis very

complex. *E. coli* GGT was treated with **2a**-**A** under varying conditions, and the small subunit from each experiment was separated by HPLC.[70] The isolated small subunit (GGTS, ca. 20,000 Da) was analyzed by electrospray ionization mass spectroscopy (ESI-MS) according to our previous method for detecting the catalytic residue (Thr391 in GGTS) of *E. coli* GGT.[40] The untreated *E. coli* GGT was also processed in the same manner as reference. The results are shown in Fig. 5. The GGTS from the untreated enzyme gave a peak at *m*/*z* 20,014 (Fig. 5A) that corresponds to the expected molecular weight (20,010) of the unmodified small subunit calculated from its amino acid sequence.[70] The small subunit obtained from the enzyme treated under a weak reaction condition (1.7 mM **2a**-**A**, 20 min) gave two peaks at *m*/*z* 20,015.5 and 20,323 (Fig. 5B). Finally, the small subunit from the enzyme with a stronger treatment (4 mM **2a**-**A**, 3 h) gave a single peak at *m*/*z* 20,323 (Fig. 5C). The mass difference (307.5) between the two peaks corresponded well to the calculated mass (308) of the inhibitor that is bound covalently to the catalytic Thr Oγ through a P-O bond with concomitant loss of the phenoxy group as shown in Scheme 1C. These results clearly indicated that the inhibitor **2a**-**A** reacted with the active site of the enzyme to leave the Cys-Gly mimicking peptidyl moiety bound to the active site of GGT. Therefore the peptidyl phosphonate inhibitors **2a-f** are highly promising as a ligand for X-ray structural analysis of GGT for defining the Cys-Gly binding site.

3. Conclusions

In this study, we designed and synthesized an array of novel peptidyl phosphorus esters as potent mechanism-based inhibitors of human and *E. coli* GGTs. The most active compound **2a** with a L-Abu-Gly analogous peptidyl chain was more than 700 times as active as acivicin toward human GGT, if the stereoisomeric composition is considered. As a close mimic of the transition

state (TS-1) for glutathione degradation, the peptidyl phosphorus esters served as a good chemical probe to define hitherto unidentified Cys-Gly binding site of mammalian and bacterial GGTs. So far the substrate specificity of GGT for the Cys-Gly binding site have been estimated by using γ-glutamyl acceptor substrates[3] with the assumption that the acceptor substrates AA-Gly bind to the same site as the Cys-Gly moiety of the γ-glutamyl donor substrates (glutathione). However, the approximation of substrate specificity by acceptor substrates might be misleading, because the apparent activity is significantly dependent on the extent of deprotonation or the pK_a of the amino group of the dipeptidyl acceptor substrates (AA-Gly).[58] In this regard, the phosphonate esters with a peptidyl side chain in the present study precisely reflect the properties of the Cys-Gly binding site; human GGT has a specific binding site for Cys-Gly to recognize strictly its stereochemistry (L) and the size of its side chain up until the sulfur atom, but no recognition is expected for any group beyond the sulfur (γ-position). This mode of substrate recognition seems quite reasonable from the proposed physiological function of mammalian GGT; the enzyme accepts glutathione (unsubstituted L-Cys) as a primary natural substrate, but at the same time has to accommodates structurally diverse glutathione *S*-conjugates (*S*-substituted L-Cys) for catabolism of xenobiotics. These apparently contradictory demands can be met nicely by strict recognition around the α, β and γ-position (S atom) of the Cys residue, while any groups beyond the γ-position remain unbound, probably by sticking out of the active site. The stereochemical preference of GGT for the chiral phosphorus (tentatively assigned as S_P*) shown in this study gives insights into the stereochemical course of the catalyzed reaction with glutathione. In this context, the peptidyl phosphorus diesters such as **2a**-**A** is an ideal ligand for X-ray structural analysis of GGT to define the putative Cys-Gly binding site. This line of work is in progress, and the results will be published in due course.

Finally, the irreversible nature of the present phosphonate inhibitors is highly advantageous over reversible inhibitors for needs to eliminate the GGT activities completely until new enzymes are *de novo* synthesized. This is exactly the major reason why acivicin, a rather weak and nonselective inhibitor of mammalian GGT, has been and is still being used widely, irrespective of its potent cyto- and neurotoxicity. Therefore the properties of the Cys-Gly binding site as probed by the present peptidyl phosphorus esters should give a basis for future molecular design for potent and irreversible inhibitors specific for human GGT, as well as for bacterial enzymes.

4. Experimental Procedures

4.1. General methods

All chemicals were obtained commercially and used without further purification unless otherwise sated. 7-(γ-L-Glutamylamino)-4-methylcoumarin (γ-Glu-AMC) was purchased from Sigma. Racemic benzyl 2-(*N*-benzyloxycarbonylamino)-4-phosphonobutanoate (**12**) was synthesized as previously described.[39] Dry CH_2Cl_2 and dry triethylamine (Et_3N) were prepared by distillation from CaH_2 and stored over Molecular Sieves 4Å. *E. coli* γ-glutamyl transpeptidase was purified from the periplasmic fraction of a recombinant strain of *E. coli* K-12 (SH642).[63] Human γ-glutamyl transpeptidase HC-GTP (T-72) was a generous gift from Asahi Kasei Corporation (Osaka, Japan). The enzyme preparation contained a significant amount of bovine serum albumin (BSA) as a stabilizer (GGT content < 1%), but the enzyme was catalytically homogeneous and was used without further purification.

Melting points (mp) were recorded using a Mettler FP62 melting point apparatus and are corrected. ^1H and ^{31}P NMR spectra were recorded on a JEOL JNM-AL300 (300 MHz for ^1H)

and JOEL JNM-AL400 (400 MHz for ^{1}H) spectrophotometers. ^{1}H chemical shifts (δ_H) are reported downfield of tetramethylsilane as an internal reference (δ_H 0.0), except for measurements in D_2O for which sodium 3-(trimethylsilyl)propanesulfonate was used as an internal standard (δ_H 0.0). Splitting patterns are abbreviated as follows: s, singlet; d, doublet; t, triplet, q; quartet; m, multiplet. ^{31}P chemical shifts (δ_P) are reported downfield of 85% H_3PO_4 as an external standard (δ_P 0.0). Infrared spectra were recorded on a HORIBA FT-720 Fourier Transform infrared spectrophotometer. Mass spectra were obtained with a JEOL JMS-700 spectrometer. Mass spectra of the enzyme were recorded on a Sciex API-3000 mass spectrometer (PE Sciex) interfaced with an ion-spray ion source. Elemental analyses were performed on a Yanaco MT-5. Reactions were monitored by analytical thin layer chromatography (TLC) on silica gel 60 F_{254} plates (Merck, #5715, 0.25 mm). Compounds were purified by flash column chromatography on silica gel 60 N (Kanto Kagaku, spherical and neutral, 40-50 mm, No. 37563-79) or by medium-pressure reversed-phase column chromatography using a Yamazen YFLC System with ODS-SM-50B column (Yamazen Co., Osaka, Japan). The spectral data (^{1}H NMR and/or IR) for known compounds **7a, c, e-g**, **9b**, **11**, **13** and **18** are described in Supplementary data. The spectral data supported the structure or are in good agreement with those reported (references cited in Supplemental data).

4.2. Synthesis

4.2.1. (*S*)-2-Hydroxybutanoic acid (7a).

(*S*)-2-Aminobutanoic acid (**6a**) (4.00 g, 38.8 mmol) was dissolved in 0.5 M H_2SO_4 (155 mL, 77.6 mmol). This solution was cooled to 0 °C and a solution of $NaNO_2$ (16.0 g, 233 mmol) in H_2O (40 mL) was added slowly with stirring. After stirring at this temperature for 3 h, the

reaction was allowed to warm to room temperature and was stirred for further 2 h. The reaction mixture was extracted 7 times with ethyl acetate (7 × 150 mL). The combined organic extracts were washed with sat. NaCl and dried over anhydrous Na_2SO_4. The solvent was removed *in vacuo* to afford the hydroxy acid **7a** (3.98 g) as an oil. This product was used for the preparation of compound **9a** without purification.

4.2.2. (*R*)-3-Chloro-2-hydroxypropanoic acid (7c).

A solution of (*R*)-(+)-3-chloropropane-1,2-diol (**10**) (5.0 g, 45.5 mmol) in water (5 mL) was placed in a 100 mL test tube. To the bottom of this test tube, 60% (13N) nitric acid (6.99 mL, 90.9 mmol) was injected slowly by a pipette, keeping the layers separated. The mixture was allowed to stand at room temperature without mixing for 3 days until the two layers became a clear homogeneous solution. Nitrogen oxide gas (Caution! toxic) was removed from the reaction mixture by argon stream in a fuming hood, and the solvent was removed *in vacuo* to afford crude (*R*)-3-chloro-2-hydroxypropanoic acid (**7c**) (6.83 g) as a colorless oil. The product was used for the preparation of compound **9c** without purification.

4.2.3. (*S*)-2-Hydroxy-3-phenylpropanoic acid (7e).

Compound **7e** was prepared from L-phenylalanine (10.0 g, 60.5 mmol) by the same procedure as described for the synthesis of **7a** and was purified by recrystallization from a mixture of diethyl ether and hexane (1:10) to yield pure **7e** as a colorless power (6.18 g, 61%). Mp 123.8-124.6 ^{o}C. $[\alpha]_D^{27}$ –21.3 (*c* 1.04, H_2O) [lit.[49] $[\alpha]_D^{20}$ = –21.3 (*c* 2.35, H_2O)]. Anal. calcd for $C_9H_{10}O_3$: C, 65.05; H, 6.07. Found: C, 64.98; H, 6.04.

4.2.4. (*S*)-2-Hydroxyhexanoic acid (7f).

Compound **7f** was prepared from L-norleucine (8.00 g, 61.0 mmol) by the same procedure as described for the synthesis of **7a** and was purified by recrystallization from a mixture of diethyl ether and hexane (1:12) to give pure **7f** as a highly hygroscopic colorless crystalline mass (3.18 g, 39%). $[\alpha]_D^{30}$ –16.2 (*c* 1.02, 1 M NaOH) [lit.[49] $[\alpha]_D^{20}$ = –16.3 (*c* 3.92, 1 M NaOH)]. Anal. calcd for $C_6H_{12}O_3$: C, 54.53; H, 9.15. Found: C, 54.33; H, 9.12.

4.2.5. (*R*)-2-Hydroxyhexanoic acid (7g).

Compound **7g** was prepared from D-norleucine (8.00 g, 61.0 mmol) by the same procedure as described for the synthesis of **7a** and was purified by recrystallization from a mixture of diethyl ether and hexane (1:12) to yield pure **7g** as highly hygroscopic colorless crystalline mass (4.19 g, 52%). $[\alpha]_D^{30}$ +16. 8 (*c* 0.99, 1 M NaOH) [lit.[49] $[\alpha]_D^{20}$ = –16.3 (*c* 3.92, 1 M NaOH) for the *S* enantiomer]. Anal. calcd for $C_6H_{12}O_3$: C, 54.53; H, 9.15. Found: C, 54.11; H, 9.11. HRMS (FAB, glycerol): Calcd for $C_6H_{13}O_3$ (MH^+) 133.0865; found 133.0865.

4.2.6. (*S*)-Benzyl 2-(2-hydroxybutanamido)acetate (9a).

Glycine benzyl ester monotosylate (**8**) (19.3 g, 57.3 mmol), 1-hydroxybenzotriazole monohydrate (HOBt·H_2O) (7.75 g, 57.3 mmol) and *N*-methylmorpholine (NMM) (6.59 g, 65.1 mmol) were dissolved in CH_2Cl_2 (150 mL). This solution was cooled to 0 °C. To this solution were added the hydroxy acid **7a** (3.98 g, 38.3 mmol) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) (11.0 g, 57.3 mmol), successively. After stirring at room temperature for 18 h, the reaction mixture was concentrated *in vacuo* to remove CH_2Cl_2. Ethyl acetate (250 mL) was added to the residue, and the resultant

mixture was washed successively with 1 N HCl, sat. $NaHCO_3$ and sat. NaCl , and was dried over anhydrous Na_2SO_4. After removal of solvent *in vacuo*, the residue was purified by flash column chromatography (40-60% ethyl acetate in hexane) to afford **9a** as a pale yellow oil (4.93 g, 51%). $[\alpha]_D^{27}$ –26.2 (*c* 0.80, EtOH). ^{1}H-NMR (300 MHz, $CDCl_3$): δ_H 7.39-7.30 (m, 5H, Ph), 7.08 (br, 1H, NH), 5.18 (s, 2H, PhC*H*$_2$O), 4.18-4.02 [m, 3H, C*H*(OH)CONH and Glyα-*H*], 3.08 (br, 1H, OH), 1.88 (dqd, *J* = 14.4, 7.5 and 4.2 Hz, 1H, $CH_3C\mathit{H}_aH_b$), 1.69 (dqd, *J* = 14.4, 7.5 and 7.2 Hz, 1H, $CH_3CH_a\mathit{H}_b$), 0.98 (t, *J* = 7.5 Hz, 3H, CH_3). IR (NaCl) 3396 (br), 1747, 1655, 1533, 1194, 1128, 1057, 1032, 984, 741, 698 cm^{-1}. Anal. calcd for $C_{13}H_{17}NO_4 \cdot 0.2H_2O$: C, 61.26; H, 6.88; N, 5.50. Found: C, 61.43; H, 7.07; N, 5.57. HRMS (FAB, glycerol): Calcd for $C_{13}H_{18}NO_4$ (MH^+) 252.1236; found 252.1238.

4.2.7. Benzyl 2-(2-hydroxyacetamido)acetate (9b).

Compound **9b** was prepared from glycolic acid (**7b**) (2.28 g, 30.0 mmol) and glycine derivative **8** (4.87 g, 14.5 mmol) by the same procedure as described for the synthesis of **9a**, and was purified by flash column chromatography (50% acetone in hexane) to give **9b** as a pale yellow oil (2.36 g, 73%). Anal. calcd for $C_{11}H_{13}NO_4 \cdot 0.2H_2O$: C, 58.25; H, 5.95; N, 6.17. Found: C, 58.26; H, 5.98; N, 6.05. HRMS (FAB, glycerol): Calcd for $C_{11}H_{14}NO_4$ (MH^+) 224.0923; found 224.0924.

4.2.8. (*R*)-Benzyl 2-(3-chloro-2-hydroxypropanamido)acetate (9c).

Compound **9c** was prepared from crude (*R*)-3-chloro-2-hydroxypropanoic acid (**7c**) (45.2 mmol) and glycine derivative **8** (18.4 g, 54.6 mmol) by the same procedure as described for the synthesis of **9a** and was purified by flash column chromatography (50% ethyl acetate in hexane),

followed by recrystallization from ethyl acetate and hexane (1:12) to afford **9c** as a colorless powder (6.85 g, 56%). Mp 87.7-88.8 ^{o}C; $[\alpha]_D^{27}$ −35.4 (*c* 1.08, EtOH). ^{1}H-NMR (300 MHz, $CDCl_3$): δ_H 7.39-7.31 (m, 5H, Ph), 7.24 (br, 1H, NH), 5.20 (s, 2H, PhC*H*$_2$O), 4.41 [dd, *J* = 6.0 and 3.6 Hz, 1H, C*H*(OH)CONH], 4.16 (dd, *J* = 18.3 and 5.7 Hz, 1H, Glyα-*H*$_a$), 4.09 (dd, *J* = 18.3 and 5.7 Hz, 1H, Glyα-*H*$_b$), 3.91 (dd, *J* = 11.4 and 3.6 Hz, 1H, ClC*H*$_a$H$_b$), 3.84 (dd, *J* = 11.4 and 6.0 Hz, 1H, ClCH$_a$*H*$_b$). IR (KBr) 3384 (br), 1736, 1655, 1541, 1221, 1088, 953, 762, 704 cm^{-1}. Anal. calcd for $C_{12}H_{14}ClNO_4$: C, 53.05; H, 5.19; N, 5.16. Found: C, 52.78; H, 5.12; N, 5.33.

4.2.9. (*R*)-Benzyl 2-(2-hydroxy-3-iodopropanamido)acetate (9d).

The chloride **9c** (4.00 g, 14.8 mmol) and sodium iodide (22.0 g, 148 mmol) were dissolved in acetonitrile (200 mL). The solution was stirred at 80 °C for 3 days. Insoluble salt (NaBr) was removed by filtration, and the filtrate was evaporated. The residue was dissolved in ethyl acetate (300 mL), washed with sat. NaCl (2 × 200 mL) and was dried over anhydrous Na_2SO_4. After removal of the solvent *in vacuo*, the residue was purified by flash column chromatography (60% ethyl acetate in hexane), followed by recrystallization from a mixture of ethyl acetate and hexane (1:20) to afford **9d** as a colorless powder (2.41 g, 45%). Mp 84.0-85.1 ^{o}C. $[\alpha]_D^{27}$ −36.2 (*c* 1.01, EtOH). ^{1}H-NMR (300 MHz, $CDCl_3$): δ_H 7.39-7.33 (m, 5H, Ph), 7.21 (br, 1H, NH), 5.20 (s, 2H, PhC*H*$_2$O), 4.21 [ddd, *J* = 6.3, 5.7 and 3.9 Hz, 1H, C*H*(OH)CONH], 4.16 (dd, *J* = 18.3 and 6.0 Hz, 1H, Glyα-*H*$_a$), 4.11 (dd, *J* = 18.3 and 4.5 Hz, 1H, Glyα-*H*$_b$), 3.60 (dd, *J* = 10.2 and 3.9 Hz, 1H, IC*H*$_a$H$_b$), 3.53 (dd, *J* = 10.2 and 6.3 Hz, 1H, ICH$_a$*H*$_b$), 3.18 (d, *J* = 5.7 Hz, 1H, OH). IR (KBr) 3352 (br), 1741, 1662, 1535, 1392, 1242, 1201, 1078, 957, 715 cm^{-1}. Anal. calcd for $C_{12}H_{14}INO_4$: C, 39.69; H, 3.89; N, 3.86. Found: C, 39.87; H, 3.97; N 3.84.

4.2.10. (*S*)-Benzyl 2-(2-hydroxy-3-phenylpropanamido)acetate (9e).

