Phosphono Analogues of Glutathione as New Inhibitors of Glutathione S-Transferases

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Summary

Phosphono-analogues of glutathione containing the $O=P(OR)_2$ moiety in place of the cysteinyl residue CH_2SH **1a–1d** were prepared by solution phase peptide synthesis. Benzyl, benzyloxycarbonyl, and *tert*-butyl protecting groups were used to mask the individual amino acid functional groups. The formation of peptide bonds was achieved by the usual peptide synthesis via activation of carboxylic functions with cyclohexylcarbodiimide and subsequent reaction with free amino groups. The thus obtained, fully-protected peptides were each purified by normal phase column chromatography. Deprotection was accomplished by hydrogenolysis and by treatment with HBr/acetic acid yielding the desired phosphonic acid diester **1a–1d**. The inhibition of the glutathione conjugation of 1-chloro-2,4-dinitrobenzene by human placental glutathione S-transferase was studied by determining the IC_{50} values of the new glutathione analogues. The IC_{50} values were 291 μM, 139 μM, 64 μM, and 21 μM for the dimethyl, diethyl, diisopropyl, and di-*n*-butyl esters, respectively. The results clearly show that the formal substitution of the glutathione thiol function by phosphonic acid esters leads to a new class of glutathione S-transferase inhibitors. Further investigations directed at the question of whether or not these glutathione analogues are suitable for a modulation in chemotherapy are in progress.

Introduction

Glutathione S-transferases (GSTs, EC 2.5.1.18) are a family of multifunctional proteins involved in the cellular detoxification of a broad range of electrophilic xenobiotic and reactive endogenous compounds of the oxidative metabolism[1]. GSTs have been subdivided into Alpha, Mu, Pi, Theta, Sigma, and microsomal species-independent gene classes[2]. Elevated levels of class Alpha, Mu, or Pi GSTs are factors associated with the increased resistance of tumours to a variety of antineoplastic agents[3]. The resistance towards alkylating agents such as, for example, chlorambucil[4], melphalan[5], and nitrosoureas[6], is caused by a direct conjugation with reduced glutathione[3e]. Another mechanism for lowering the response to antineoplastic drugs is the repair of cellular injuries by the selenium-independent peroxidase activity of glutathione S-transferases[3e]. Anthracyclines, e.g., doxorubicin, probably act via the release of superoxide anion[7]. This leads to the formation of hydroperoxides of lipids and DNA, which could be reduced enzymatically to the corresponding alcohols by mainly class Alpha glutathione S-transferases[3e]. An effective way of overcoming drug resistance as a result of increased glutathione S-transferase activity would be the use of inhibitors before or during antineoplastic therapy. This concept of modulation cancer therapy was recently reviewed[3c, 8]. A positive effect of a combination of thiotepa and ethacrynic acid, a glutathione S-transferase inhibitor, was found in a first phase I clinical trial[9]. However, there are limitations to the use of ethacrynic acid: ethacrynic acid enhances the expression of glutathione S-transferases[10], inhibits NAD(P)H (quinone acceptor) oxidoreductases[11], induces dihydrodiol dehydrogenases[12], shows no notable isoenzyme-specific inhibition of the targeted glutathione S-transferases[13], and causes a marked diuresis and simultaneously an electrolyte imbalance *in vivo* in humans[9]. Another drug used as a modulator in the chemotherapy of malignant tumours is sulfasalazine[14]. The results are somewhat contradictory for the response of cancer patients[14]. Hence there is still a need for isoenzyme specific inhibitors with minor side effects.

Crystallographic studies of glutathione S-transferases indicate that the proteins are organised generally into two domains, a GSH-binding domain at the N-terminus (G-site) and a hydrophobic substrate-binding domain (H-site) composed of the C-terminal two-thirds of the protein[15].

The possibility for the formation of hydrogen bonds between a tyrosine residue of the active centre and the thiol function of glutathione to form a thiolate anion is considered to be an important catalytic mechanism[16]. In an earlier study, the structural requirements for binding at the G-site were investigated by determining the substrate specificities and inhibition constants of a series of glutathione analogues[17]. These studies were considered to constitute a rational approach for the development of new glutathione S-transferase inhibitors. It was assumed that glutathione analogues containing an $O=P(OR)_2$ moiety in place of the cysteinyl residue CH_2SH would bind to the G-site of glutathione S-transferases. On account of the absence of a free thiol function, these analogues should inhibit glutathione S-transferases. Both the interactions with the backbone of the tripeptide and the possibility for the formation of hydrogen bonds with an oxygen atom of the phosphonic acid ester should lead to low binding constants.