Compound **9e** was prepared from **7e** (6.13 g, 36.9 mmol) and glycine derivative **8** (14.9 g, 44.2 mmol) by the same procedure as described for the synthesis of **9a** and was purified by recrystallization from ethyl acetate (80 mL) to give **9e** as a colorless powder (8.57 g, 74%). Mp 106.7-107.9 °C. $[\alpha]_D^{27}$ –66.7 (*c* 1.00, EtOH). ^{1}H-NMR (300 MHz, $CDCl_3$): δ_H 7.77 (d, *J* = 9.3 Hz) and 7.44-7.22 (m) (10H, 2 × Ph), 7.28 (br t, 1H, NH), 5.19 (s, 2H, PhC*H*$_2$O), 4.37 [dd, *J* = 8.7 and 3.6 Hz, 1H, C*H*(OH)CONH], 4.13 (dd, *J* = 18.6 and 5.7 Hz) and 4.06 (dd, *J* = 18.6 and 5.7 Hz) (2H, Glyα-*H*), 3.27 (dd, *J* = 13.8 and 3.6 Hz, 1H, PhC*H*$_a$H$_b$), 2.87 (dd, *J* = 13.8 and 8.7 Hz, 1H, PhCH$_a$*H*$_b$), 2.61 (br, 1H, OH). IR (KBr) 3309 (br), 1759, 1660, 1541, 1456, 1385, 1362, 1186, 1092, 1036, 964, 746, 704, 588 cm^{-1}. Anal. calcd for $C_{18}H_{19}NO_4$: C, 68.99; H, 6.11; N, 4.47. Found: C, 68.75; H, 6.15; N, 4.35.

4.2.11. (*S*)-Benzyl 2-(2-hydroxyhexanamido)acetate (9f).

Compound **9f** was prepared from **7f** (2.03 g, 15.4 mmol) and glycine derivative **8** (6.22 g, 18.4 mmol) by the same procedure as described for the synthesis of **9a** and was purified by flash column chromatography (30 to 50% ethyl acetate in hexane) to give **9f** as a colorless oil (4.17 g, 97%). $[\alpha]_D^{31}$ -30.3 (*c* 1.03, EtOH). ^{1}H-NMR (300 MHz, $CDCl_3$): δ_H 7.40-7.28 (m, 5H, Ph), 7.17 (br t, 1H, NH), 5.17 (s, 2H, PhC*H*$_2$O), 4.14 [m, 1H, C*H*(OH)CONH], 4.13 (dd, *J* = 18.0 and 5.7 Hz, 1H, Glyα-*H*$_a$), 4.03 (dd, *J* = 18.0 and 5.7 Hz, 1H, Glyα-*H*$_b$), 3.43 (d, *J* = 5.7 Hz, 1H, OH), 1.89-1.77 [m, 1H, $CH_3(CH_2)_2$C*H*$_a$H$_b$], 1.70-1.57 [m, 1H, $CH_3(CH_2)_2$CH$_a$*H*$_b$], 1.47-1.26 (m, 4H, CH_3C*H*$_2$C*H*$_2$), 0.90 (t, *J* = 7.1 Hz, 3H, CH_3). IR (NaCl) 3398 (br), 2956, 1747, 1657, 1533, 1456, 1387, 1358, 1194, 1132, 962, 908 cm^{-1}. HRMS (FAB, glycerol): Calcd for $C_{15}H_{22}NO_4$ (MH^+)

280.1549; found 280.1550. Anal. calcd for $C_{15}H_{21}NO_4$: C, 64.50; H, 7.58; N 5.01. Found: C, 63.94; H, 7.58; N 4.93.

4.2.12. (*R*)-Benzyl 2-(2-hydroxyhexanamido)acetate (9g).

Compound **9g** was prepared from **7g** (2.20 g, 16.7 mmol) and glycine derivative **8** (6.76 g, 20.0 mmol) by the same procedure as described for the synthesis of **9a** and was purified by flash column chromatography (30% ethyl acetate in hexane) to give **9g** as a clear oil (4.50 g, 97%). $[\alpha]_D^{27}$ +30.7 (*c* 0.94, EtOH). ^{1}H-NMR (300 MHz, $CDCl_3$): δ_H 7.40-7.28 (m, 5H, Ph), 7.00 (br t, 1H, NH), 5.19 (s, 2H, PhC*H*$_2$O), 4.17 [m, 1H, C*H*(OH)CONH], 4.15 (dd, *J* = 14.4 and 5.4 Hz, 1H, Glyα-*H*$_a$), 4.08 (dd, *J* = 14.4 and 5.4 Hz, 1H, Glyα-*H*$_b$), 2.68 (d, *J* = 5.1 Hz, 1H, OH), 1.91-1.79 [m, 1H, $CH_3(CH_2)_2CH_aH_b$], 1.71-1.58 [m, 1H, $CH_3(CH_2)_2CH_aH_b$], 1.47-1.26 (m, 4H, $CH_3CH_2CH_2$), 0.91 (t, *J* = 6.9 Hz, 3H, CH_3). IR (NaCl) 3404 (br), 2956, 1749, 1652, 1533, 1456, 1387, 1358, 1192, 1132, 960, 908 cm^{-1}. HRMS (FAB, glycerol): Calcd for $C_{15}H_{22}NO_4$ (MH^+) 280.1549; found 280.1548. Anal. calcd for $C_{15}H_{21}NO_4 \cdot 0.2H_2O$: C, 63.68; H, 7.62; N, 4.95. Found: C, 63.77; H, 7.67; N 4.91.

4.2.13. (*S*)-Methyl 2-hydroxyhexanoate (11).

Thionyl chloride (2.00 g, 16.8 mmol) was added dropwise to methanol (20 mL) at 0˚C, and the mixture was allowed to warm to room temperature. After stirring at room temperature for 30 min, the hydroxy acid **7f** (1.39 g, 10.5 mmol) was added to the solution. After stirring at room temperature for 1.5 h, the mixture was concentrated *in vacuo*. The resulting residue was purified by flash column chromatography (40% ethyl acetate in hexane) to afford the methyl ester **11** as a colorless oil (1.30 g, 84%). $[\alpha]_D^{27}$ –5.50 (*c* 0.92, EtOH). Anal. calcd for $C_7H_{14}O_3$: C, 57.51; H, 9.65. Found: C, 56.78; H 9.47.

4.2.14. (*R*,*S*)-Benzyl 2-(*N*-benzyloxycarbonylamino)-4-(dichlorophosphoryl)butanoate (13).

Oxalyl chloride (1.41 g, 11.1 mmol) was added to a solution of racemic benzyl 2-(benzyloxycarbonylamino)-4-phosphonobutanoate (**12**) (2.01 g, 4.93 mmol) in dry CH_2Cl_2 (10 mL) in the presence of a catalytic amount (1 drop) of DMF. Evolution of gas (CO and CO_2) was observed immediately. The reaction was complete in 2 h at 30˚C. Volatiles were removed by vacuum evaporation, followed by removal of trace of HCl by flushing Ar flow to give the phosphonic dichloride **13** as an yellow oil. Compound **13** was used fresh for the next reaction without purification.

4.2.15. (2*R*, *S*)-Benzyl 2-(*N*-benzyloxycarbonylamino)-4-{(*S*)-1-[*N*-(benzyloxycarbonylmethyl)carbamoyl]propyl(phenyl)-(R_P,S_P)-phosphoryl}butanoate (14a).

Phenol (748 mg, 7.94 mmol) in dry CH_2Cl_2 (5 mL) was added to a solution of the phosphonic dichloride **13** (3.52 g, 7.94 mmol) in dry CH_2Cl_2 (20 mL), and the solution was cooled to −65 °C. Dry Et_3N (804 mg, 7.94 mmol) was added dropwise to the cold solution over 15 min, and the mixture was stirred at −65 °C for 30 min. The mixture was then allowed to warm to room temperature. After stirring at room temperature for 2 h, the consumption of **13** (δ_P 49.2) and the formation of monochloro monophenyl ester (δ_P 39.6) were confirmed with ^{31}P NMR. A solution of the alcohol **9a** (1.33 g, 5.30 mmol) in dry CH_2Cl_2 (5 mL) was then added to the mixture at 0 °C, followed by the addition of Et_3N (536 mg, 5.30 mmol) to initiate the reaction. The mixture was stirred at 0 °C for 30 min and was allowed to warm to room temperature. After stirring at room temperature for 4 h, the reaction mixture was concentrated *in vacuo* to remove CH_2Cl_2. To the resulting residue was added ethyl acetate (40 mL), and the insoluble salt

was removed by filtration. The crude product contained the desired product **14a** (49%, δ_P 28.8, 28.6, 28.4 and 28.2) along with the corresponding diphenyl phosphonate (13%, δ_P 24.5) and dialkyl phosphonate (38%, δ_P 39.5) as byproducts (^{31}P NMR). The filtrate was concentrated, and the crude product was purified by flash column chromatography (60% ethyl acetate in toluene) to afford the phosphonate diester **14a** as a mixture of four diastereoisomers (ca. 2:2:1:1) with respect to the chiral α-carbon and phosphorus (1.99 g, 53%, a pale yellow oil). ^{1}H-NMR (300 MHz, $CDCl_3$): δ_H 7.37-7.10 (m, 20H, 4 × Ph), 7.20-6.78 (4 × br t, 1H, amide NH), 5.79 (br d, *J* = ca. 7 Hz) and 5.59 (d, *J* = 7.6 Hz) (1H, carbamate NH), 5.23-5.04 (m, 6H, 3 × PhC*H*$_2$O), 4.85-4.75 [m, 1H, C*H*(OP)CONH], 4.55-4.37 [m, 1H, NH(CO)C*H*$(CH_2)_2$P], 4.15-3.95 and 3.80-3.65 (m, 2H, Glyα-*H*), 2.40-1.70 (m, 6H, CH_2CH_2P and CH_3CH_2), 0.93 (2 × t, *J* = 7.6 Hz) and 0.82 (2 × t, *J* = 7.6 Hz) (3H, CH_3). ^{31}P-NMR (121 MHz, $CDCl_3$): δ_P 28.76, 28.57, 28.40, 28.14. IR (NaCl) 3292 (br), 1743, 1684, 1529, 1313, 1201, 933, 738 cm^{-1}. Anal. calcd for $C_{38}H_{41}N_2O_{10}P$: C, 63.68; H, 5.77; N, 3.91. Found: C, 63.54; H, 5.78; N 3.74.

4.2.16. (2*R*,*S*)-Benzyl 2-(*N*-benzyloxycarbonylamino)-4-{[*N*-(benzyloxycarbonylmethyl) carbamoyl]methyl(phenyl)-(R_P,S_P)-phosphono}butanoate (14b).

Compound **14b** was synthesized from the phosphonic dichloride **13** (2.18 g, 4.91 mmol) and the alcohol **9b** (1.10 g, 4.91 mmol) by the same procedure as described for the synthesis of **14a** and was purified by flash column chromatography (45% acetone in hexane) to yield pure **14b** as a mixture of two diastereoisomers (1.31 g, 39%, a pale yellow oil). ^{1}H-NMR (300 MHz, $CDCl_3$): δ_H 7.37-7.13 (m, 20H, 4 × Ph), 7.04 (br, 1H, amide NH), 5.66 (br, 1H, carbamate NH), 5.18, 5.15 and 5.08 (3 × s, 2H, PhC*H*$_2$O), 4.66-4.46 [m, 1H, NH(CO)C*H*$(CH_2)_2$P] and 4.46 [dd, $^3J_{HP}$ = 15.2 Hz, *J* = 8.0 Hz, 3H, C*H*$_2$(OP)CONH], 4.12-3.92 (m, 2H, Glyα-*H*), 2.43-2.2 (m) and 2.15-1.80 (m) (4H, CH_2CH_2P). ^{31}P-NMR (121 MHz, $CDCl_3$): δ_P 29.12, 29.04. IR (NaCl) 3298 (br), 1745,

1685, 1535, 1255, 1200, 1065, 937, 741, 696 cm^{-1}. Anal. calcd for $C_{36}H_{37}N_2O_{10}P{\cdot}0.5H_2O$: C, 61.98; H, 5.49; N, 4.02. Found: C, 62.11; H, 5.44; N 4.02.

4.2.17. (2*R*,*S*)-Benzyl 2-(*N*-benzyloxycarbonylamino)-4-{(*R*)-1-[*N*-(benzyloxycarbonyl-methyl)carbamoyl]-2-chloroethyl(phenyl)-(R_P,S_P)-phosphono}butanoate (14c).

Compound **14C** was synthesized from the phosphonic dichloride **13** (3.26 g, 7.37 mmol) and the alcohol **9c** (1.33 g, 4.91 mmol) by the same procedure as described for the synthesis of **14a** and was purified by flash column chromatography (25% ethyl acetate in toluene) to afford pure **14c** as a mixture of four diastereoisomers (1.45 g, 40%, a pale yellow oil). ^{1}H-NMR (300 MHz, $CDCl_3$): δ_H 7.37-7.16 (m, 20H, 4 × Ph), 7.02 and 6.84 (2 × br t, 1H, amide NH), 5.81 (br d, J = ca. 6 Hz) and 5.56 (d, J = 7.6 Hz) (1H, carbamate NH), 5.21-5.00 [m, 7H, 3 × PhCH_2O and C*H*(OP)CONH], 4.68-4.34 [m, 1H, NH(CO)C*H*$(CH_2)_2$P], 4.16 (dd, $^3J_{HP}$ = 18.3 Hz and J = 6.3 Hz) and 3.96 (dd, $^3J_{HP}$ = 18.3 Hz and J = 4.8 Hz) (2H, Glyα-*H*), 4.10-3.73 and 3.56-3.48 (2 × m, 2H, and $ClCH_2$), 2.50-2.25 and 2.23-1.96 (2 × m, 4H, CH_2CH_2P). ^{31}P-NMR (121 MHz, $CDCl_3$): δ_P 29.86, 29.75, 29.51. IR (NaCl) 3292 (br), 1747, 1684, 1533, 1255, 1200, 1061, 939, 741, 696 cm^{-1}. Anal. calcd for $C_{37}H_{38}ClN_2O_{10}P$: C, 60.29; H, 5.20; N, 3.80. Found: C, 60.22; H, 5.33; N, 3.68.

4.2.18. (2*R*,*S*)-Benzyl 2-(*N*-benzyloxycarbonylamino)-4-{(*R*)-1-[*N*-(benzyloxycarbonyl-methyl)carbamoyl]-2-iodoethyl(phenyl)-(R_P,S_P)-phosphono}butanoate (14d).

Compound **14d** was prepared from the phosphonic dichloride **13** (2.38 g, 5.37 mmol) and the alcohol **9d** (1.30 g, 3.58 mmol) by the same procedure as described for the synthesis of **14a** and was purified by flash column chromatography (25% ethyl acetate in toluene) to afford pure **14d** as a mixture of the diastereoisomers (538 mg, 18%, a pale yellow oil). ^{1}H-NMR (300 MHz,

$CDCl_3$): δ_H 7.37-6.95 (m, 21H, 4 × Ph and amide NH), 5.81 (br, 1H, carbamate NH), 5.22-5.03 (m, 6H, 3 × PhC*H*$_2$O), 4.97-4.88 [m, 1H, C*H*(OP)CONH], 4.60-4.44 [m, 1H, NH(CO)C*H*$(CH_2)_2$P], 4.14 (dd, $^3J_{HP}$ = 18.3 Hz and J = 6.0 Hz) and 3.97 (dd, $^3J_{HP}$ = 18.3 Hz and J = 4.5 Hz) (2H, Glyα-*H*), 3.64-3.50 and 3.21-3.19 (2 × m, 2H, IC*H*$_2$), 2.47-1.96 (m, 4H, CH_2CH_2P). ^{31}P-NMR (121 MHz, $CDCl_3$): δ_P 29.59, 29.47, 29.32. IR (NaCl) 3288 (br), 1743, 1682, 1489, 1456, 1255, 1200, 1053, 939, 754, 696 cm^{-1}. Anal. calcd for $C_{37}H_{38}IN_2O_{10}P$: C, 53.63; H, 4.62; N, 3.38. Found: C, 54.04; H, 4.72; N, 3.33. HRMS (FAB, glycerol): Calcd for $C_{37}H_{39}IN_2O_{10}P$ (MH^+) 829.1387; found 829.1375.

4.2.19. (2*R*,*S*)-Benzyl 2-(*N*-benzyloxycarbonylamino)-4-{(*S*)-1-[*N*-(benzyloxycarbonyl-methyl) carbamoyl]-2-phenylethyl(phenyl)-(R_P,S_P)-phosphono}butanoate (14e).

Compound **14e** was prepared from the phosphonic dichloride **13** (2.18 g, 4.91 mmol) and the alcohol **9e** (1.54 g, 4.91 mmol) by the same procedure as described for the synthesis of **14a** and was purified by flash column chromatography (30% ethyl acetate in toluene) to afford pure **14e** as a mixture of four diastereoisomers (804 mg, 21%, a pale yellow oil). ^{1}H-NMR (300 MHz, $CDCl_3$): δ_H 7.39-6.97 (m, 25H, 5 × Ph), 6.57 (br t, 1H, amide NH), 5.57 (br d, 1H, carbamate NH), 5.28-5.04 [m, 7H, 3 × PhC*H*$_2$O and C*H*(OP)CONH], 4.41 and 4.28 [2 × m, 1H, NH(CO)C*H*$(CH_2)_2$P], 4.02-3.83 (m) and 3.62 (dd, J = 18.3 and 4.8 Hz) (2H, Glyα-*H*), 3.33 (d, J = 12.6 Hz) and 3.25-2.89 (m) (2H, PhC*H*$_2$), 2.29-1.76 (m), 1.75-1.50 (m), 1.49-1.27 (m) (4H, CH_2CH_2P). ^{31}P-NMR (121 MHz, $CDCl_3$): δ_P 29.98, 29.85, 28.99, 28.78. IR (NaCl) 3294 (br), 1745, 1687, 1529, 1456, 1387, 1346, 1254, 1200, 935, 752, 698, 580 cm^{-1}. Anal. calcd for $C_{43}H_{43}N_2O_{10}P$: C, 66.32; H, 5.57; N, 3.60. Found: C, 66.21; H, 5.55; N 3.62.