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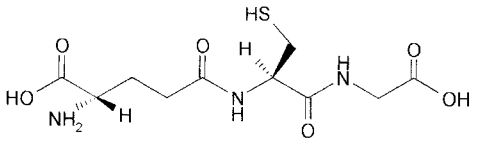
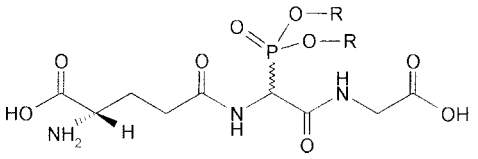


γ-L-Glu-L-Cys-Gly, glutathione



1	a	b	c	d
R	Me	Et	*i*Pr	*n*Bu

Scheme 1

In this paper the first preparation of phosphono-glutathione analogues with the structures given in Scheme 1 is described and their inhibitory properties towards human class Pi glutathione S-transferase are examined.

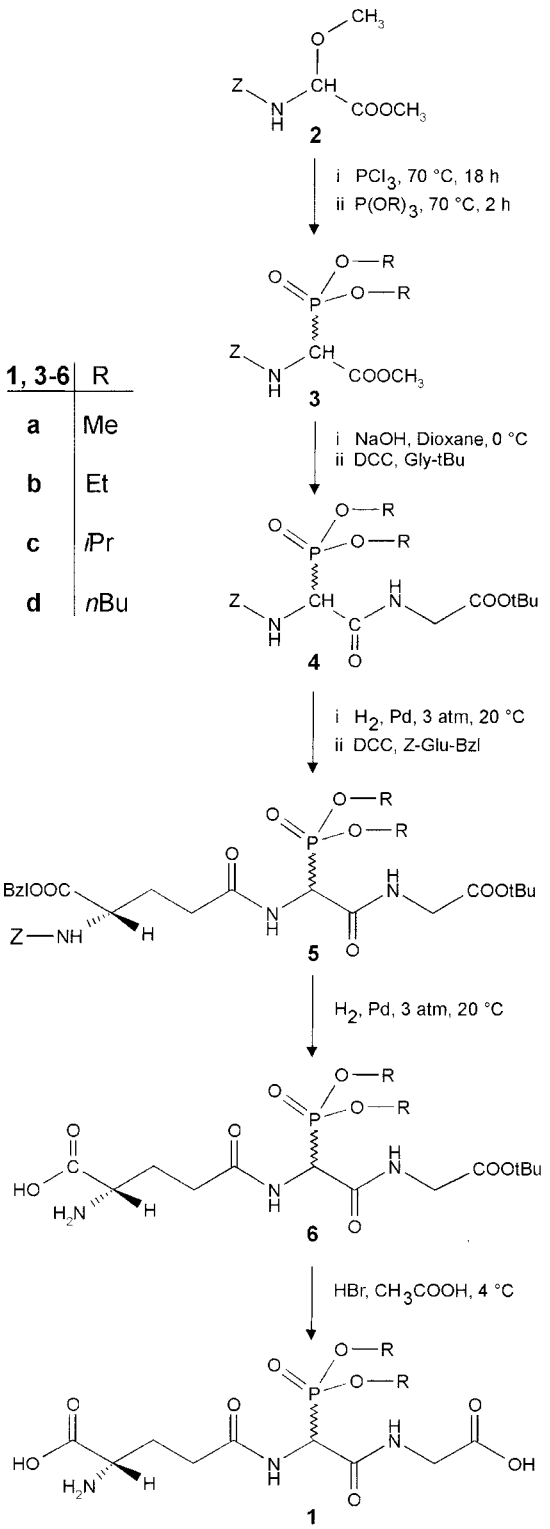
Results and Discussion

Chemistry. To obtain the desired phosphono-glutathione analogues **1a–1d**, a synthetic route was chosen using the methyl *Z*-amino-(dialkyloxyphosphinyl)-acetates **3a–3d** as the central amino acid of the tripeptide.

Compounds **3a–3d** were readily synthesised starting from inexpensive glyoxylic acid and benzyl carbamate via methyl 2-benzyloxycarbonylamino-2-methoxyacetate (**2**) by chlorination and a subsequent by Michaelis-Arbusov reaction with the respective trialkyl phosphites[18]. This multistep reaction could be extended to more lipophilic phosphonic acid esters, e.g. **3d**, without any significant changes in the reaction rate or the occurrence of side reactions. Hence it may be assumed that this reaction route could be used to obtain a greater variety of these amino acid derivatives.

Two routes starting from **3a–3d** are available for the construction of the tripeptides. Either the C-terminal amino acid is introduced after deprotection of the amino functions of **3a–3d** or it is linked with *tert*-butyl glycinate after hydrolysis of the methyl esters **3a–3d**. Since the hydrolysis of the carboxylic ester exhibits the lowest selectivity, this critical step was placed at the beginning of the synthetic sequence and, accordingly, the dipeptides **4a–4d** were prepared first (Scheme 2).

The syntheses of the desired glutathione analogues were performed by solution phase peptide methods comprising hydrogenolytic removal of the Z group and coupling with DCC. The addition of reagents such as 1-hydroxybenzotriazole, used frequently to decrease racemisation[19], was not necessary since no α-amino acids were directly involved (Scheme 2). The introduction of the (S)-glutamoyl residue leads to the diastereomers **5a–5d** in a diastereomeric ratio of about 50 : 50. They were not further separated. A selective cleavage of the protecting groups of the glutamoyl residue was achieved by hydrogenolysis (Scheme 2). Treatment of the *tert*-butyl glycinates **6a–6d**



1, 3-6	R
a	Me
b	Et
c	*i*Pr
d	*n*Bu

Scheme 2

with HBr/acetic acid yielded the desired tripeptides **1a–1d**. The purities of all compounds were checked by HPLC with UV detection or with fluorescence detection using precolumn OPA derivatisation[20]. Under the chosen reaction conditions, no appreciable hydrolysis of the phosphonic esters was observed. The stability of these tripeptides in aqueous solutions was tested at neutral pH. Over a

Table 1. Chromatographic behaviour of the dialkylphosphonates **1**, **3**–**6**.

Cmpd.	t_R values/min ± *s*				Method[a]
	dimethyl	diethyl	diisopropyl	di-*n*-butyl	
3	11.77 ± 0.02	14.82 ± 0.03	17.58 ± 0.04	21.33 ± 0.04	A
4	15.32 ± 0.04	17.64 ± 0.03	19.93 ± 0.06	23.14 ± 0.03	A
5[b]	19.60 ± 0.05 19.71 ± 0.05	21.05 ± 0.05 21.17 ± 0.06	22.56 ± 0.02 22.68 ± 0.02	25.09 ± 0.02 25.21 ± 0.02	A
6	15.57 ± 0.05	16.31 ± 0.02	17.30 ± 0.02	18.69 ± 0.02	B
1[b]	10.41 ± 0.04 10.97 ± 0.04	11.65 ± 0.03 12.15 ± 0.03	13.12 ± 0.02 13.56 ± 0.02	15.47 ± 0.04 15.78 ± 0.03	B

[a] For details see experimental section.
b) Diastereomers were separated, diastereomeric ratios are given in the experimental section.

period of 48 h no remarkable decomposition (>10 %) could be detected by HPLC (method B). The chromatographic behaviour of the synthesised dialkylphosphonates are summarized in Table 1.

Enzyme inhibition. The glutathione analogues **6a–6d** and **1a–1d** were tested for inhibition of the conjugation of 1-chloro-2,4-dinitrobenzene with glutathione catalysed by human placental glutathione S-transferase. The results are given in Table 2. Glutathione S-transferase P1-1 is the predominant form of placental tissue[1a]; thus, it is to be expected that the IC_{50} values obtained from the commercially available enzyme preparation, presented in Table 2, would be in good agreement with those of the purified enzyme.

Table 2. Inhibition of human placental glutathione S-transferases by phosphono analogues of glutathione.