4.2.20. (2*R,S*)-Benzyl 2-(*N*-benzyloxycarbonylamino)-4-{(*S*)-1-[*N*-(benzyloxycarbonyl-methyl)carbamoyl]pentyl(phenyl)-(R_P,S_P)-phosphono}butanoate (14f).

Compound **14f** was prepared from the phosphonic dichloride **13** (3.26 g, 7.37 mmol) and the alcohol **9f** (1.37 g, 4.91 mmol) by the same procedure as described for the synthesis of **14a** and was purified by flash column chromatography (35% ethyl acetate in toluene) to afford pure **14f** as a mixture of four diastereoisomers (1.96 g, 54%, a pale yellow oil). ^{1}H-NMR (300 MHz, $CDCl_3$): δ_H 7.36-7.15 (m, 20H, 4 × Ph), 7.05-6.76 (4 × br t, 1H, amide NH), 5.83 (br d, J = 5.1 Hz) and 5.60 (d, J = 7.8 Hz) (1H, carbamate NH), 5.23-5.04 (m, 6H, 3 × PhCH_2O), 4.97-4.87 [m, 1H, C*H*(OP)CONH], 4.57-4.43 [m, 1H, NH(CO)C*H*$(CH_2)_2$P], 4.10 (dd, $^3J_{HP}$ = 18.3 Hz and J = 6.0 Hz), 3.96 (dd, $^3J_{HP}$ = 18.3 Hz and J = 4.8 Hz) and 4.15-3.77 (m) (2H, Glyα-*H*), 2.42-2.25 (m), 2.25-1.94 (m) and 1.93-1.63 (m) [6H, CH_2CH_2P and $CH_3(CH_2)_2CH_2$], 1.31 and 1.17 (2 × m, 4H, $CH_3CH_2CH_2$), 0.86 (m) and 0.78 (t, J = 7.1 Hz) (3H, CH_3). ^{31}P-NMR (121 MHz, $CDCl_3$): δ_P 28.80, 28.59, 28.37, 28.11. IR (NaCl) 3290 (br), 2956, 1747, 1684, 1591, 1533, 1489, 1456, 1248, 1201, 1047, 1005, 935 cm^{-1}. Anal. calcd for $C_{40}H_{45}N_2O_{10}P$: C, 64.51; H, 6.09; N, 3.76. Found: C, 64.57; H, 6.08; N 3.70.

4.2.21. (2*R,S*)-Benzyl 2-(*N*-benzyloxycarbonylamino)-4-{(*R*)-1-[*N*-(benzyloxycarbonyl-methyl)carbamoyl]pentyl(phenyl)-(R_P,S_P)-phosphono}butanoate (14g).

Compound **14g** was prepared from the phosphonic dichloride **13** (3.67 g, 8.28 mmol) and the alcohol **9g** (2.08 g, 7.45 mmol) by the same procedure as described for the synthesis of **14a** and was purified by flash column chromatography (40% ethyl acetate in toluene) to afford pure **14g** as a mixture of four diastereoisomers (1.57 g, 28%, a pale yellow oil). ^{1}H-NMR (300 MHz, $CDCl_3$): δ_H 7.37-7.12 (m, 20H, 4 × Ph), 7.05-6.76 (4 × br t, 1H, amide NH), 5.81 (br d, J = ca. 7

Hz) and 5.58 (d, *J* = 7.5 Hz) (1H, carbamate NH), 5.21-5.04 (m, 6H, 3 × PhC*H*$_2$O), 4.98-4.85 [m, 1H, C*H*(OP)CONH], 4.59-4.44 [m, 1H, NH(CO)C*H*(CH$_2$)$_2$P], 4.10 (dd, $^3J_{HP}$ = 18.0 Hz and *J* = 6.0 Hz), 3.97 (dd, $^3J_{HP}$ = 18.0 Hz and *J* = 5.4 Hz) and 4.15-3.93 (m) (2H, Glyα-*H*), 2.42-2.23 (m), 2.20-1.94 (m) and 1.93-1.63 (m) [6H, CH$_2$CH$_2$P and CH$_3$(CH$_2$)$_2$C*H*$_2$], 1.31 and 1.17 (2 × m, 4H, CH$_3$C*H*$_2$C*H*$_2$), 0.86 and 0.78 (2 × m, 3H, CH$_3$). ^{31}P-NMR (121 MHz, CDCl$_3$): δ_P 28.80, 28.59, 28.37, 28.11. IR (NaCl) 3290 (br), 2956, 1745, 1682, 1589, 1529, 1491, 1454, 1254, 1201, 1047, 1005, 935 cm^{-1}. Anal. calcd for $C_{40}H_{45}N_2O_{10}P\cdot 0.5H_2O$: C, 63.74; H, 6.15; N, 3.72. Found: C, 63.62; H, 6.16; N, 3.59. HRMS (FAB, glycerol): Calcd for $C_{40}H_{46}N_2O_{10}P$ (MH^+) 745.2890; found 745.2894.

4.2.22. (2*R*,*S*)-Benzyl 2-(*N*-benzyloxycarbonylamino)-4-{(*S*)-1-methoxycarbonylpentyl-(phenyl)-(R_P,S_P)-phosphono}butanoate (15).

Compound **15** was prepared from the phosphonic dichloride **13** (2.24 g, 5.07 mmol) and the alcohol **11** (494 mg, 3.38 mmol) by the same procedure as described for the synthesis of **14a** and was purified by flash column chromatography (40% ethyl acetate in hexane) to afford pure **15** as a mixture of four diastereoisomers (763 mg, 37%, a pale yellow oil). ^{1}H-NMR (300 MHz, CDCl$_3$): δ_H 7.38-7.12 (m, 15H, 3 × Ph), 5.61 (br) and 5.50 (br d, *J* = ca. 7 Hz) (1H, carbamate NH), 5.23-5.07 (m, 6H, 3 × PhC*H*$_2$O), 4.96-4.82 [m, 1H, C*H*(OP)CO$_2$Me], 4.57-4.38 [m, 1H, NH(CO)C*H*(CH$_2$)$_2$P], 3.68 and 3.60 (2 × s, 3H, CH$_3$O), 2.44-2.25 (m) and 2.25-1.90 (m) (4H, CH$_2$CH$_2$P), 1.90-1.75 (m) and 1.74-1.60 (m) [2H, CH$_3$(CH$_2$)$_2$C*H*$_2$], 1.32 and 1.14 (2 × m, 4H, CH$_3$C*H*$_2$C*H*$_2$), 0.88 (t, *J* = 6.3 Hz) and 0.77 (t, *J* = 6.6 Hz) (3H, CH$_3$). ^{31}P-NMR (121 MHz, CDCl$_3$): δ_P 29.61, 29.51, 28.05, 27.94. IR (NaCl) 3265 (br), 2956, 1753, 1724, 1591, 1493, 1454, 1344, 1255, 1205, 1053, 1026, 931, 756, 696 cm^{-1}. Anal. calcd for $C_{32}H_{38}NO_9P$: C, 62.84; H,

6.26; N, 2.29. Found: C, 62.71; H, 6.26; N, 2.26.

4.2.23. (2*R*,*S*)-Benzyl 2-(*N*-benzyloxycarbonylamino)-4-{(*S*)-1-[*N*-(benzyloxycarbonyl-methyl)carbamoyl]propyl(methyl)-(R_P,S_P)-phosphono}butanoate (16).

Compound **16** was prepared from the phosphonic dichloride **13** (3.18 g, 7.17 mmol), dry methanol (230 mg, 7.17 mmol) and the alcohol **9a** (1.20 g, 4.78 mmol) by the same procedure as described for the synthesis of **14a** and was purified by flash column chromatography (40% ethyl acetate in diethyl ether) to afford pure **16** as a mixture of four diastereoisomers (624 mg, 20%, a pale yellow oil). ^{1}H-NMR (300 MHz, $CDCl_3$): δ_H 7.40-7.19 (m, 15H, 3 × Ph), 7.11 (br, 1H, amide NH), 5.79 (br t, *J* = ca. 8 Hz, 1H, carbamate NH), 5.20-5.06 (m, 6H, 3 × PhC*H*$_2$O), 4.87-4.75 [m, 1H, C*H*(OP)CONH], 4.55-4.35 [m, 1H, NH(CO)C*H*$(CH_2)_2$P], 4.15-3.94 and 3.82-3.63 (2 × m, 2H, Glyα-*H*), 3.71 and 3.59 (2 × d, $^3J_{HP}$ = 11.1 Hz, 3H, CH_3O), 2.33-2.10 (m) and 2.10-1.71 (m) (6H, CH_2CH_2P and CH_3CH_2), 0.95 (t, *J* = 7.5 Hz, 3H, CH_3). ^{31}P-NMR (121 MHz, $CDCl_3$): δ_P 33.24, 33.05, 32.79. IR (NaCl) 3275 (br), 2952, 1743, 1724, 1682, 1531, 1456, 1244, 1215, 1196, 1043, 995, 739, 698 cm^{-1}. HRMS (FAB, glycerol): Calcd for $C_{33}H_{40}O_{10}N_2P$ (MH^+) 655.2421; found 655.2422.

4.2.24.

(2*R*,*S*)-2-Amino-4-{(*S*)-1-[*N*-(carboxymethyl)carbamoyl]propyl(phenyl)-(S_P*)-phosphono}-butanoic acid (2a-A).

(2*R*,*S*)-2-Amino-4-{(*S*)-1-[*N*-(carboxymethyl)carbamoyl]propyl(phenyl)-(R_P*)-phosphono}-butanoic acid (2a-B).

The phosphonate diester **14a** (227 mg, 0.317 mmol, a mixture of four diastereoisomers) was dissolved in a mixture of methanol (20 mL) and H_2O (4 mL) containing acetic acid (95.0 mg,

1.58 mmol). Hydrogen gas was passed through the solution in the presence of 5% Pd/C for 2 h at room temperature. The consumption of **14a** was monitored by TLC (ethyl acetate/hexane, 2:1, phosphomolybdate, R_f = 0.5), and the formation of product was confirmed by TLC (butan-1-ol/acetic acid/H_2O, 5:2:2, ninhydrin, R_f = 0.55). Pd/C was removed by filtration through Celite, and the filtrate was evaporated. The residue was purified by medium-pressure reversed-phase column chromatography on ODS-S-50B (Yamazen Co., Osaka, Japan). The column was eluted (6 mL/min) with a linear gradient of 40 to 60% methanol in H_2O. The UV-positive (254 nm) fractions containing the product (TLC, butan-1-ol/acetic acid/H_2O, 5:2:2, ninhydrin) were collected and lyophilized to afford the S_P* isomer (**2a-A**) (41.3 mg, 33%, slow-eluted isomer, eluted at 50% methanol) and the R_P* isomer (**2a-B**) (17.0 mg, 13%, fast-eluted isomer, eluted at 40% methanol) each as a colorless solid.

Compound **2a-A** (a mixture of two diastereoisomers). ^{1}H-NMR (300 MHz, D_2O): δ_H 7.47 (t, *J* = 8.0 and 7.8 Hz, 2H), 7.33 (t, *J* = 7.8 Hz, 1H) and 7.26 (d, *J* = 7.8 Hz, 2H) (Ph), 4.98-4.86 [m, 1H, C*H*(OP)CONH], 4.00 (d, *J* = 18.0 Hz, 1H, Glyα-H_a), 3.93 (d, *J* = 18.0 Hz, 1H, Glyα-H_b), 3.92-3.85 [m, 1H, $NH_2(CO_2H)C$*H*$(CH_2)_2P$], 2.48-2.19 (m, 4H, CH_2CH_2P), 1.90-1.76 (m, 2H, CH_3CH_2), 0.85 (t, *J* = 7.5 Hz, 3H, CH_3). ^{31}P-NMR (121 MHz, D_2O): δ_P 30.91. IR (KBr) 3435 (br), 2976, 1668, 1489, 1205, 1051, 1007, 943, 769, 690 cm^{-1}. Anal. calcd for $C_{16}H_{23}N_2O_8P \cdot 1.25H_2O$: C, 45.23; H, 6.05; N, 6.59. Found: C, 45.28; H, 5.90; N, 6.74. HRMS (FAB, glycerol): Calcd for $C_{16}H_{24}N_2O_8P$ (MH^+) 403.1270; found 403.1263.

Compound **2a-B** (a mixture of two diastereoisomers). ^{1}H-NMR (400 MHz, D_2O): δ_H 7.44 (t, *J* = 8.1 Hz), 7.32 (t, *J* = 8.1 Hz), 7.21 (d, *J* = 8.4 Hz), 6.98 (t, *J* = 7.6 Hz) and 6.91 (d, *J* = 8.0 Hz, 1H) (5H, Ph), 4.92-4.85 (m) and 4.61 (ddd, $^3J_{HP}$= 9.4 Hz, *J* = 4.9 and 4.9 Hz) [1H, C*H*(OP)CONH], 3.99 (s, 1H, Glyα-H_a), 3.78 (s, 1H, Glyα-H_b), 3.88 (t, *J* = 5.4 Hz) and 3.82 (dd,

J = 11.6 and 5.4 Hz) [1H, $NH_2(CO_2H)CH(CH_2)_2P$], 2.47-2.31 (m), 2.35-2.20 (m) and 2.20-2.05 (m) (4H, CH_2CH_2P), 1.95-1.76 (m, 2H, CH_3CH_2), 0.96 and 0.93 (2 × t, *J* = 7.4 Hz, 3H, CH_3). ^{31}P-NMR (121 MHz, D_2O): δ_P 30.71. IR (KBr) 3429 (br), 2978, 1668, 1489, 1202, 1051, 1009, 941, 769, 690 cm^{-1}. HRMS (FAB, glycerol): Calcd for $C_{16}H_{24}N_2O_8P$ (MH^+) 403.1270; found 403.1268.

4.2.25. (2*R*,*S*)-2-Amino-4-{[*N*-(carboxymethyl)carbamoyl]methyl(phenyl)-(R_P*,S_P*)-phosphono}butanoic acid (2b).

Compound **2b** was prepared from **14b** (500 mg, 0.727 mmol, a mixture of two diastereoisomers) by the same procedure as described for the synthesis of **2a** and was purified by medium-pressure reversed-phase column chromatography (ODS-S-50B) with a linear gradient of 5 to 35% methanol in H_2O. The UV-positive (254 nm) fractions containing the product (TLC, butan-1-ol/acetic acid/H_2O, 5:2:2, ninhydrin) eluted at 30% methanol were collected and lyophilized to give compound **2b** as a colorless solid (79.2 mg, 29%) (a mixture of four stereoisomers). ^{1}H-NMR (300 MHz, D_2O): δ_H 7.47 (dd, *J* = 7.8 and 7.4 Hz, 2H), 7.33 (t, *J* = 7.4 Hz, 1H) and 7.26 (d, *J* = 7.8 Hz, 2H) (Ph), 4.80-4.68 [m, 2H, CH_2(OP)CONH], 3.97 (s, 2H, Glyα-*H*), 3.91 [br t, 1H, $NH_2(CO_2H)CH(CH_2)_2P$], 2.48-2.22 (m, 4H, CH_2CH_2P). ^{31}P-NMR (121 MHz, D_2O): δ_P 31.73, 31.70. IR (KBr) 3419 (br), 2943, 1670, 1489, 1228, 1201, 1070, 1024, 941, 769, 690 cm^{-1}. Anal. calcd for $C_{14}H_{19}N_2O_8P \cdot 1.5H_2O$: C, 41.90; H, 5.53; N, 6.98. Found: C, 41.97; H, 5.50; N 7.10. HRMS (FAB, glycerol): Calcd for $C_{14}H_{20}N_2O_8P$ (MH^+) 375.0957; found 375.0959.

4.2.26.

(2*R*,*S*)-2-Amino-4-{(*R*)-1-[*N*-(carboxymethyl)carbamoyl]-2-chloroethyl(phenyl)-(S_P*)-

phosphono}butanoic acid (2c-A).

(2*R*,*S*)-2-Amino-4-{(*R*)-1-[*N*-(carboxymethyl)carbamoyl]-2-chloroethyl(phenyl)-(R_P*)-phosphono}butanoic acid (2c-B).

Compound **2c** was prepared from **14c** (379 mg, 0.515 mmol) by the same procedure as described for the synthesis of **2a** and was purified by medium-pressure reversed-phase column chromatography (ODS-S-50B) with a linear gradient of 30 to 50% methanol in H_2O. The UV-positive (254 nm) fractions containing the product (TLC, butan-1-ol/acetic acid/H_2O, 5:2:2, ninhydrin) were collected and lyophilized to afford the S_P* isomer (**2c-A**) (72.9 mg, 34%, slow-eluted isomer, eluted at 50% methanol) and the R_P* isomer (**2c-B**) (28.9 mg, 13%, fast-eluted isomer, eluted at 35% methanol) each as a colorless solid.

Compound **2c-A** (a mixture of two diastereoisomers). ^{1}H-NMR (400 MHz, D_2O): δ_H 7.47 (t, *J* = 8.0 Hz), 7.34 (t, *J* = 8.0 Hz), 7.29 (d, *J* = 8.0 Hz), 6.98 (t, *J* = 7.6 Hz) and 6.91 (d, *J* = 8.4 Hz) (5H, Ph), 5.29 and 4.96 [2 × m, 1H, C*H*(OP)CONH], 4.10-3.89 (m, 4H, $ClCH_2$ and Glyα-*H*), 3.84-3.78 [m, 1H, $NH_2(CO_2H)CH(CH_2)_2P$], 2.53-2.11 (m, 4H, CH_2CH_2P). ^{31}P-NMR (121 MHz, D_2O): δ_P 31.42. IR (KBr) 3427 (br), 2944, 1674, 1491, 1228, 1203, 1070, 947, 768, 690 cm^{-1}. Anal. calcd for $C_{15}H_{20}ClN_2O_8P \cdot 0.43H_2O$: C, 41.85; H, 4.88; N, 6.51. Found: C, 42.12; H, 4.97; N, 6.21. HRMS (FAB, glycerol): Calcd for $C_{15}H_{21}{}^{35}ClN_2O_8P$ (MH^+) 423.0724; found 423.0722. Calcd for $C_{15}H_{21}{}^{37}ClN_2O_8P$ (MH^+) 425.0695; found 425.0698.