Cmpd.	IC_{50} values/μM			
	dimethyl	diethyl	diisopropyl	di-*n*-butyl
6	912	829	756	688
1	291	139	64	21

Since the inhibition of glutathione S-transferases is highly stereospecific towards the α-carbon of the respective cysteinyl residue[17a, 17d], it is assumed that the IC_{50} values of the "right" diastereomer of **1a–1d** will be about 2-fold lower than those of the diastereomeric mixture. Work by Askelöf et al.[21] had previously shown that the potency of S-functionalised glutathione analogues as inhibitors of rat GSTs correlated positively with the increasing length of the *n*-alkyl chains bonded to the sulfur. These findings are in harmony with the inhibition characteristics of the phosphono analogous of glutathione **6a–6d** and **1a–1d** presented in this paper (Table 2). An addition interaction of the alkyl group of the phosphonic ester unit with the H-site of glutathione S-transferase must most certainly be taken into consideration to explain the high affinities of the lipophilic derivatives. It needs to be clarified by e.g. molecular modelling studies whether this interaction can make a decisive contribution to the inhibition. The generally much improved binding of **1a–1d** in comparison with **6a–6d**, resulting from the cleavage of the *tert*-butyl group, is in contrast to the methyl and ethyl esters of other glutathione analogues whose interactions with the enzyme are only slightly influenced[17a]. A steric hindrance caused by the bulky *tert*-butyl residue might be a possible explanation.

The most effective of the compounds in the series studied in this work is the di-*n*-butyl derivative **1d** with an IC_{50} value in the lower μmolar region (Table 2). This inhibitory potency against human glutathione S-transferase P1-1 is comparable to other glutathione analogues that have shown the ability to enhance chlorambucil toxicity in HT-29 human colon adenocarcinoma cells[22]. The overexpression of GST P1-1 is strongly related to, e.g., adriamycin-resistance of MCF-7 breast cancer cells[23], to cisplatin-resistance of human gastric cancer cells[24], and to chlorambucil of HT-29 cells. Thus, inhibitors of this particular isoenzyme are good candidates for modulation of the drug resistance of these tumours. Whether or not the here presented phosphono analogues can, at least partially, overcome a drug resistance remains to be elucidated. In this context two major problems has to be taken into consideration: first, could these analogues pass the cell membranes? and second, are these analogues stable enough to withstand a degradation by e.g. γ-glutamyl transferase? Additional investigations on the mechanism of inhibition and on the isoenzyme specificity of these glutathione analogues are in progress.

Abbreviations

CDNB, 1-chloro-2,4-dinitrobenzene; H-site, hydrophobic substrate binding site of glutathione S-transferases; G-site, glutathione binding site of glutathione S-transferases; GSH, reduced glutathione; GST, glutathione S-transferase.

Acknowledgements

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Experimental

General: Melting points: Büchi 510 apparatus (uncorr.).– IR data: Perkin Elmer Fourier FTIR 16PC spectrophotometer.– ^{1}H and ^{13}C NMR spectra: Bruker ARX 300 and Bruker AM 400 spectrometer, solvent as indicated, internal standard TMS; ^{13}C NMR spectra were ^{1}H decoupled and assignment of signals was done with the assistance of DEPT135 and GATED experiments.– Thermospray-MS: HP 5989A, solution of compounds in 0.1 M ammonium acetate/methanol 75:25 (*V/V*).– Elemental analysis: CHN-Autoanalyzer, Hewlett-Packard.– Preparative silica gel column chromatography was carried out using Merck 7734 (70–230 mesh) silica gel.– TLC analyses: Macherey-Nagel Polygram SIL G/UV$_{254}$ precoated plastic sheets.– HPLC analyses was performed with Merck-Hitachi equipment with the exception of a Waters 470 fluorescence detector: Method A for protected amino acids and peptides: UV/VIS detection at λ = 210 nm, column dimension 125 × 3 mm, stationary phase Nucleosil 120-5 C18, eluent A water/MeCN 80:20 (*V/V*), eluent B MeCN, linear gradient from 0% B to 100% B, 30 min, flow rate 0.5 ml min^{-1}, Method B for deprotected and peptides: fluorescence detection at λ_{ex} 330 nm and λ_{em} 450 nm, prepacked column as mentioned above, eluent A acetate buffer (50 mM, pH 7.0)/MeOH 80:20 (*V/V*), eluent B MeOH, linear gradient from 0% B to 100% B, 20 min, flow rate 0.5 ml min^{-1}, precolumn derivatization with *ortho*-phthaldialdehyde as described by Graser et al.[20]. (2RS)-Methyl 2-benzyloxycarbonylamino-2-(dimethoxyphosphinyl)-acetate (**3a**) was synthesised by the method of Schmidt et al.[25].

Enzymic Assays

The glutathione S-transferase activity towards 1-chloro-2,4-dinitrobenzene (CDNB) was measured at 25 °C, containing max. 2% ethanol[26]. IC$_{50}$ values were determined by measuring the reaction rate in the presence and in the absence of inhibitor. The concentration of inhibitor giving 50% inhibition, the IC$_{50}$ value, was determined by nonlinear fits of the data to the hyberbolic function: $V_I/V_0 = IC_{50}/(IC_{50} + [I])$[27] where V_0 is the observed activity without inhibitor and V_I is the activity in the presence of inhibitor. The program used was SigmaPlot from Jandel Scientific, Erkrath, Germany. Inhibitor solutions were diluted appropriately before addition to the assay solution in order to maintain a constant concentration of organic solvent (DMSO) when the inhibitor concentration was varied. The reaction was started generally by addition of enzyme preparations.

*General Method for the Preparation of methyl 2-benzyloxycarbonylamino-2-(dialkoxyphosphinyl)-acetates (**3b–3d**)*[28]

0.1 mol of methyl 2-benzyloxycarbonylamino-2-methoxyacetate (**2**)[18a] was dissolved in 100 ml toluene at 70 °C, phosphorus(III) chloride (0.1 mol, 8.75 ml) and the mixture was kept at 70 °C for 18h. The respective trialkyl phosphite (0.1 mol) was subsequently added dropwise to the stirred mixture at 70 °C and stirring was continued for further 2h at 70 °C. After removal of the solvent *in vacuo*, the oil-like residue was redissolved in ethyl acetate, washed with saturated sodium hydrogen carbonate solution (3 × 30 ml), and dried with sodium sulfate. Crystallization of the product was achieved by adding *n*-hexane to this solution.

*(2RS)-(±)-Methyl 2-benzyloxycarbonylamino-2-(diethoxyphosphinyl)-acetate (**3b**)*[29]

Yield 30.8g (86 %) colourless powder.– mp 80 °C (ref.[29c]: 79–81 °C).– IR (KBr): ν = 3228 cm^{-1}, 3038, 2982, 2965, 1751, 1712, 1541, 1329, 1267, 1236, 1208.– ^{1}H-NMR (300.13 MHz, CDCl$_3$): δ = 1.26–1.32 ppm (m, 6H, OCH$_2$C*H*$_3$), 3.80 (s, 3H, OC*H*$_3$), 4.08–4.18 (m, 4H, OC*H*$_2$CH$_3$), 4.82 (dd, J_1 = 9.1 Hz, J_2 = 22.5 Hz, 1H, C*H*P), 5.11, 5.16 (d, $^2J_{ab}$ = 12.2 Hz, 2H, C*H*$_2$Ph), 5.61 (br. d, J = 9.2 Hz 1H, NH), 7.35 (s, 5H, Ph).– ^{13}C-NMR (100.62 MHz, CDCl$_3$): δ = 16.2 (CH$_2$*C*H$_3$), 52.6 (d, $^1J_{C,P}$ = 147.5 Hz, *C*HP), 53.2 (O*C*H$_3$), 63.9 (d, $^2J_{C,P}$ = 5.9 Hz, *C*H$_2$CH$_3$), 67.5 (*C*H$_2$Ph), 128.1, 128.3, 128.5 (H-bonded aromatic C), 136.0 (alkyl-bonded aromatic C), 155.7 (d, $^3J_{C,P}$ = 8.5 Hz, NH*C*OO), 167.4 (*C*OOCH$_3$).– MS (100 eV): *m/z* (%) = 377 (100) [M+NH$_4^+$], 360 (78) [M+H$^+$].– Anal. (C$_{15}$H$_{22}$NO$_7$P).