Compound **2c-B** (a mixture of two diastereoisomers). ^{1}H-NMR (400 MHz, D_2O): δ_H 7.45 (t, *J* = 8.4 Hz), 7.32 (t, *J* = 8.4 Hz), 7.24 (d, *J* = 8.4 Hz), 6.98 (t, *J* = 7.6 Hz) and 6.91 (d, *J* = 8.4 Hz) (5H, Ph), 5.24-5.18 (m) and 4.96 (m) [1H, C*H*(OP)CONH], 4.04-3.79 [m, 5H, $ClCH_2$, Glyα-*H* and $NH_2(CO_2H)CH(CH_2)_2P$], 2.54-2.09 (m, 4H, CH_2CH_2P). ^{31}P-NMR (121 MHz, D_2O): δ_P 31.31; IR (KBr) 3427 (br), 2945, 1674, 1491, 1228, 1203, 1070, 947, 768, 690 cm^{-1}. Anal. calcd for $C_{15}H_{20}ClN_2O_8P \cdot H_2O$: C, 40.87; H, 5.03; N, 6.36. Found: C, 40.73; H, 4.89; N, 6.35. HRMS

(FAB, glycerol): Calcd for $C_{15}H_{21}{}^{35}ClN_2O_8P$ (MH^+) 423.0724; found 423.0732. Calcd for $C_{15}H_{21}{}^{37}ClN_2O_8P$ (MH^+) 425.0695; found 425.0686.

4.2.27.

(2*R*,*S*)-2-Amino-4-{(*R*)-1-[*N*-(carboxymethyl)carbamoyl]-2-iodoethyl(phenyl)-(S_P*)-phosphono}butanoic acid (2d-A).

To a solution of the phosphonate diester **14d** (257 mg, 0.310 mmol) in dry nitromethane (4 mL) was added anisole (403 mg, 3.72 mmol) and aluminum chloride (248 mg, 1.86 mmol) at room temperature. After stirring at room temperature for 1 h, the reaction mixture was diluted with 1 N HCl (20 mL) and washed with diethyl ether (3 × 30 mL) to remove anisole and nitromethane. The aqueous layer was concentrated *in vacuo* to ca. 10 mL (do not dry up) and was directly applied to the medium-pressure reversed-phase column chromatography on ODS-S-50B. The column was eluted (6 mL/min) with a linear gradient of 30 to 50% methanol in H_2O. The UV-positive (254 nm) fractions containing the product (TLC, butan-1-ol/acetic acid/H_2O, 5:2:2, ninhydrin) were collected and lyophilized to afford the S_P* isomer (**2d-A**) (35.0 mg, 22%, slow-eluted isomer, eluted at 50% methanol) as colorless solid and the R_P* isomer (**2s-B**) (trace, detected by TLC, fast-eluted isomer, eluted at 40% methanol).

Compound **2c-A** (a mixture of two diastereoisomers). ^{1}H-NMR (400 MHz, D_2O): δ_H 7.48 (t, *J* = 7.8 Hz), 7.34 (t, *J* = 7.8 Hz), 7.31 (d, *J* = 7.8 Hz), 6.98 (t, *J* = 7.6 Hz) and 6.91 (d, *J* = 7.6 Hz, 1H) (5H, Ph), 5.07 (ddd, $^3J_{HP}$= 8.0 Hz, *J* = 4.0 and 4.0 Hz) and 4.73-4.67 (m) [1H, C*H*(OP)CONH], 4.12-3.91 [m, 3H, NH(CO)C*H*(CH_2)$_2$P and Glyα-*H*], 3.66-3.38 (m, 2H, ICH_2), 2.52-2.11 (m, 4H, CH_2CH_2P). ^{31}P-NMR (121 MHz, D_2O): δ_P 31.19. IR (KBr) 3435 (br), 2945, 1668, 1633, 1489, 1228, 1201, 1053, 497, 768, 690 cm^{-1}. Anal. calcd for $C_{15}H_{20}IN_2O_8P{\cdot}H_2O$: C, 33.85; H, 4.17; N, 5.26. Found: C, 33.89; H, 3.97; N 5.25. HRMS (FAB, glycerol): Calcd for

$C_{15}H_{21}IN_2O_8P$ (MH^+) 515.0080; found 515.0068.

4.2.28.
(2*R*,*S*)-2-Amino-4-{(*S*)-1-[*N*-(carboxymethyl)carbamoyl]-2-phenylethyl(phenyl)-(S_P*)-phosphono}butanoic acid (2e-A).

(2*R*,*S*)-2-Amino-4-{(*S*)-1-[*N*-(carboxymethyl)carbamoyl]-2-phenylethyl(phenyl)-(R_P*)-phosphono}butanoic acid (2e-B).

Compound **2e** was prepared from **14e** (253 mg, 0.325 mmol)) by the same procedure as described for the synthesis of **2a** and was purified by medium-pressure reversed-phase column chromatography (ODS-S-50B) with a linear gradient of 40 to 60% methanol in H_2O. The UV-positive (254 nm) fractions containing the product (TLC, butan-1-ol/acetic acid/H_2O, 5:2:2, ninhydrin) were collected and lyophilized to afford the S_P* isomer (**2c-A**) (59.3 mg, 39%, slow-eluted isomer, eluted at 60% methanol) and the R_P* isomer (**2c-B**) (33.8 mg, 22%, fast-eluted isomer, eluted at 55% methanol) each as a colorless solid.

Compound **2e-A** (a mixture of two diastereoisomers). ^{1}H-NMR (300 MHz, D_2O): δ_H 7.40 (t, *J* = 7.8 Hz, 2H), 7.36-7.25 (m, 4H), 7.23-7.17 (m, 2H) and 7.03 (d, *J* = 8.1 Hz, 2H) (2 × Ph), 5.12 [m, 1H, C*H*(OP)CONH], 3.88 (d, *J* = 17.4 Hz) and 3.80 (d, *J* = 17.4 Hz) (2H, Glyα-*H*), 3.80-3.75 [m, 1H, $NH_2(CO_2H)CH(CH_2)_2P$], 3.18 (dd, *J* = 13.8 and 4.2 Hz) and 3.10 (dd, *J* = 13.8 and 8.1 Hz) (2H, PhCH_2), 2.28-2.07 (m, 4H, CH_2CH_2P). ^{31}P-NMR (121 MHz, D_2O): δ_P 30.79. IR (KBr) 3446 (br), 3064, 1668, 1491, 1252, 1211, 1053, 1007, 947, 758, 690 cm^{-1}. Anal. calcd for $C_{21}H_{25}N_2O_8P \cdot 0.5H_2O$: C, 53.28; H, 5.54; N, 5.92. Found: C, 53.45; H, 5.44; N, 5.99. HRMS (FAB, glycerol): Calcd for $C_{21}H_{26}N_2O_8P$ (MH^+) 465.1427; found 465.1428.

Compound **2e-B** (a mixture of two diastereoisomers). ^{1}H-NMR (300 MHz, D_2O): δ_H 7.45-7.24 (m, 8H) and 7.14 (d, *J* = 8.4 Hz, 2H) (2 × Ph), 5.12 [m, 1H, C*H*(OP)CONH], 3.71 (s,

2H, Glyα-*H*), 3.74-3.64 [m, 1H, $NH_2(CO_2H)CH(CH_2)_2P$], 3.25 (dd, *J* = 14.4 and 3.6 Hz) and 3.11 (dd, *J* = 14.4 and 8.4 Hz) (2H, $PhCH_2$), 2.18-1.81 (m, 4H, CH_2CH_2P). ^{31}P-NMR (121 MHz, D_2O): δ_P 30.64. IR (KBr) 3419 (br), 3064, 1668, 1491, 1456, 1404, 1340, 1250, 1203, 1055, 1009, 964, 941, 764, 700 cm^{-1}. Anal. calcd for $C_{21}H_{25}N_2O_8P \cdot 1.5H_2O$: C, 51.32; H, 5.74; N, 5.70. Found: C, 51.26; H, 5.86; N, 5.66. HRMS (FAB, glycerol): Calcd for $C_{21}H_{26}N_2O_8P$ (MH^+) 465.1427; found 465.1436.

4.2.29.

(2*R*,*S*)-2-Amino-4-{(*S*)-1-[*N*-(carboxymethyl)carbamoyl]pentyl(phenyl)-(S_P*)-phosphono}-butanoic acid (2f-A).

(2*R*,*S*)-2-Amino-4-{(*S*)-1-[*N*-(carboxymethyl)carbamoyl]pentyl(phenyl)-(R_P*)-phosphono}-butanoic acid (2f-B).

Compound **2f** was prepared from **14f** (447 mg, 0.601 mmol) by the same procedure as described for the synthesis of **2a** and was purified by medium-pressure reversed-phase column chromatography (ODS-S-50B) with a linear gradient of 20 to 60% methanol in H_2O. The UV-positive (254 nm) fractions containing the product (TLC, butan-1-ol/acetic acid/H_2O, 5:2:2, ninhydrin) were collected and lyophilized to afford the S_P* isomer (**2f-A**) (157 mg, 56%, slow-eluted isomer, eluted at 60% methanol) and the R_P* isomer (**2f-B**) (71.0 mg, 25%, fast-eluted isomer, eluted at 60% methanol) each as a colorless solid.

Compound **2f-A** (a mixture of two diastereoisomers). ^{1}H-NMR (300 MHz, D_2O): δ_H 7.47 (t, *J* = 7.8 Hz, 2H), 7.33 (t, *J* = 7.8 Hz, 1H) and 7.26 (d, *J* = 7.8 Hz, 2H) (Ph), 4.97-4.87 [m, 1H, C*H*(OP)CONH], 4.02 (d, *J* = 18.0 Hz, 1H, Glyα-H_a), 3.95 (d, *J* = 18.0 Hz, 1H, Glyα-H_b), 3.93-3.86 [m, 1H, $NH_2(CO_2H)CH(CH_2)_2P$], 2.45-2.18 (m, 4H, CH_2CH_2P), 1.86-1.65 [m, 2H, $CH_3(CH_2)_2CH_2$], 1.24-1.11 (m, 4H, $CH_3CH_2CH_2$), 0.78 (t, *J* = 6.3 Hz, 3H, CH_3). ^{31}P-NMR (121

MHz, D_2O): δ_P 30.90. IR (KBr) 3435 (br), 1664, 1491, 1207, 1041, 1007, 941, 766, 690 cm^{-1}. Anal. calcd for $C_{18}H_{27}N_2O_8P \cdot H_2O$: C, 48.21; H, 6.52; N, 6.25. Found: C, 48.39; H, 6.30; N, 6.27. HRMS (FAB, glycerol): Calcd for $C_{18}H_{28}N_2O_8P$ (MH^+) 431.1583; found 431.1571.

Compound **2f-B** (a mixture of two diastereoisomers). ^{1}H-NMR (300 MHz, D_2O): δ_H 7.44 (dd, *J* = 7.8 and *J* = 7.4 Hz, 2H), 7.31 (t, *J* = 7.4 Hz, 1H) and 7.21 (d, *J* = 7.8 Hz, 2H) (Ph), 4.97-4.87 [m, 1H, C*H*(OP)CONH], 3.88 [t, *J* = 5 Hz, 1H, $NH_2(CO_2H)CH(CH_2)_2P$], 3.80 (s, 2H, Glyα-*H*), 2.44-2.21 (m, 4H, CH_2CH_2P), 1.93-1.83 [m, 2H, $CH_3(CH_2)_2CH_2$], 1.42-1.26 (m, 4H, $CH_3CH_2CH_2$), 0.87 (t, *J* = 6.9 Hz, 3H, CH_3). ^{31}P-NMR (121 MHz, D_2O): δ_P 30.60 and 30.56. IR (KBr) 3431 (br), 2958, 1664, 1491, 1205, 1047, 1018, 937, 764, 690 cm^{-1}. Anal. calcd for $C_{18}H_{27}N_2O_8P \cdot 1.5H_2O$: C, 47.26; H, 6.61; N, 6.12. Found: C, 47.35; H, 6.35; N, 6.17. HRMS (FAB, glycerol): Calcd for $C_{18}H_{28}N_2O_8P$ (MH^+) 431.1583; found 431.1583.

4.2.30.
(2*R*,*S*)-2-Amino-4-{(*R*)-1-[*N*-(carboxymethyl)carbamoyl]pentyl(phenyl)-(R_P*)-phosphono}-butanoic acid (2g-A).
(2*R*,*S*)-2-Amino-4-{(*R*)-1-[*N*-(carboxymethyl)carbamoyl]pentyl(phenyl)-(S_P*)-phosphono}-butanoic acid (2g-B).

Compound **2g** was prepared from **14g** (300 mg, 0.403 mmol) by the same procedure as described for the synthesis of **2a** and was purified by medium-pressure reversed-phase column chromatography (ODS-S-50B) with a linear gradient of 40 to 60% methanol in H_2O. The UV-positive (254 nm) fractions containing the product (TLC, butan-1-ol/acetic acid/H_2O, 5:2:2, ninhydrin) were collected and lyophilized to afford the R_P* isomer (**2g-A**) (97.3 mg, 52%, slow-eluted isomer, eluted at 60% methanol) and the S_P* isomer (**2g-B**) (46.9 mg, 25%, fast-eluted isomer, eluted at 60% methanol) each as a colorless solid.

Compound **2g-A** (a mixture of two diastereoisomers). ^{1}H-NMR (300 MHz, D_2O): δ_H 7.47 (dd, *J* = 8.1 and 7.5 Hz, 2H), 7.33 (t, *J* = 7.5 Hz, 1H) and 7.26 (d, *J* = 8.1 Hz, 2H) (Ph), 4.97-4.87 [m, 1H, C*H*(OP)CONH], 4.01 (d, *J* = 17.7 Hz, 1H, Glyα-H_a), 3.94 (d, *J* = 17.7 Hz, 1H, Glyα-H_b), 3.93-3.85 [m, 1H, $NH_2(CO_2H)CH(CH_2)_2P$], 2.47-2.18 (m, 4H, CH_2CH_2P), 1.85-1.66 [m, 2H, $CH_3(CH_2)_2CH_2$], 1.24-1.11 (m, 4H, $CH_3CH_2CH_2$), 0.78 (t, *J* = 6.0 Hz, 3H, CH_3). ^{31}P-NMR (121 MHz, D_2O): δ_P 30.85. IR (KBr) 3419 (br), 2956, 1668, 1491, 1207, 1039, 1007, 941, 767, 690 cm^{-1}. Anal. calcd for $C_{18}H_{27}N_2O_8P\cdot 0.5H_2O$: C, 49.20; H, 6.42; N, 6.38. Found: C, 49.12; H, 6.33; N, 6.42. HRMS (FAB, glycerol): Calcd for $C_{18}H_{28}N_2O_8P$ (MH^+) 431.1583; found 431.1593.

Compound **2g-B** (a mixture of two diastereoisomers). ^{1}H-NMR (300 MHz, D_2O): δ_H 7.44 (dd, *J* = 7.8 and 7.4 Hz, 2H), 7.31 (t, *J* = 7.4 Hz, 1H) and 7.21 (d, *J* = 7.8 Hz, 2H) (Ph), 4.97-4.87 [m, 1H, CH(OP)CONH], 3.89 [t, *J* = 5 Hz, 1H, $NH_2(CO_2H)CH(CH_2)_2P$], 3.82 (s, 2H, Glyα-*H*), 2.44-2.21 (m, 4H, CH_2CH_2P), 1.93-1.83 [m, 2H, $CH_3(CH_2)_2CH_2$], 1.42-1.25 (m, 4H, $CH_3CH_2CH_2$), 0.87 (t, *J* = 6.9 Hz, 3H, CH_3). ^{31}P-NMR (121 MHz, D_2O): δ_P 30.56 and 30.53. IR (KBr) 3429 (br), 2958, 1668, 1491, 1203, 1045, 1009, 939, 766, 690 cm^{-1}. HRMS (FAB, glycerol): Calcd for $C_{18}H_{28}N_2O_8P$ (MH^+) 431.1583; found 431.1583.

4.2.31. (2*R*,*S*)-2-Amino-4-{(*S*)-1-methoxycarbonylpentyl(phenyl)-(S_P*)-phosphono}butanoic acid (3-A).

(2*R*,*S*)-2-Amino-4-{(*S*)-1-methoxycarbonylpentyl(phenyl)-(R_P*)-phosphono}butanoic acid (3-B).

Compound **3** was prepared from **15** (479 mg, 0.784 mmol) by the same procedure as described for the synthesis of **2a** and was purified by medium-pressure reversed-phase column chromatography (ODS-S-50B) with a linear gradient of 40 to 60% methanol in H_2O. The

UV-positive (254 nm) fractions containing the product (TLC, butan-1-ol/acetic acid/H_2O, 5:2:2, ninhydrin) were collected and lyophilized to afford the S_P* isomer (**3-A**) (140 mg, 46%, slow-eluted isomer, eluted at 60% methanol) and the R_P* isomer (**3-B**) (67.3 mg, 22%, fast-eluted isomer, eluted at 60% methanol) each as a colorless solid.

Compound **3-A** (a mixture of two diastereoisomers). ^{1}H-NMR (300 MHz, D_2O): δ_H 7.46 (dd, *J* = 7.5 and 7.5 Hz, 2H), 7.32 (t, *J* = 7.5 Hz, 1H) and 7.24 (d, *J* = 7.5 Hz, 2H) (Ph), 5.00 [m, 1H, C*H*(OP)CO_2Me], 3.85 [m, 1H, $NH_2(CO_2H)$C*H*$(CH_2)_2$P], 3.79 (s, 3H, CH_3O), 2.45-2.17 (m, 4H, CH_2CH_2P), 1.84-1.68 [m, 2H, $CH_3(CH_2)_2$C*H*$_2$], 1.24-1.07 (m, 4H, CH_3C*H*$_2$C*H*$_2$), 0.78 (t, *J* = 6.6 Hz, 3H, CH_3). ^{31}P-NMR (121 MHz, D_2O): δ_P 31.36. IR (KBr) 3467 (br), 2958, 1747, 1631, 1592, 1491, 1406 ,1348, 1288, 1207, 1080, 1024, 935, 769, 690, 542 cm^{-1}. Anal. calcd for $C_{17}H_{26}NO_7P\cdot 0.5H_2O$: C, 51.51; H, 6.87; N, 3.53. Found: C, 51.40; H, 6.88; N. 3.64. HRMS (FAB, glycerol): Calcd for $C_{17}H_{27}NO_7P$ (MH^+) 388.1525; found 388.1519.