*(2RS)-(±)-Methyl 2-benzyloxycarbonylamino-2-(diisopropoxyphosphinyl)-acetate (**3c**)*

Yield 30.5 g (75 %) colourless powder.– mp 56 °C.– IR (KBr): ν = 3241 cm^{-1}, 3048, 2979, 2939, 1746, 1707, 1545, 1458, 1312, 1263, 1245, 1222.– ^{1}H-NMR (300.13 MHz, CDCl$_3$): δ = 1.28–1.35 ppm (m, 12H, CHC*H*$_3$), 3.80 (s, 3H, OC*H*$_3$), 4.73 (sept, J = 6.5 Hz, 2H, C*H*CH$_3$), 4.82 (dd, J_1 = 9.2 Hz, J_2 = 22.8 Hz, 1H, C*H*P), 5.10, 5.16 (d, $^2J_{ab}$ = 12.2 Hz, 2H, C*H*$_2$Ph), 5.50 (br. d, J = 6.2 Hz, 1H, NH), 7.35 (s, 5H, Ph).– ^{13}C-NMR (100.62 MHz, CDCl$_3$): δ = 23.7, 24.0, (CH*C*H$_3$), 53.0 (O*C*H$_3$), 53.3 (d, $^1J_{C,P}$ = 147.5 Hz, *C*HP), 67.5 (*C*H$_2$Ph), 72.0 (d, $^2J_{C,P}$ = 6.8 Hz, *C*HCH$_3$), 72.8 (d, $^2J_{C,P}$ = 5.9 Hz, *C*HCH$_3$), 128.2, 128.3, 128.5 (H-bonded aromatic C), 136.0 (alkyl-bonded aromatic C), 155.7, (d, $^3J_{C,P}$ = 8.5 Hz, NH*C*OO), 167.7 (*C*OOCH$_3$).– MS (100 eV): *m/z* (%) = 388 (21) [M+H$^+$], 178 (100).– Anal. (C$_{17}$H$_{26}$NO$_7$P).

*(2RS)-(±)-Methyl 2-benzyloxycarbonylamino-2-(di-n-butoxyphosphinyl)-acetate (**3d**)*

Yield 36.0 g (87 %) colourless powder.– mp 56 °C.– IR (KBr): ν = 3253 cm^{-1}, 3035, 2960, 2936, 1753, 1725, 1533, 1456, 1312, 1269.– ^{1}H-NMR (300.13 MHz, CDCl$_3$): δ = 0.89–0.95 ppm (m, 6H, CH$_2$C*H*$_3$), 1.35–1.41 ppm (m, 4H, C*H*$_2$CH$_3$), 1.60–1.66 ppm (m, 4H, OCH$_2$C*H*$_2$), 3.81 (s, 3H, OC*H*$_3$), 4.02–4.12 ppm (m, 4H, OC*H*$_2$CH$_2$), 4.89 (dd, J_1 = 9.2 Hz, J_2 = 22.4 Hz, 1H, C*H*P), 5.10, 5.16 (d, $^2J_{ab}$ = 12.2 Hz, 2H, C*H*$_2$Ph), 5.60 (br. d, J = 7.5 Hz 1H, NH), 7.35 (s, 5H, Ph).– ^{13}C-NMR (100.62 MHz, CDCl$_3$): δ = 13.5 (CH$_2$*C*H$_3$), 18.6 (*C*H$_2$CH$_3$), 32.3 (OCH$_2$*C*H$_2$), 52.5 (d, $^1J_{C,P}$ = 147.5 Hz, *C*HP), 53.1 (O*C*H$_3$), 65.5 (O*C*H$_2$CH$_2$), 67.5 (*C*H$_2$Ph), 128.1, 128.3, 128.6 (H-bonded aromatic C), 135.9 (alkyl-bonded aromatic C), 155.7 (d, $^3J_{C,P}$ = 7.6 Hz, NH*C*OO), 167.4 (*C*OOCH$_3$).– MS (100 eV): *m/z* (%) = 416 (100) [M+H$^+$].– Anal. (C$_{19}$H$_{30}$NO$_7$P).

*General Method for the Preparation of N-[2-benzyloxycarbonylamino-2-(dialkoxyphosphinyl)-acetyl]-glycine tert-butyl ester (**4a–4d**)*[25]

30 mmol of the corresponding methyl 2-benzyloxycarbonylamino-2-(dialkoxyphosphinyl)-acetate (**3a–3d**) was dissolved in 30 ml dioxane, the solution was cooled to 0 °C and 15 ml of ice cold 2 normal aqueous sodium hydroxide was added subsequently to the mixture. The hydrolysis was controlled by TLC and when completed the dioxane was removed under reduced pressure. The remaining aqueous solution was washed with ethyl acetate, acidified with 6 normal hydrochloric acid and extracted 2 times with ethyl acetate. The combined extracts were dried with sodium sulfate and the solvent was evaporated to give a oil-like or crystalline residue, that was weighted for the calculation of the appropriate amount of coupling reagents (yields: 50–60 %), but not further characterised. A stream of dry gaseous ammonia was passed through a stirred ice-cooled suspension of *tert*-butyl glycinate hydrochloride (1 equiv. refered to the free acids of **3a–3d** respectively) in 80 ml dichloromethane. The precipitate was filtered off and the solvent was carefully removed under reduced pressure. The residue and the respective free acid of **3a–3d** were combined and dissolved in 20 ml dry acetonitrile and solid dicyclohexylcarbodiimide (1.1 equiv.) was added with stirring at 0 °C. Stirring was continued at 0 °C for 1 h and subsequently at room temperature for 14 h. The precipitated urea was filtered off, the filtrate was concentrated *in vacuo* and redissolved in ethyl acetate (100 ml). The solution was washed with 1 normal potassium hydrogen sulfate (30 ml) and with saturated sodium hydrogen carbonate solution (30 ml), dried with sodium sulfate, and evaporated. The oil-like crude products were purified by column silica gel chromatography (eluent: ethylacetat/*n*-hexane 8:2 (*V/V*), column dimension: 500 × 40 mm) and followed by crystallisation from ethyl acetate

*N-[(2RS)-(±)-2-Benzyloxycarbonylamino-2-(dimethoxyphosphinyl)-acetyl]-glycine tert-butyl ester (**4a**)*

Yield 7.3 g (57 %) colourless crystals.– mp 105 °C.– IR (KBr): ν = 3347 cm^{-1}, 3264, 3075, 2980, 2960, 1739, 1712, 1676, 1557, 1522, 1377, 1266, 1244.– ^{1}H-NMR (300.13 MHz, CDCl$_3$): δ = 1.47 ppm (s, 9H, C(C*H*$_3$)$_3$), 3.79, 3.84 (d, J = 10.9 Hz, 6H, POC*H*$_3$), 3.96 (d, J = 5.2 Hz, 2H, Gly-C$_\alpha$*H*$_2$), 4.88 (dd, J_1 = 8.5 Hz, J_2 = 20.5 Hz, 1H, C*H*P), 5.14 (s, 2H, OC*H*$_2$C$_6$H$_5$), 5.72 (br. d, J = 8.0 Hz,1H, NH), 7.20 (br. s, 1H, NH), 7.35 (s, 5H, Ph).– ^{13}C-NMR (100.62 MHz, CDCl$_3$): δ = 28.0 (C(*C*H$_3$)$_3$), 42.5 (Gly-*C*$_\alpha$H$_2$), 51.7 (d, $^1J_{C,P}$ = 150.0 Hz, *C*HP), 54.0, 54.3 (POCH$_3$), 67.5

(CH_2Ph), 82.3 ($C(CH_3)_3$), 128.1, 128.2, 128.5 (H-bonded aromatic C), 136.0 (alkyl-bonded aromatic C), 156.0 (NHCOO), 164.7 (CONH), 168.1 ($COOC(CH_3)_3$).– MS (100 eV): *m/z* (%) = 431 (95) [M+H$^+$], 375 (100).– Anal. ($C_{18}H_{27}N_2O_8P$).

N-[(2RS)-(±)-2-Benzyloxycarbonylamino-2-(diethoxyphosphinyl)-acetyl]-glycine tert-butyl ester (**4b**)