Compound **3-B** (a mixture of two diastereoisomers). ^{1}H-NMR (300 MHz, D_2O): δ_H 7.46 (dd, *J* = 7.8 and 7.8 Hz, 2H), 7.32 (t, *J* = 7.8 Hz, 1H) and 7.23 (d, *J* = 7.8 Hz, 2H) (Ph), 5.06 [m, 1H, C*H*(OP)CO_2Me], 3.83 [m, 1H, $NH_2(CO_2H)$C*H*$(CH_2)_2$P], 3.69 and 3.68 (2 × s, 3H, CH_3O), 2.45-2.14 (m, 4H, CH_2CH_2P), 1.96-1.83 [m, 2H, $CH_3(CH_2)_2$C*H*$_2$], 1.45-1.24 (m, 4H, CH_3C*H*$_2$C*H*$_2$), 0.87 (t, *J* = 6.9 Hz, 3H, CH_3). ^{31}P-NMR (121 MHz, D_2O): δ_P 30.84. IR (KBr) 3467 (br), 2954, 1749, 1616, 1592, 1492, 1410, 1348 ,1261 ,1211, 1085, 1055, 1028, 933, 766, 692, 555 cm^{-1}. Anal. calcd for $C_{17}H_{26}NO_7P\cdot 0.5H_2O$: C, 51.51; H, 6.87; N, 3.53. Found: C, 51.31; H, 6.66; N 3.59. HRMS (FAB, glycerol): Calcd for $C_{17}H_{27}NO_7P$ (MH^+) 388.1525; found 388.1530.

4.2.32.
(2*R*,*S*)-2-Amino-4-{(*S*)-1-[*N*-(carboxymethyl)carbamoyl]propyl(methyl)-(S_P*)-phosphono}-

butanoic acid (4-A).

(2*R*,*S*)-2-Amino-4-{(*S*)-1-[*N*-(carboxymethyl)carbamoyl]propyl(methyl)-(R_P*)-phosphono}-butanoic acid (4-B).

Compound **4** was prepared from **16** (324 mg, 0.495 mmol) by the same procedure as described for the synthesis of **2a** and was purified by medium-pressure reversed-phase column chromatography (ODS-S-50B) with a linear gradient of 5 to 55% methanol in H_2O. The ninhydrin-positive fractions (TLC, butan-1-ol/acetic acid/H_2O, 5:2:2) were collected and lyophilized to afford the S_P* isomer (**4-A**) (68.1 mg, 41%, slow-eluted isomer, eluted at 30% methanol) and the R_P* isomer (**4-B**) (21.5 mg, 13%, fast-eluted isomer, eluted at 10% methanol) each as a colorless solid.

Compound **4-A** (a mixture of two diastereoisomers). ^{1}H-NMR (300 MHz, D_2O): δ_H 4.92-4.79 [m, 1H, C*H*(OP)CONH], 4.05 (d, *J* = 18.0 Hz, 1H, Glyα-H_a), 3.98 (d, *J* = 18.0 Hz, 1H, Glyα-H_b), 3.90-3.82 [m, 1H, $NH_2(CO_2H)CH(CH_2)_2P$], 3.82 (d, $^3J_{HP}$ = 12.0 Hz, 3H, CH_3OP), 2.27-2.01 (m, 4H, CH_2CH_2P), 1.93 (dq, *J* = 7.2 and 6.6 Hz, 2H, CH_3CH_2), 0.98 (t, *J* = 7.2 Hz, 3H, CH_3). ^{31}P-NMR (121 MHz, D_2O): δ_P 35.09. IR (KBr) 3419 (br), 2976, 1732, 1664, 1539, 1448, 1406, 1346, 1219, 1043, 1001, 893, 827, 775 cm^{-1}. Anal. calcd for $C_{11}H_{21}N_2O_8P\cdot 0.8H_2O$: C, 37.25; H, 6.42; N, 7.90. Found: C, 37.21; H, 6.55; N, 7.98. HRMS (FAB, glycerol): Calcd for $C_{11}H_{22}N_2O_8P$ (MH^+) 341.1114; found 341.1114.

Compound **4-B** (a mixture of two diastereoisomers). ^{1}H-NMR (300 MHz, D_2O): δ_H 4.90-4.78 [m, 1H, C*H*(OP)CONH], 4.04 (d, *J* = 17.7 Hz, 1H, Glyα-H_a), 3.98 (d, *J* = 17.7 Hz, 1H, Glyα-H_b), 3.89-3.83 [m, 1H, $NH_2(CO_2H)CH(CH_2)_2P$], 3.79 (d, $^3J_{HP}$ = 11.1 Hz, 3H, CH_3OP), 2.26-2.02 (m, 4H, CH_2CH_2P), 1.91 (dq, *J* = 7.5 and 6.6 Hz, 2H, CH_3CH_2), 0.97 (t, *J* = 7.5 Hz, 3H, CH_3). ^{31}P-NMR (121 MHz, D_2O): δ_P 35.04. IR (KBr) 3428 (br), 2978, 1728, 1658, 1540,

1448, 1406, 1348, 1223, 1040, 1003, 895, 827, 773 cm^{-1}. HRMS (FAB, glycerol): Calcd for $C_{11}H_{22}N_2O_8P$ (MH^+) 341.1114; found 341.1116.

4.2.33. (*S*)- Benzyl 2-(benzyloxycarbonyl)but-3-enoate (18).[55]

Detailed synthetic procedure and the spectral data are written in Supplementary data.

4.2.34. (*S*)-Benzyl 2-(*N*-benzyloxycarbonylamino)-4-[2-(*tert*-butoxycarbonyl)-ethylhydroxyphosphinyl]butanoate (20).

To a solution of the (*S*)-vinylglycine derivative **18** (1.30 g, 4.00 mmol) and ammonium hypophosphite ($NH_4^+H_2PO_2^-$, 830 mg, 10.0 mmol) in methanol (9.3 mL) was added triethylborane (4.00 mL of 1 M solution in THF, 4.00 mmol) at room temperature. The mixture was stirred vigorously open to air. After stirring for 5 h, the reaction was concentrated *in vacuo* to remove the solvent. The residual oil was partitioned between 10% aqueous $KHSO_4$ (30 mL) and ethyl acetate (40 mL). The aqueous layer was extracted with ethyl acetate (2 × 40 mL), and the combined the organic layers were dried over anhydrous Na_2SO_4. The solvent was removed *in vacuo* to afford the phosphinic acid **19** (1.51 g, 97%) as a colorless oil. TLC (CH_2Cl_2/methanol/acetic acid, 80:20:1, cerium sulfate) R_f = 0.4, single spot. ^{1}H-NMR (300 MHz, $CDCl_3$): δ_H 11.5 (br s, 1H, P-OH), 7.41-7.19 (m, 10H, 2 × Ph), 6.95 (d, $^1J_{HP}$ = 533 Hz, 1H, P-H), 5.14 and 5.07 (2 × s, 4H, 2 × PhC*H*$_2$O), 4.42 (br t, 1H, NH), 2.05-1.53 (m, 4H, CH_2CH_2P). ^{31}P-NMR (121 MHz, $CDCl_3$): δ_P 36.39. The crude phosphinic acid **19** was used for the next reaction without further purification.

Compound **19** was dissolved in dry CH_2Cl_2 (15 mL) under argon. *N*,*O*-Bis (trimethylsilyl)acetamide (2.50 g, 12.0 mmol) and *tert*-butyl acrylate (564 mg, 4.40 mmol) was added successively to the solution, and the mixture was stirred at room temperature for 22 h. The

reaction mixture was cooled to 0 °C and quenched with ethanol (3 mL) plus two drops of trifluoroacetic acid. The mixture was allowed to warm to room temperature. After stirring at room temperature for 30 min, the solvent was removed *in vacuo*. Ethyl acetate (70 mL) was added to the residue, and the resulting solution was washed successively with 10% aqueous $KHSO_4$ and sat. NaCl, and was dried over anhydrous Na_2SO_4. The solvent was removed *in vacuo* to afford the crude dialkylphosphinic acid **20** (2.43 g) as a colorless oil. TLC (CH_2Cl_2/methanol/acetic acid, 80:20:1, cerium sulfate) R_f = 0.6 (ca. 80% purity). ^{1}H-NMR (300 MHz, $CDCl_3$): δ_H 8.22 (br, 1H, P-OH), 7.38-7.24 (m, 10H, 2 × Ph), 5.15 and 5.08 (2 × s, 4H, 2 × PhC*H*$_2$O), 4.40 [m, 1H, NH(CO)C*H*$(CH_2)_2$P], 2.45 (dt, $^3J_{HP}$ = 11.4 Hz, *J* = 8.4 Hz, 2H, PCH$_2$C*H*$_2$CO$_2{}^t$Bu), 2.14 and 1.92 (2 × m, 2H, CHC*H*$_2$CH$_2$P), 1.90 (m, 2H, C*H*$_2$CO$_2{}^t$Bu), 1.66 (m, 2H, CHCH$_2$C*H*$_2$P), 1.42 (s, 9H, ^{t}Bu). ^{31}P-NMR (121 MHz, $CDCl_3$): δ_P 57.72. IR (NaCl) 3356 (br), 2978, 1728, 1533, 1456, 1367, 1250, 1215, 1155, 1049, 976, 847, 739, 698 cm^{-1}. HRMS (FAB, NBA): Calcd for $C_{26}H_{35}NO_8P$ (MH^+) 520.2100; found 520.2106. The crude **20** was used for the next reaction without further purification.

4.2.35. (*S*)-Benzyl 2-(*N*-benzyloxycarbonylamino)-4-{2-[*N*-(benzyloxycarbonylmethyl)-carbamoyl]ethyl(hydrogen)phosphino}butanoate (23).

Crude compound **20** (2.43 g, ca. 4.0 mmol) was dissolved in a mixture of trifluoroacetic acid (10 mL) and CH_2Cl_2 (10 mL) at 0 °C. The solution was stirred at room temperature for 7 h. Trifluoroacetic acid was removed *in vacuo*, followed by two cycles of azeotropic evaporation with toluene. The resulting residue was dissolved in ethyl acetate (80 mL), washed successively with 1 N HCl and sat. NaCl, and died over anhydrous Na_2SO_4. The solvent was removed *in vacuo* to afford the carboxylic acid **22** (1.88 g, 102%) as a pale yellow oil. To a solution of crude

22 in CH_2Cl_2 (50 mL), was added glycine benzyl ester monotosylate (**8**) (1.48 g, 4.40 mmol), 1-hydroxybenzotriazole monohydrate (HOBt·H_2O) (594 mg, 4.40 mmol) and *N*,*N*-diisopropylethylamine (2.07 g, 16.0 mmol) at 0 °C with stirring. Reaction was initiated by adding EDC (3.07 g, 16.0 mmol). After stirring at room temperature for 5 h, the reaction mixture was concentrated *in vacuo* to remove CH_2Cl_2. Ethyl acetate (100 mL) was added to the residue, and the resultant solution was washed successively with 1 N HCl, sat. $NaHCO_3$ and sat. NaCl, and was dried over anhydrous Na_2SO_4. After removal of the solvent *in vacuo*, the residue was purified by flash column chromatography (4% methanol and 0.5% acetic acid in CH_2Cl_2) to afford **23** (1.23 g, 50% from **18**) as a pale yellow oil. TLC (CH_2Cl_2/methanol/acetic acid, 80:20:1, cerium sulfate, R_f = 0.5). ^{1}H-NMR (300 MHz, $CDCl_3$): δ_H 10.23 (br, 1H, OH), 7.38-7.00 (m, 16H, 3 × Ph and NH), 5.14 and 5.11 (2 × s, 4H, 2 × PhCH_2O), 5.08 and 5.03 (2 × d, J = 12 Hz, 2H, PhC$H_a$$H_b$OCONH), 4.39 [m, 1H, NH(CO)CH(CH_2)$_2$P], 3.99 and 3.97 (2 × s, 2H, Glyα-$H_a$$H_b$), 2.53 (dt, $^3J_{HP}$ = 14.7 Hz and J = 7.8 Hz, 2H, PCH_2CH_2CONH), 2.14 and 1.95 (2 × m, 2H, CHCH_2CH_2P), 1.95 (m, 2H, PCH_2CH_2CONH), 1.72 (m, 2H, CHCH_2CH_2P). ^{31}P-NMR (121 MHz, $CDCl_3$): δ_P 54.60. IR (NaCl) 3321 (br), 2949, 1743, 1718, 1533, 1456, 1394, 1340, 1254, 1192, 1047, 970, 825, 739, 698 cm^{-1}. Anal. calcd for $C_{31}H_{35}N_2O_9P$: C, 60.98; H, 5.78; N, 4.59. Found: C, 60.69; H, 5.83; N, 4.57.

4.2.36. (*S*)-Benzyl 2-(*N*-benzyloxycarbonylamino)-4-{2-[*N*-(benzyloxycarbonylmethyl)-carbamoyl]ethyl(phenyl)-(R_P,S_P)-phosphino}butanoate (24).

Oxalyl chloride (699 mg, 5.51 mmol) was added to a solution of **23** (840 mg, 1.38 mmol) and one drop of DMF in dry CH_2Cl_2 (10 mL) at 0 °C. After stirring at room temperature for 1 h, toluene was added to the reaction, and the volatiles were evaporated. The resulting residue was

dissolved in dry CH_2Cl_2 (10 mL), and phenol (259 mg, 2.75 mmol) in dry CH_2Cl_2 (2 mL) was added to the solution. The solution was cooled to 0 °C, and dry Et_3N (209 mg, 2.06 mmol) was added to initiate the reaction. The consumption of **23** was monitored by TLC (CH_2Cl_2/methanol/acetic acid, 80:20:1, cerium sulfate, R_f = 0.5), and the formation of product was confirmed by TLC (ethyl acetate/hexane, 5:1, cerium sulfate, R_f = 0.4). After stirring at room temperature for 2 h, the reaction mixture was concentrated *in vacuo*. The residue was dissolved in ethyl acetate (100 mL) and was washed successively with 1 N HCl and sat. NaCl, and was dried over anhydrous Na_2SO_4. After removal of the solvent *in vacuo*, the residue was purified by flash column chromatography (3% methanol in CH_2Cl_2) to afford the phosphinate ester **24** as a mixture of two diastereoisomers (524 mg, 55%, a pale yellow oil). ^{1}H-NMR (300 MHz, $CDCl_3$): δ_H 7.37-7.08 (m, 20H, 4 × Ph), 6.61 (br t, 1H, amide NH), 5.92 (br d, *J* = 7.8 Hz) and 5.84 (br d, *J* = 7.5 Hz) (1H, carbamate NH), 5.18-5.06 (m, 6H, 3 × PhC*H*$_2$O), 4.40 [m, 1H, NH(CO)C*H*$(CH_2)_2$P], 4.07-3.90 (m, 2H, Glyα-*H*), 2.54 [m, 2H, PC*H*$_2$CH$_2$CONH], 2.28-1.76 [m, 6H, PC*H*$_2$CH$_2$CONH and CHC*H*$_2$C*H*$_2$P]. ^{31}P-NMR (121 MHz, $CDCl_3$): δ_P 56.68 and 56.48. IR (NaCl) 3276 (br), 2949, 1747, 1718, 1541, 1491, 1456, 1254, 1196, 1045, 924, 825, 741, 696 cm^{-1}. Anal. calcd for $C_{37}H_{39}N_2O_9P$: C, 64.72; H, 5.72; N, 4.08. Found: C, 64.44; H, 5.49; N 4.05.

4.2.37. (*S*)-2-amino-4-{2-[*N*-(carboxymethyl)carbamoyl]ethyl(phenyl)-(R_P*,S_P*)-phosphino}butanoic acid (5).

The phosphinate ester **24** (211 mg, 0.307 mmol) was dissolved in a mixture of methanol (20 mL) and H_2O (4 mL) containing acetic acid (92.3 mg, 1.54 mmol). Hydrogen gas was passed through the solution in the presence of 5% Pd/C for 2.5 h at room temperature. The consumption of **24** was monitored by TLC (ethyl acetate/hexane, 5:1, cerium sulfate, R_f = 0.3), and the

formation of product was confirmed by TLC (butan-1-ol/acetic acid/H_2O, 5:2:2, ninhydrin, R_f = 0.35). The reaction mixture was filtered through Celite, and the filtrate was evaporated. The residue was purified by medium-pressure reversed-phase column chromatography on ODS-S-50B. The column was eluted (6 mL/min) with a linear gradient of 5 to 55% methanol in H_2O. The UV-positive (254 nm) fractions containing the product (TLC, butan-1-ol/acetic acid/H_2O, 5:2:2, ninhydrin) were collected and lyophilized to afford **5** as a mixture of two diastereoisomers (22.8 mg, 20%, a colorless solid). ^{1}H-NMR (300 MHz, D_2O): δ_H 7.46 (dd, *J* = 7.5 and 7.5 Hz, 2H), 7.31 (t, *J* = 7.5 Hz, 1H) and 7.22 (d, *J* = 7.5 Hz, 2H) (Ph) 3.80 [t, *J* = 5.1 Hz, 1H, NH(CO)C*H*$(CH_2)_2$P], 3.73 (s, 2H, Glyα-*H*), 2.64 [m, 2H, PCH_2CH_2CONH], 2.39 [dt, $^2J_{HP}$ = 13.5 Hz, *J* = 8.1 Hz, 2H, PCH_2CH_2CONH], 2.32-2.03 (m, 4H, $CHCH_2CH_2P$). ^{31}P-NMR (121 MHz, D_2O): δ_P 62.53 and 62.48. IR (KBr) 3431 (br), 3068, 1647, 1491, 1406, 1200, 1072, 1024, 928, 833, 769, 692 cm^{-1}. HRMS (FAB, glycerol): Calcd for $C_{15}H_{22}N_2O_7P$ (MH^+) 373.1165; found 373.1168.

4.3. Enzyme Assay

4.3.1. General

The hydrolytic activity of human GGT was measured by using 4.0 μM 7-(*N*-γ-glutamylamino)-4-methylcoumarin (γ-Glu-AMC) as substrate at 25˚C and pH 5.5.[39] Assays were initiated by adding 10 μL enzyme stock solution to 100 mM succinate-NaOH buffer (pH 5.5) in a total volume of 1 mL containing 100 μL of γ-Glu-AMC stock solution (40 μM in water) at 25˚C (the final γ-Glu-AMC concentration of 4.0 μM). The release of AMC was monitored continuously for 10 min with a Hitachi F-2000 spectrophotometer (350 nm excitation, 440 nm emission). AMC concentrations were calculated using a standard calibration curve of

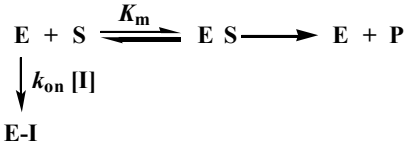
fluorescence intensity (F) versus AMC concentration (C): $\Delta F/\Delta C$ = 0.11 nM^{-1}. The fluorescence intensity was proportional to the concentration of AMC up to 2.0 μM. The Michaelis constant (K_m) for γ-Glu-AMC of human GGT was determined as 12.6 μM under these conditions. The hydrolytic activity of *E. coli* GGT was measured under the same conditions, except that the final substrate concentration was 0.2 μM. The K_m value for γ-Glu-AMC of *E. coli* GGT was determined as 0.2 μM under the same conditions.

4.3.2. Time-dependent inhibition assay

The inhibition of GGT was measured under pseudo-first order rate conditions. Reaction was initiated by adding enzyme to a preincubated mixture of varying concentrations of the inhibitor and the substrate (the final concentrations of 4.0 and 0.2 μM of γ-Glu-AMC for human and *E. coli* GGT, respectively) in 100 mM sodium succinate buffer (pH 5.5) at 25°C. Time-dependent inhibition of the enzyme was followed by monitoring the release of AMC continuously for 10 min. The resulting concave progress curves were analyzed by fitting the data to the first-order rate equation (1) to calculate the observed pseudo-first-order rate constants for enzyme inactivation (k_{obs}) using the KaleidaGraph v.3.5 program package (Synergy Software).

$$[P] = [P]_\infty[1-\exp(-k_{obs}\,t)] \qquad (1)$$

where [P] and $[P]_\infty$ are the concentrations of AMC formed at time t and at time approaching infinity, respectively. Since the plot of k_{obs} vs. inhibitor concentration ([I]) exhibited no saturation under standard inhibitor concentrations, the second-order rate constant for enzyme inactivation (k_{on}) was calculated according to the equation (2) derived from the kinetic mechanism described below:



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

where S is the substrate 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).

4.4. Ion-Spray Mass Spectroscopy.

The mass spectra were recorded on a Sciex API-3000 mass spectrometer (PE Sciex) interfaced with an ion-spray ion source. The *E. coli* GGT (600 μg) was inactivated by incubation with **2a**-**A** (1.7 mM) in 50 mM Tris-HCl buffer (pH 8.0) (total volume 100 μL) at 37 ˚C for 20 min or with **2a**-**A** (4 mM) for 3 h. The activity of the partially or completely inactivated enzyme was measured by hydrolysis of γ-Glu-AMC. The small subunit of the inactivated enzyme was isolated by reversed-phase HPLC (COSMOSIL 5C18-AR-300, 4.6 × 150 mm) and was lyophilized as previously described.[70] The lyophilized small subunit was dissolved in a mixture of 100 μL of 0.1% formic acid and 100 μL of acetonitrile, and was injected directly into mass spectrometer using a syringe (1.46 mm inside diameter) at a flow rate of 2.5 μL/min. The quadrupole was scanned over a range of 500-2000 atomic mass units (amu) with a step size of 0.1 amu and a dwell time of 0.2 ms. Ion-spray voltage was set at 5 kV, and orifice potential was 60 V. Data analysis were performed using the BioMultiView program (PE Sciex).

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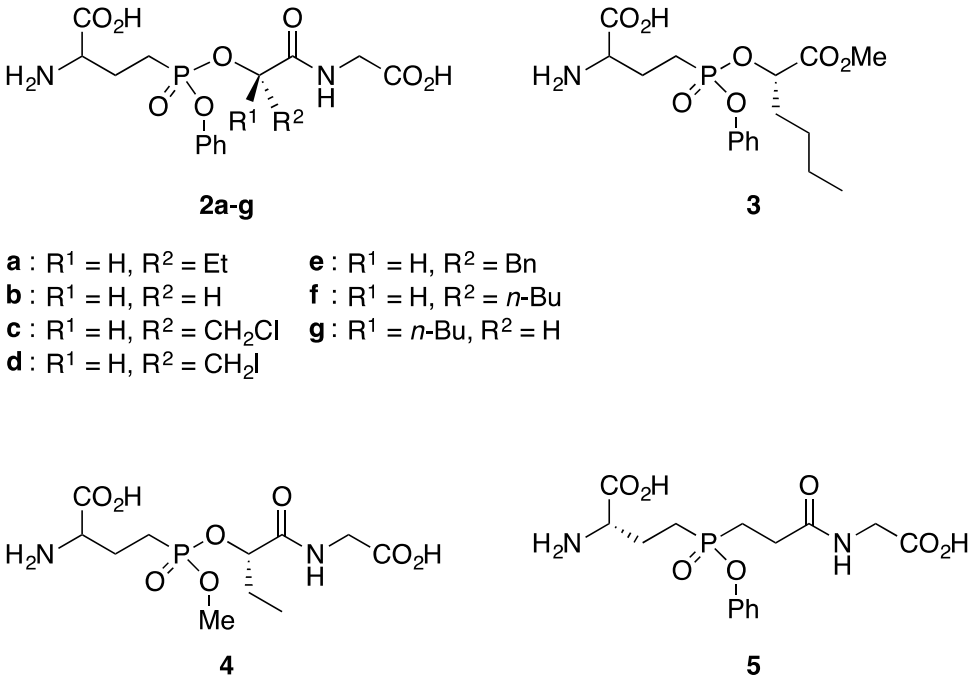


Figure 1. Peptidyl phosphorus esters **2a-g**, **3**, **4** and **5** as mechanism-based inhibitors of GGT.

Figure 1.

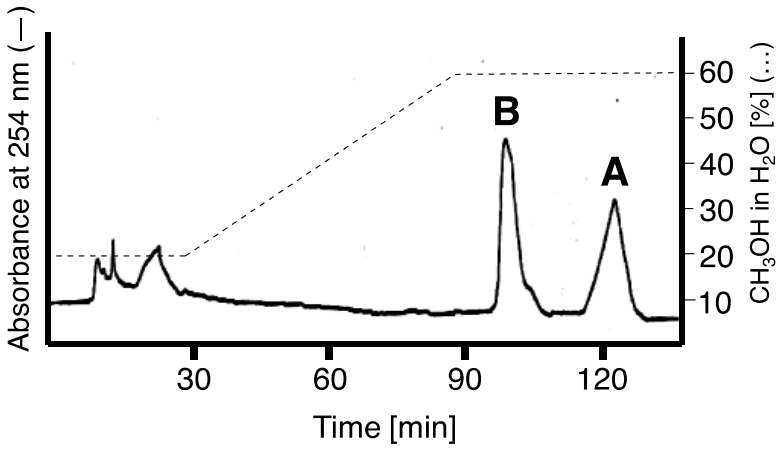


Figure 2. Separation of diastereoisomeric pairs (A, B) of **2f** with MPLC. See Experimental Procedures for conditions of chromatography.

Figure 2.

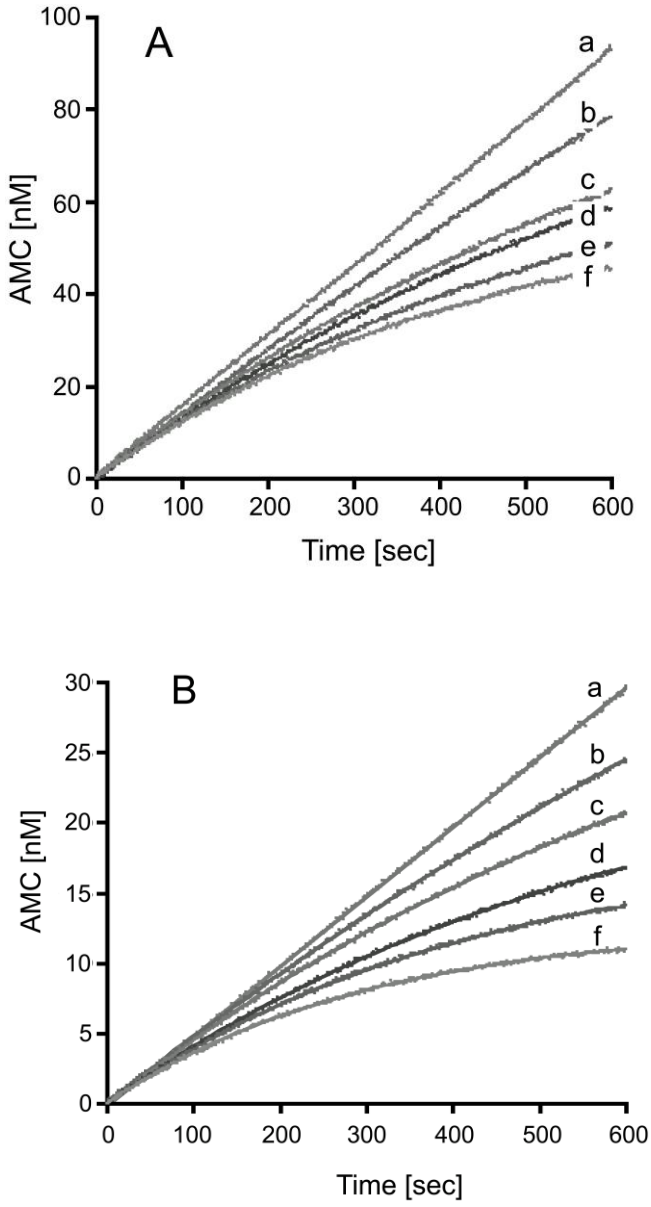


Figure 3. Typical time-dependent inhibition of GGT by **2a**-**A** at pH 5.5 with γ-Glu-AMC as substrate. (A) Progress curves for reactions catalyzed by human GGT with 4.0 μM γ-Glu-AMC in the presence of varying concentration of **2a**-**A**: (a) 0, (b) 4, (c) 10, (d) 12, (e) 16 and (f) 20 μM. (B) Progress curves for reactions catalyzed by *E. coli* GGT with 0.2 μM γ-Glu-AMC in the presence of varying concentration of **2a**-**A**: (a) 0, (b) 10, (c) 20, (d) 30, (e) 40 and (f) 60 μM. The formation of product AMC was monitored continuously by fluorescence (see 4.3 Enzyme Assay).

Figure 3.

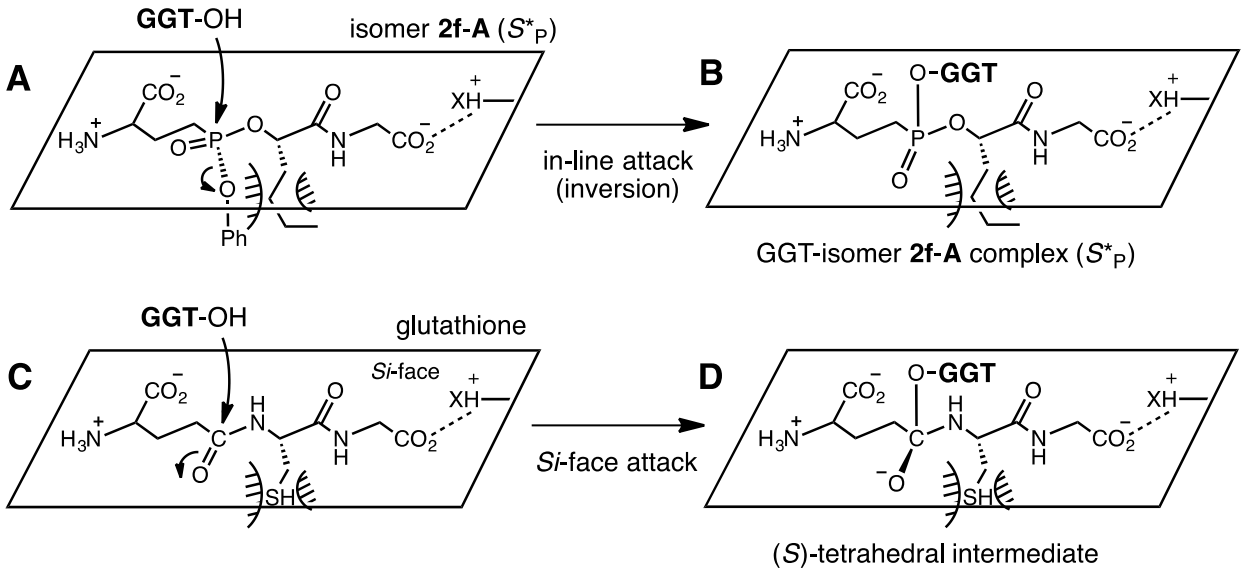


Figure 4. Stereochemistry of the attack of the catalytic nucleophile of human GGT (Thr residue) on the chiral phosphorus of the inhibitor **2f-A** (A to B) as a model for catalyzed reaction of glutathione (B to D).

Figure 4.

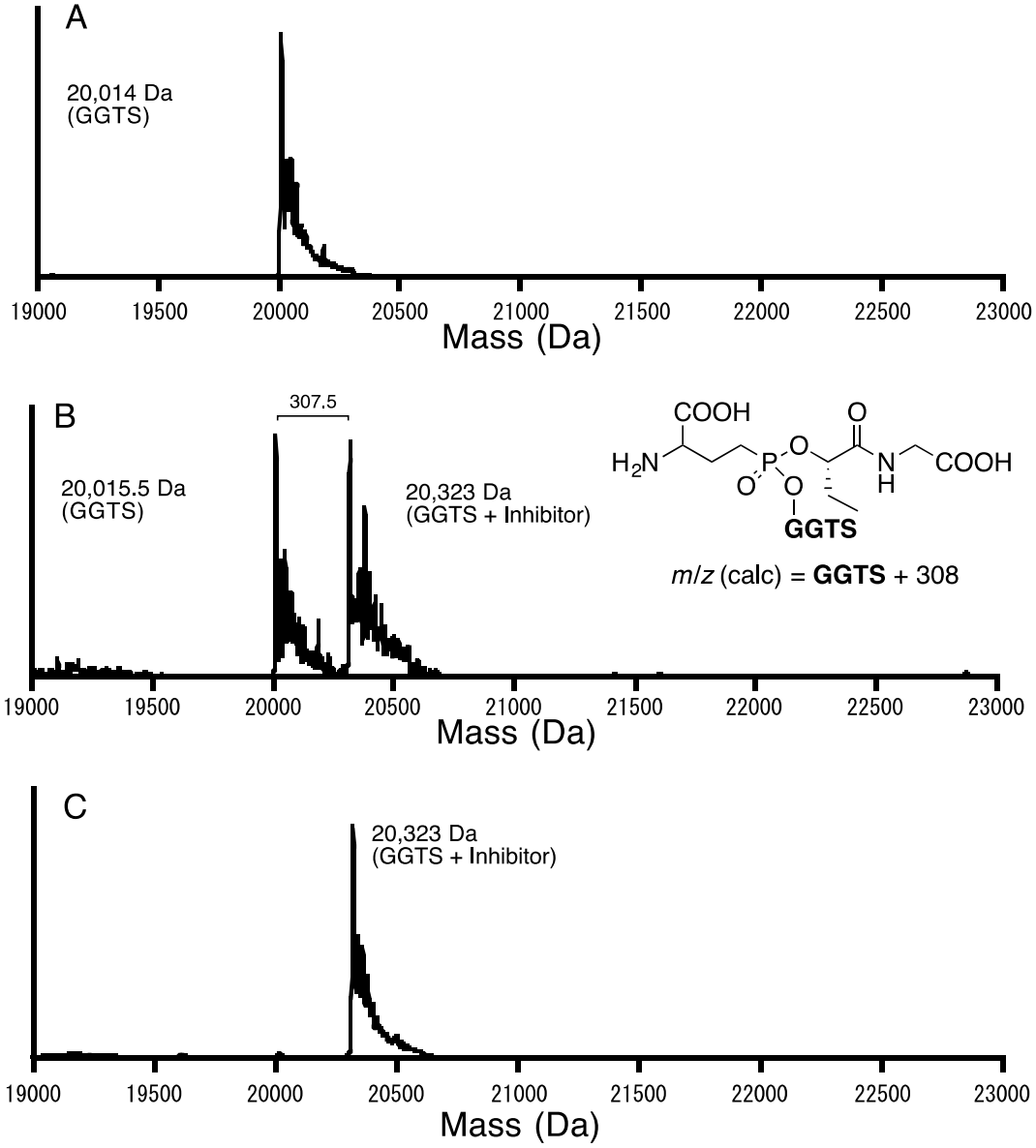
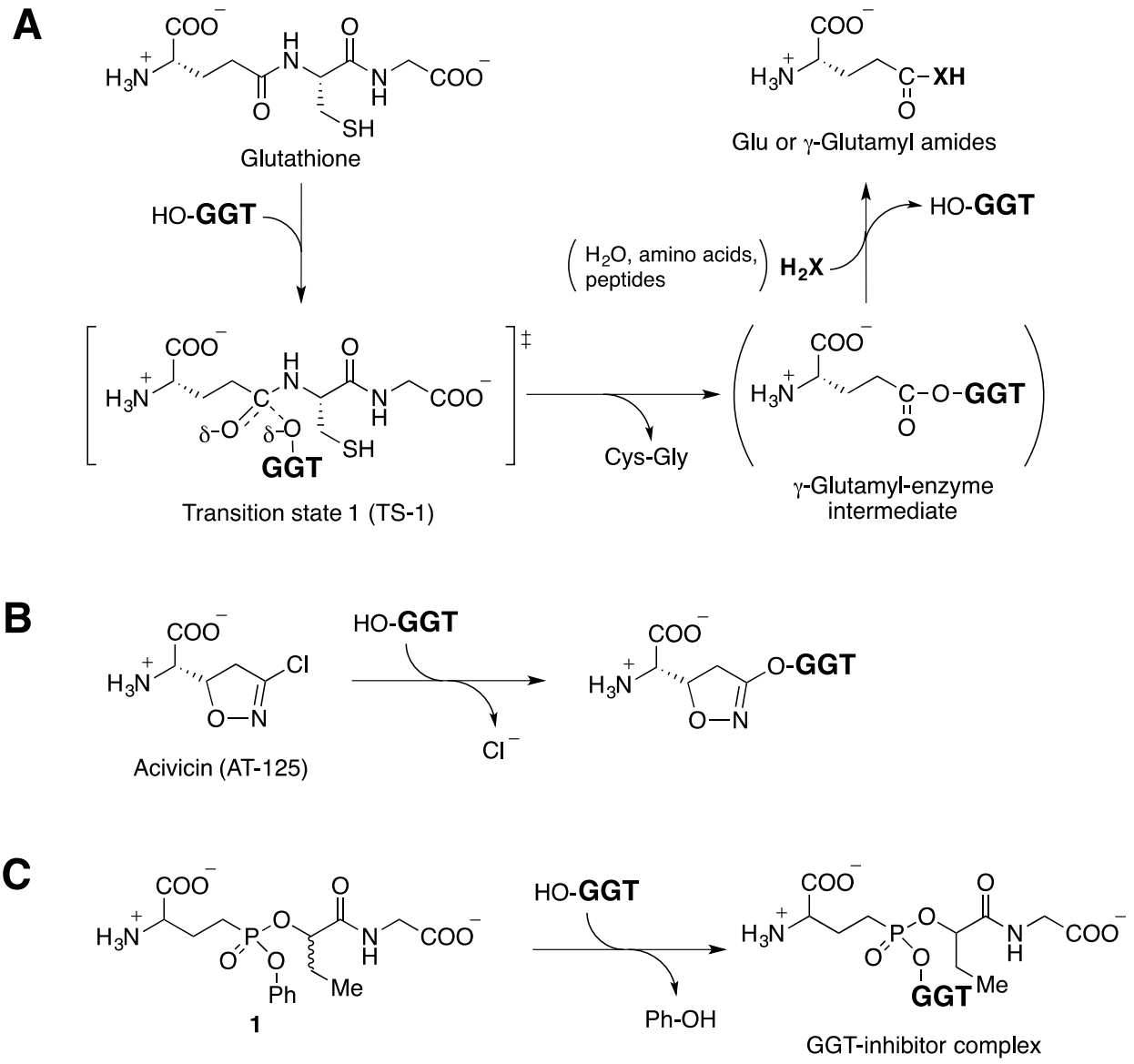


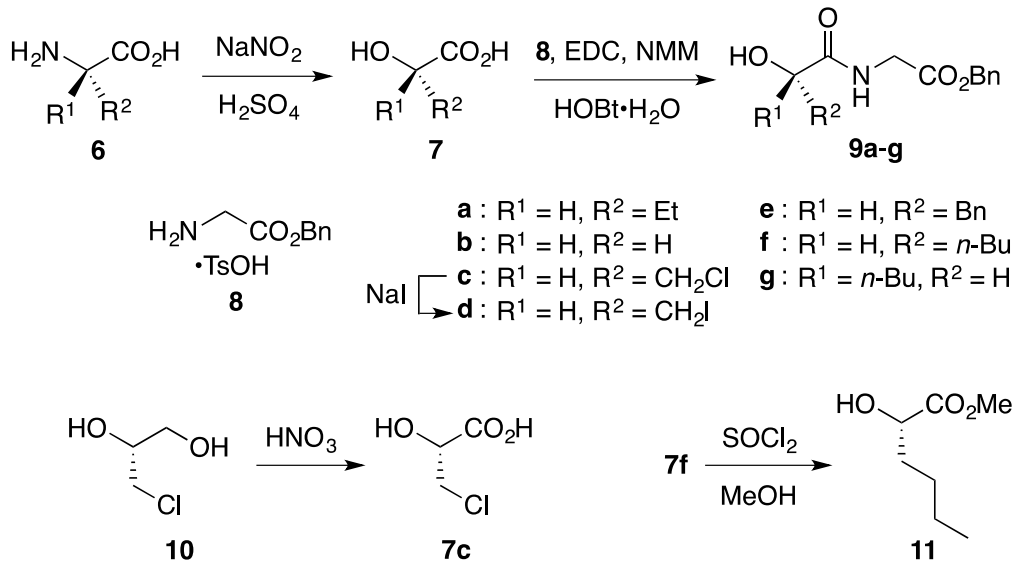
Figure 5. Mass spectra of *E. coli* GGT small subunit (GGTS) incubated with **2a-A**. (A) 0 mM, (B) 1.7 mM, 20 min, (C) 4.0 mM, 3 h.

Figure 5.



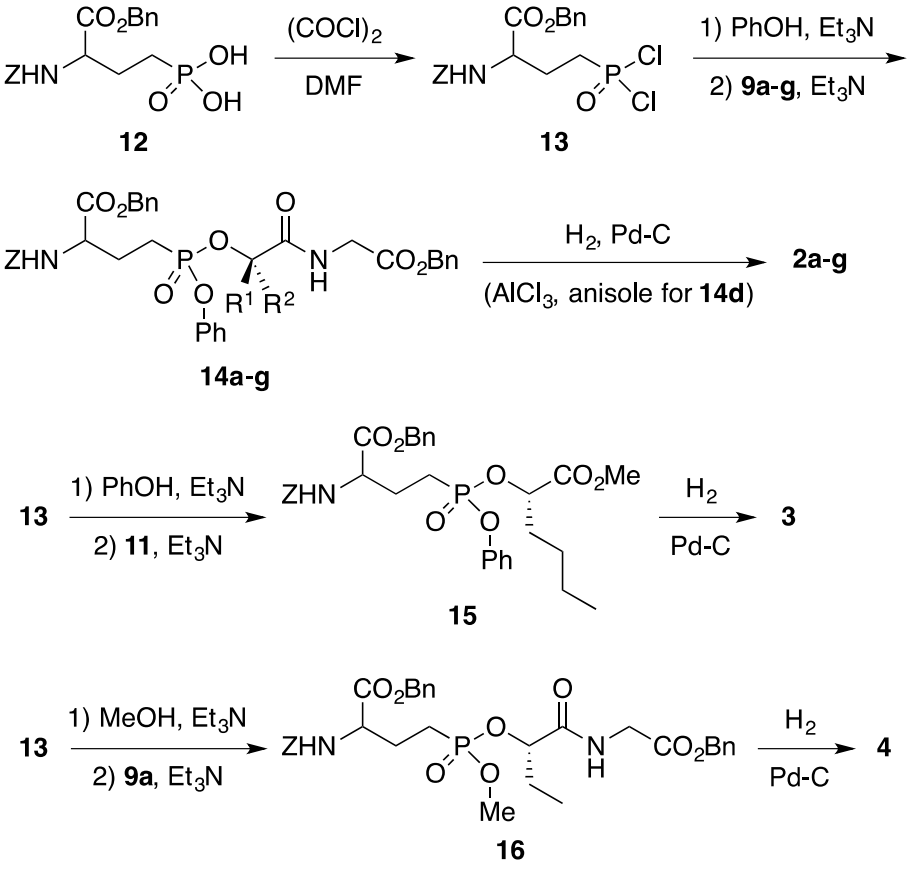
Scheme 1: Proposed catalytic mechanism of GGT (A) and inhibition of GGT by the mechanism-based inhibitor **1** (B). Compound **1** is a mixture of stereoisomers with respect to the ethyl side chain (see also Table 1).

Scheme 1.



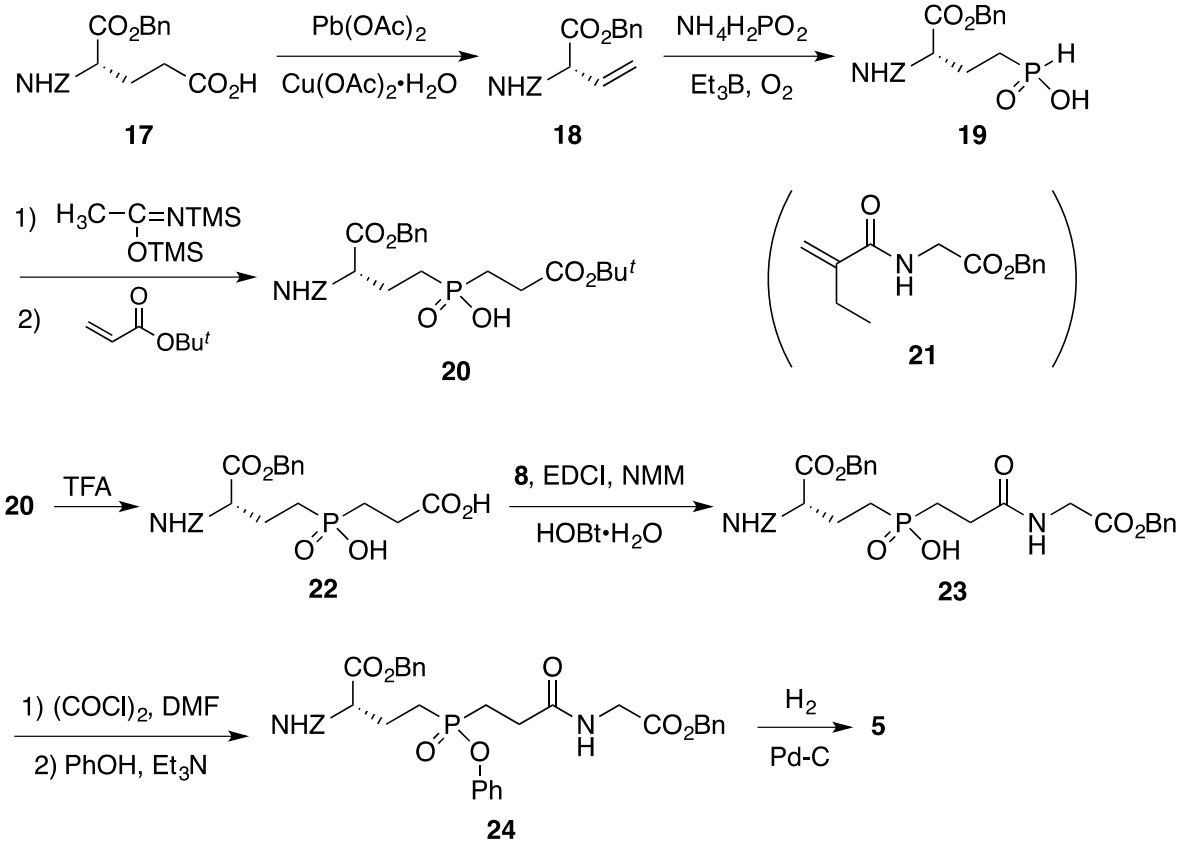
Scheme 2. Synthesis of α-hydroxycarboxamides **9a**-**g** and α-hydroxyester **11**.

Scheme 2.



Scheme 3. Synthesis of phosphonate diesters **2a**-**g**, **3** and **4**.

Scheme 3.



Scheme 4. Synthesis of phosphinate ester **5**.

Scheme 4

Table 1. Composition of stereoisomers of **1**, **2a**-**g**, **3**, **4** and **5** and their stereochemistry.

Compound		Composition[a]	Stereochemistry[b]
1		Four stereoisomers[c]	**(L$_{\alpha}$, S_P*, L$_{\alpha'}$)**, (L$_{\alpha}$, R_P*, D$_{\alpha'}$), **(D$_{\alpha}$, S_P*, L$_{\alpha'}$)**, (D$_{\alpha}$, R_P*, D$_{\alpha'}$)
2a, **c**-**f**, **3** and **4**	Peak A	Two diastereoisomers	**(L$_{\alpha}$, S_P*, L$_{\alpha'}$), (D$_{\alpha}$, S_P*, L$_{\alpha'}$)**
	Peak B	Two diastereoisomers	(L$_{\alpha}$, R_P*, L$_{\alpha'}$), (D$_{\alpha}$, R_P*, L$_{\alpha'}$)
2b		Four stereoisomers	**(L$_{\alpha}$, S_P*)**, (L$_{\alpha}$, R_P*), **(D$_{\alpha}$, S_P*)**, (D$_{\alpha}$, R_P*)
2g	Peak A	Two diastereoisomers	(L$_{\alpha}$, R_P*, D$_{\alpha'}$), (D$_{\alpha}$, R_P*, D$_{\alpha'}$)
	Peak B	Two diastereoisomers	(L$_{\alpha}$, S_P*, D$_{\alpha'}$), (D$_{\alpha}$, S_P*, D$_{\alpha'}$)
5		Two diastereoisomers	**(L$_{\alpha}$, S_P*)**, (L$_{\alpha}$, R_P*)

[a] The ratio of stereoisomers is almost equal (1 : 1) in each case.

[b] Stereochemistry [ex. (L$_{\alpha}$, S_P*, L$_{\alpha'}$)] is indicated in the order of the distal α-carbon of the glutamate moiety (L$_{\alpha}$ or D$_{\alpha}$), the chiral phosphorus (S_P* or R_P*) and the proximal α-carbon of the peptidyl moiety (L$_{\alpha'}$ or D$_{\alpha'}$). Inhibitory active stereoisomers [ex. (L$_{\alpha}$, S_P*, L$_{\alpha'}$)] are shown in bold (see Sections 2.5 and 2.6).

[c] See Section 2.5.

Table 2. Inhibitory activities of peptidyl phosphonate diesters **1**,[39] **2a**-**g**, **3**, **4** and phosphinate ester **5** toward human and *E. coli* GGTs.[a]

Compound	Peptidyl moiety mimicking	k_{on} ($M^{-1}s^{-1}$)[b]	
		Human GGT	*E. coli* GGT
1	DL-Abu-Gly	75 ± 3[c]	80 ± 2[c]
2a	L-Abu-Gly	145 ± 5.0	109 ± 2.9
2b	Gly-Gly	44.5 ± 2.5	80.6 ± 1.2
2c	L-β-ClAla-Gly	40.3 ± 0.59	188 ± 14
2d	L-β-IAla-Gly	43.6 ± 2.0	98.4 ± 2.9
2e	L-Phe-Gly	4.44 ± 0.28	389 ± 24
2f	L-Nle-Gly	63.2 ± 0.76	50.7 ± 0.66
		NI[d, e]	1.7[e, f]
2g	D-Nle-Gly	NI[d]	1.3[f]
		NI[d, e]	NI[d, e]
3	L-Nle-OMe	NI[d]	8.83 ± 0.19
4	L-Abu-Gly	NI[d]	NI[d]
5	Gly-Gly	1.35 ± 0.11	NI[g]
acivicin	–	0.40 ± 0.02[h]	4200 ± 100[h]

[a] The activities of peak A (a mixture of two diastereoisomers, Table 1) are shown for **2a**, **2c**-**g**, **3** and **4**. The inhibitory activities of peak B are also shown for **2f** and **2g** (footnote e). The inhibitory activities of peak B were extremely low (see Sections 2.5 and 2.6). For compound **2b**,

the activities of a mixture of four stereoisomers (two sets of enantiomers, Table 1) were determined. Compound **5**, the phosphinate version of **2b**, was a mixture of two diastereoisomers (Table 1).

[b] Second-order rate constant for enzyme inactivation (see Section 4.3.2).

[c] Ref. 39.

[d] No inactivation at 1 mM.

[e] Inhibitory activity of Peak B (Table 1)

[f] Error limit was not determined due to duplicate measurements

[g] No inactivation at 1.5 mM.

[h] Ref. 38.

Figure legends

Figure 1. Peptidyl phosphorus esters **2a**-**g**, **3**, **4** and **5** as mechanism-based inhibitors of GGT.

Figure 2. Separation of diastereoisomeric pairs (A, B) of **2f** with MPLC.

For conditions of chromatography, see Section 4.2.29.

Figure 3. Typical time-dependent inhibition of GGT by **2a**-**A** at pH 5.5 with γ-Glu-AMC as substrate. (A) Progress curves for reactions catalyzed by human GGT with 4.0 μM γ-Glu-AMC in the presence of varying concentration of **2a**-**A**: (a) 0, (b) 4, (c) 10, (d) 12, (e) 16 and (f) 20 μM. (B) Progress curves for reactions catalyzed by *E. coli* GGT with 0.2 μM γ-Glu-AMC in the presence of varying concentration of **2a-A**: (a) 0, (b) 10, (c) 20, (d) 30, (e) 40 and (f) 60 μM. The formation of product AMC was monitored continuously by fluorescence (see Section 4.3.2).

Figure 4. Stereochemistry of the attack of the catalytic nucleophile of human GGT (Thr residue) on the chiral phosphorus of the inhibitor **2f-A** (A to B) as a model for catalyzed reaction of glutathione (C to D).

Figure 5. Mass spectra of *E. coli* GGT small subunit (GGTS) incubated with **2a-A**.

(A) control without inhibitor, (B) 1.7 mM of **2a-A**, 20 min, (C) 4.0 mM of **2a-A**, 3 h.

Scheme 1: Proposed catalytic mechanism of GGT (A) and inhibition of GGT by the mechanism-based inhibitor **1** (B). Compound **1** is a mixture of stereoisomers with respect to the

ethyl side chain (see also Table 1).

Scheme 2. Synthesis of α-hydroxycarboxamides **9a**-**g** and α-hydroxyester **11**.

Scheme 3. Synthesis of phosphonate diesters **2a**-**g**, **3** and **4**.

Scheme 4. Synthesis of phosphinate ester **5**.

Supplementary data

Spectral data for known compounds ***7a, c, e-g***, ***9b***, ***11***, ***13*** *and* ***18***

4.2.1. (*S*)-2-Hydroxybutanoic acid (7a).[S1]

^{1}H-NMR (300 MHz, $CDCl_3$): δ_H 4.28 [dd, *J* = 6.9 and 4.5 Hz, 1H, C*H*(OH)COOH], 1.92 (dqd, *J* = 13.8, 7.2 and 4.5 Hz, 1H, $CH_3CH_aH_b$), 1.77 (dqd, *J* = 13.8, 7.2 and 6.9 Hz, 1H, $CH_3CH_aH_b$), 1.02 (t, *J* = 7.2 Hz, 3H, CH_3).

4.2.2. (*R*)-3-Chloro-2-hydroxypropanoic acid (7c).[S2]

^{1}H-NMR (300 MHz, $CDCl_3$): δ_H 4.64 [t, *J* = 3.6 Hz, 1H, C*H*(OH)COOH], 3.94 (dd, *J* = 11.6 and 3.6 Hz, 1H, $CH_3CH_aH_b$), 3.88 (dd, *J* = 11.6 and 3.6 Hz, 1H, $CH_3CH_aH_b$).

4.2.3. (*S*)-2-Hydroxy-3-phenylpropanoic acid (7e).[S3]

^{1}H-NMR (300 MHz, $CDCl_3$): δ_H 7.35-7.21 (m, 5H, Ph), 4.52 [dd, *J* = 7.3 and 4.2 Hz, 1H, C*H*(OH)COOH], 4.27 (br, 1H, OH), 3.22 (dd, *J* = 13.9 and 4.2 Hz, 1H, $PhCH_aH_b$), 3.00 (dd, *J* = 13.9 and 7.3 Hz, 1H, $PhCH_aH_b$). IR (KBr) 3446 (br), 1734, 1311, 1243, 1192, 1091, 914, 879, 796, 702 cm^{-1}.

4.2.4. (*S*)-2-Hydroxyhexanoic acid (7f).[S3]

^{1}H-NMR (300 MHz, $CDCl_3$): δ_H 4.28 [dd, *J* = 7.5 and 4.2 Hz, 1H, C*H*(OH)COOH], 1.93-1.80 [m, 1H, $CH_3(CH_2)_2CH_aH_b$], 1.78-1.63 [m, 1H, $CH_3(CH_2)_2CH_aH_b$], 1.54-1.28 (m, 4H, $CH_3CH_2CH_2$), 0.93 (t, *J* = 7.1 Hz, 3H, CH_3). IR (NaCl) 3451 (br), 2956, 1716, 1647, 1458, 1269, 1244, 1209, 1132, 1084 cm^{-1}.

4.2.5. (*R*)-2-Hydroxyhexanoic acid (7g).[S3]

^{1}H-NMR (300 MHz, $CDCl_3$): δ_H 4.28 [dd, *J* = 7.2 and 4.2 Hz, 1H, C*H*(OH)COOH], 3.9-2.1 (br s, 1H, OH), 1.93-1.80 [m, 1H, $CH_3(CH_2)_2CH_aH_b$], 1.78-1.63 [m, 1H, $CH_3(CH_2)_2CH_aH_b$], 1.54-1.28 (m, 4H, $CH_3CH_2CH_2$), 0.93 (t, *J* = 7.2 Hz, 3H, CH_3). IR (NaCl) 3435 (br), 2958, 1732, 1635, 1458, 1273, 1247, 1216, 1134, 1086 cm^{-1}.

4.2.7. Benzyl 2-(2-hydroxyacetamido)acetate (9b).

^{1}H-NMR (300 MHz, $CDCl_3$): δ_H 7.39-7.29 (m, 5H, Ph), 7.28 (br t, 1H, NH), 5.16 (s, 2H, PhCH_2O), 4.11 [d, J = 5.7 Hz, 2H, CH_2(OH)CONH], 4.09 (d, J = 6.0 Hz, 2H, Glyα-H) 3.97 (br, 1H, OH). IR (NaCl) 3390 (br), 2941, 1747, 1660, 1539, 1196, 1124, 1078, 739, 698 cm^{-1}. The spectral data supported the structure of compound **9b**.

4.2.13. (*S*)-Methyl 2-hydroxyhexanoate (11).[S4]

^{1}H-NMR (300 MHz, $CDCl_3$): δ_H 4.19 [ddd, J = 7.2, 5.7 and 4.2 Hz, 1H, CH(OH)COOMe], 3.79 (s, 3H, CH_3O), 5.47 (d, J = 5.7 Hz, 1H, OH), 1.86-1.73 [m, 1H, $CH_3(CH_2)_2CH_aH_b$], 1.72-1.56 [m, 1H, $CH_3(CH_2)_2CH_aH_b$], 1.47-1.26 (m, 4H, $CH_3CH_2CH_2$), 0.91 (t, J = 6.6 Hz, 3H, CH_3). IR (KBr) 3523 (br), 2958, 1743, 1271, 1207, 1134, 1084, 1020, 966, 746, 696 cm^{-1}.

4.2.14. (*R,S*)-Benzyl 2-(*N*-benzyloxycarbonylamino)-4-(dichlorophosphoryl)butanoate (13).[S5]

^{1}H NMR (300 MHz, $CDCl_3$): δ_H 7.3-7.4 (m, 10H, 2 × Ph), 5.46 (br d, 1H, J = 8.1 Hz, NH), 5.24 (d, J = 12 Hz, 1H, PhC$H_a$$H_b$O), 5.18 (d, J = 12 Hz, 1H, PhC$H_a$$H_b$O), 5.12 (s, 2H, PhC$H_2$OCON), 4.53 [m, 1H, C$H$(C$H_2$)$_2$P], 2.1-2.7 (m, 4H, CH_2CH_2P). ^{31}P NMR (121 MHz, $CDCl_3$) δ_P 49.2.

4.2.33. (*S*)- Benzyl 2-(benzyloxycarbonyl)but-3-enoate (18).[S6]

Cupric acetate monohydrate (2.26 g, 11.3 mmol) was added to a suspension of (*S*)-5-benzyloxy-4-(benzyloxycarbonylamino)-5-oxopentanoic acid (**17**) (21.0 g, 56.6 mmol) in dry benzene (500 mL). The mixture was stirred at room temperature for 1 h under argon. Freshly purified lead tetraacetate (50.1 g, 113 mmol) was added, and the mixture was stirred for another 1 h at room temperature. The mixture was heated under reflux for 12 h. After filtering the reaction mixture through Celite, the filtrate was diluted with ethyl acetate (1000 mL) and was washed three times with H_2O and once with sat. NaCl. The organic layer was dried over anhydrous Na_2SO_4. After removal of solvent *in vacuo*, the residue was purified by flash column chromatography (10% ethyl acetate in hexane) to afford the (*S*)-vinylglycine derivative **18** (7.41 g, 40%) as a colorless solid. ^{1}H-NMR (300 MHz, $CDCl_3$): δ_H 7.41-7.27 (m, 10H, 2 × Ph), 5.92 [ddd, J = 16.8, 10.2 and 5.4 Hz, 1H, CHCH=CH_2] 5.47 (br d, J = 7.5, 1H, NH), 5.36 (dd, J =16.8

and 1.5 Hz) and 5.27 (dd, *J* =10.2 and 1.5 Hz) (2H, CH=CH_2), 5.19 and 5.12 (2 × s, 4H, 2 × PhCH_2O), 4.99 [dd, *J* = 7.5 and 5.4 Hz, 1H, NH(CO)C*H*CH=CH_2]. IR (NaCl) 3313 (br), 3033, 1743, 1508, 1456, 1338, 1267, 1213, 1176, 1076, 1051, 989, 924, 737, 696 cm^{-1}. Anal. calcd for $C_{19}H_{19}NO_4$: C, 70.14; H, 5.89; N, 4.31. Found: C, 70.27; H, 5.98; N. 4.29.

Supplementary references

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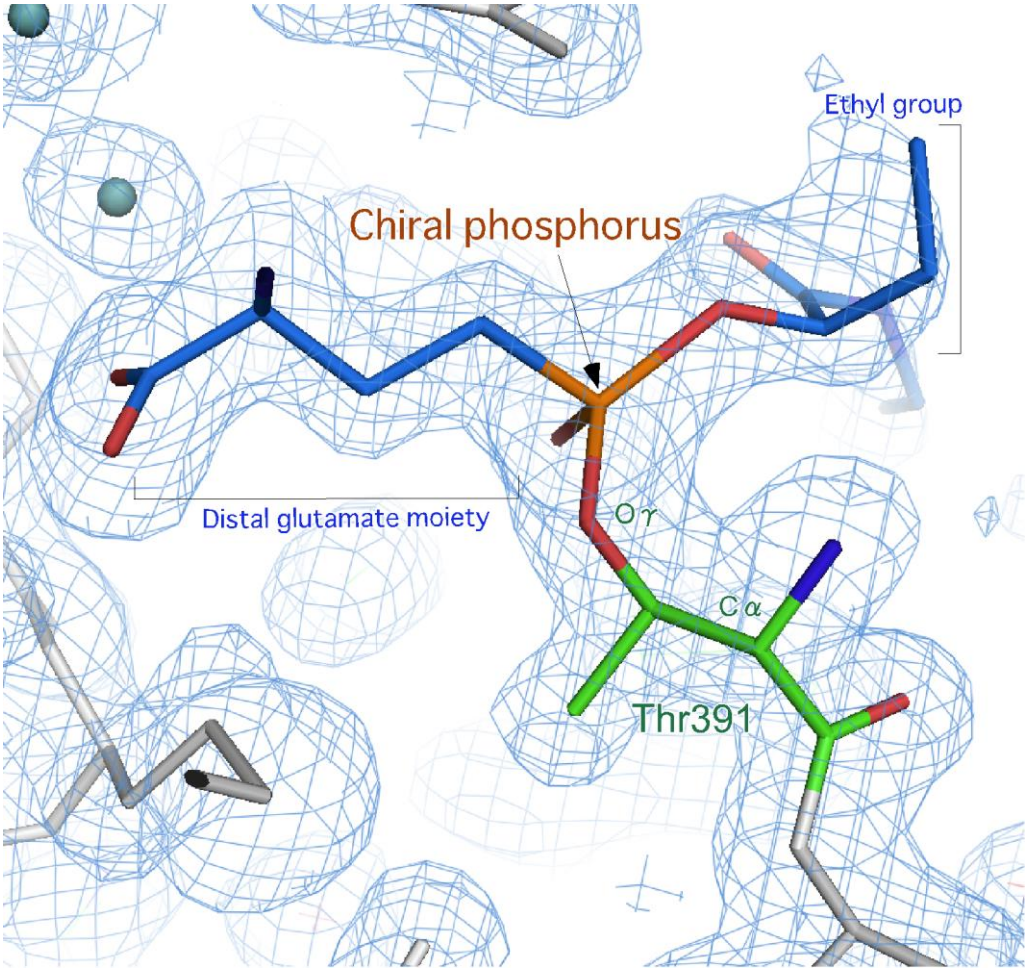

Figure S1. The electron density map around the active site of *E. coli* GGT in complex with **2a**-**A**.

A $2F_o$-F_c map at 1.0 σ (light blue) calculated from 1.9 Å resolution data contoured is overlaid on the stick model of GGT in complex with **2a**-**A**, where the R_{cryst} and R_{free} values of the model are 0.206 and 0.236, respectively. Oxygen, nitrogen, phosphorus atoms are colored in red, blue and orange, respectively. Carbon atoms in **2a**-**A** and in Thr391 are colored in light blue and green, respectively. Polypeptide chains of *E. coli GGT* except for the active site Thr391 are colored in gray, and water molecules are colored in cyan.

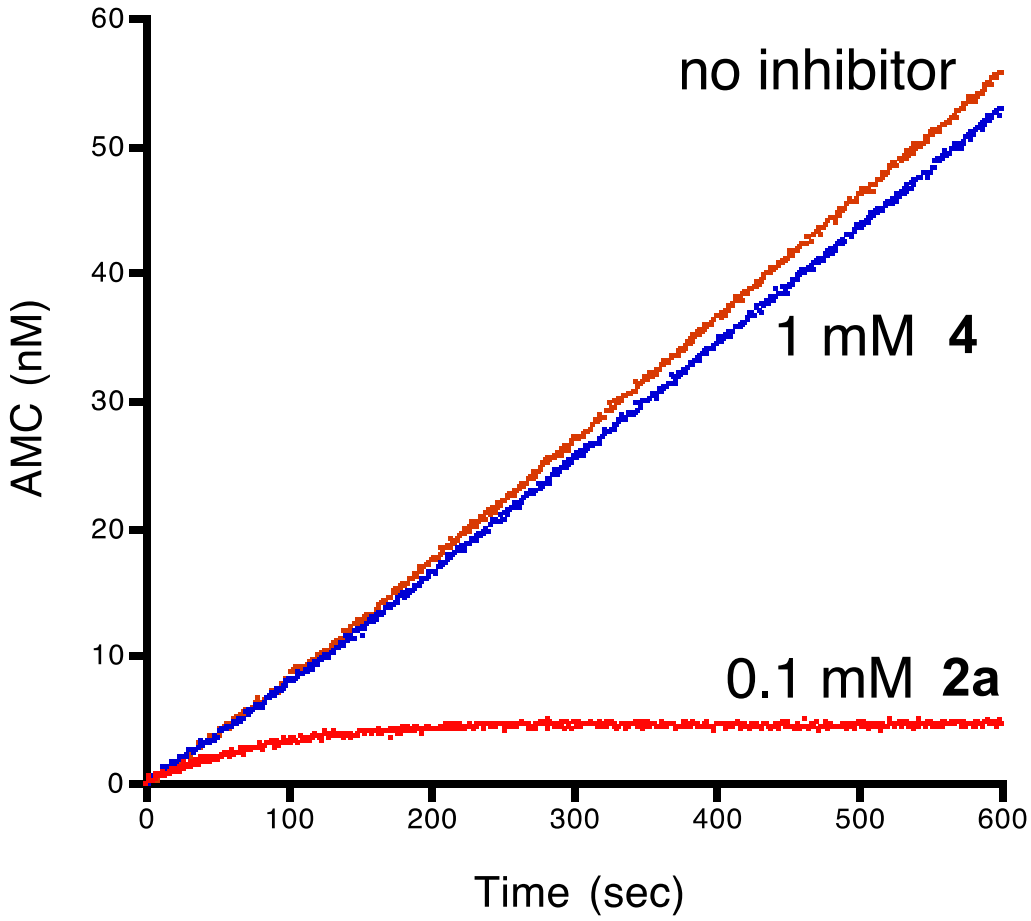

Figure S2. Inhibition of human GGT by phenyl ester **2a** and methyl ester **4**.

The enzyme reactions were conducted with 4.0 μM γ-Glu-AMC as substrate in the presence of **4** (1 mM) or **2a** (0.1 mM). The formation of product AMC was monitored continuously by fluorescence (see Section 4.3).

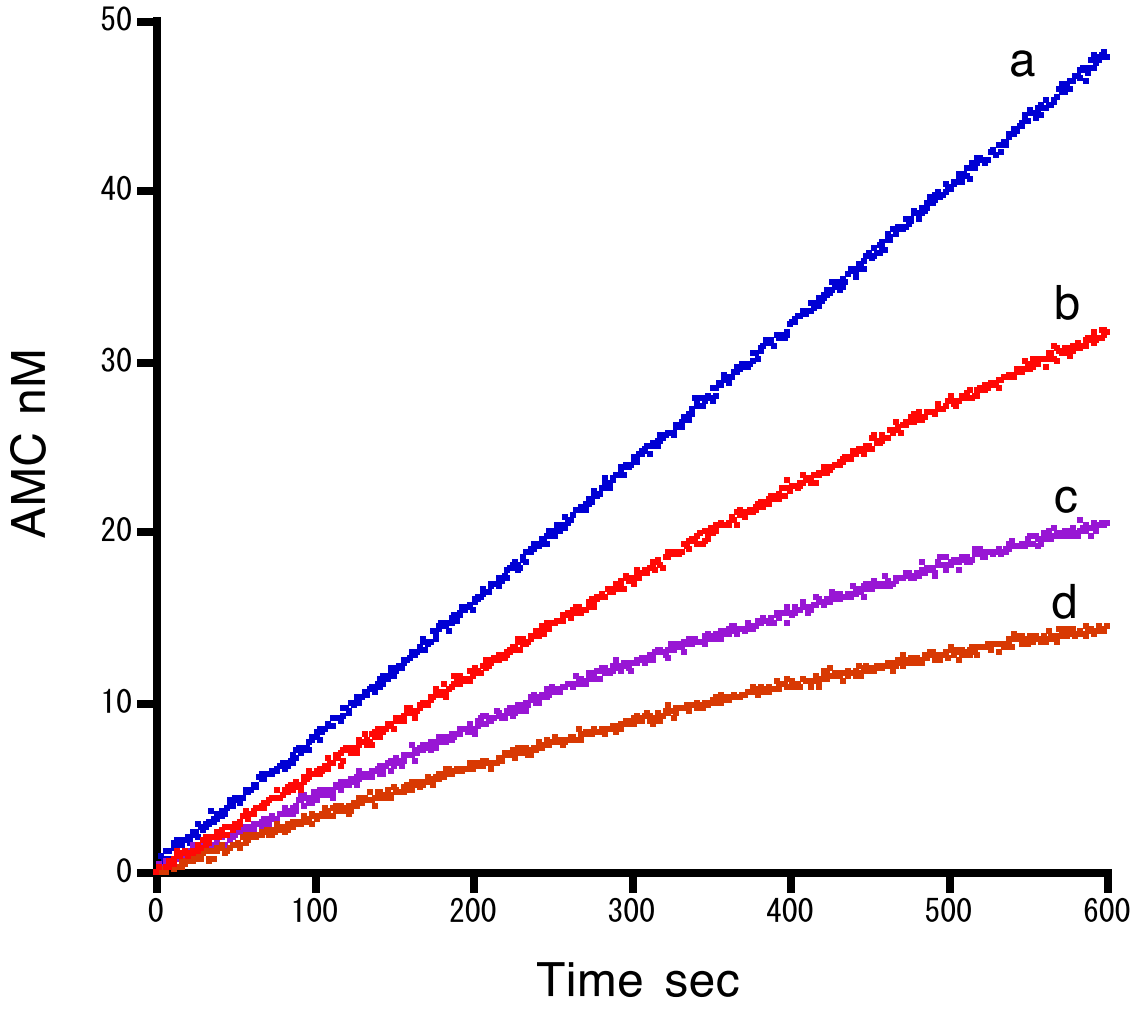

Figure S3. Time-dependent inhibition of human GGT by phosphinate **5**.

The enzyme reactions were conducted with 4.0 μM γ-Glu-AMC as substrate in the presence of varying concentration of **5**: (a) 0, (b) 500, (c) 1000, and (d) 1500 μM. The formation of product AMC was monitored continuously by fluorescence (see Section 4.3).