Yield 12 g (87 %) colourless crystals.– mp 78–79 °C.– IR (KBr): ν = 3298 cm^{-1}, 2977, 2931, 1741, 1713, 1661, 1543, 1369, 1261.– ^{1}H-NMR (300.13 MHz, $CDCl_3$): δ = 1.26–1.34 ppm (m, 6H, OCH_2CH_3), 1.47 (s, 9H, $C(CH_3)_3$), 3.95 (d, *J* = 5.1 Hz, 2H, Gly-CαH_2), 4.11–4.23 ppm (m, 4H, OCH_2CH_3), 4.89 (dd, J_1 = 8.5 Hz, J_2 = 21.1 Hz, 1H, CHP), 5.11, 5.17 (d, $^2J_{ab}$ = 12.2 Hz, 2H, CH_2Ph), 5.62 (br. d, *J* = 6.5 Hz 1H, NH), 7.20 (br. s, 1H, NH), 7.35 (s, 5H, Ph).– ^{13}C-NMR (100.62 MHz, $CDCl_3$): δ = 16.3 (CH_2CH_3), 28.0 ($C(CH_3)_3$), 42.5 (Gly-CαH_2), 52.2 (d, $^1J_{C,P}$ = 148.4 Hz, CHP), 63.7 (d, $^2J_{C,P}$ = 6.8 Hz, CH_2CH_3), 64.0 (d, $^2J_{C,P}$ = 5.1 Hz, CH_2CH_3), 67.4 (CH_2Ph), 82.2 ($C(CH_3)_3$), 128.1, 128.2, 128.5 (H-bonded aromatic C), 136.1 (alkyl-bonded aromatic C), 156.0 (NHCOO), 164.9 (CONH), 168.1 ($COOC(CH_3)_3$).– MS (100 eV): *m/z* (%) = 459 (95) [M+H$^+$], 403 (100).– Anal. ($C_{20}H_{31}N_2O_8P$).

N-[(2RS)-(±)-2-Benzyloxycarbonylamino-2-(diisopropoxyphosphinyl)-acetyl]-glycine tert-butyl ester (**4c**)

Yield 8.8 g (60.5 %) colourless powder.– mp 125 °C.– IR (KBr): ν = 3326 cm^{-1}, 3255, 2981, 1743, 1721, 1643, 1528, 1385, 1260.– ^{1}H-NMR (300.13 MHz, $CDCl_3$): δ = 1.25–1.36 ppm (m, 12H, $CHCH_3$), 1.46 (s, 9H, $C(CH_3)_3$), 3.95 (d, *J* = 5.1 Hz, 2H, Gly-CαH_2), 4.76 (sept, *J* = 6.2 Hz 2H, $CHCH_3$), 4.80 (dd, J_1 = 9.0 Hz, J_2 = 22.1 Hz, 1H, CHP), 5.10, 5.17 (d, $^2J_{ab}$ = 12.2 Hz, 2H, CH_2Ph), 5.52 (br. d, *J* = 7.8 Hz 1H, NH), 7.35 (s, 5H, Ph).– ^{13}C-NMR (100.62 MHz, $CDCl_3$): δ = 23.7, 24.0, ($CHCH_3$), 28.0 ($C(CH_3)_3$), 42.5 (Gly-CαH_2), 52.9 (d, $^1J_{C,P}$ = 150.9 Hz, CHP), 67.4 (CH_2Ph), 72.8 (d, $^2J_{C,P}$ = 6.8 Hz, $CHCH_3$), 73.1 (d, $^2J_{C,P}$ = 6.8 Hz, $CHCH_3$), 82.1 ($C(CH_3)_3$), 128.1, 128.2, 128.5 (H-bonded aromatic C), 136.1 (alkyl-bonded aromatic C), 156.1 (NHCOO), 165.1 (CONH), 168.1 ($COOC(CH_3)_3$).– MS (100 eV): *m/z* (%) = 487 (12) [M+H$^+$], 221 (100).– Anal. ($C_{22}H_{35}N_2O_8P$).

N-[(2RS)-(±)-2-Benzyloxycarbonylamino-2-(di-n-butoxyphosphinyl)-acetyl]-glycine tert-butyl ester (**4d**)

Yield 6.3 g (41 %) colourless crystals.– mp 78–79 °C.– IR (KBr): ν = 3336 cm^{-1}, 3265, 2960, 2935, 1743, 1723, 1672, 1528, 1373, 1225.– ^{1}H-NMR (300.13 MHz, $CDCl_3$): δ = 0.87–0.94 ppm (m, 6H, CH_2CH_3), 1.31–1.41 (m, 4H, CH_2CH_3), 1.46 (s, 9H, $C(CH_3)_3$), 1.59–1.67 (m, 4H, OCH_2CH_2), 3.95 (d, *J* = 5.2 Hz, 2H, Gly-CαH_2), 4.05–4.12 ppm (m, 4H, OCH_2CH_2), 4.89 (dd, J_1 = 9.1 Hz, J_2 = 22.3 Hz, 1H, CHP), 5.10, 5.17 (d, $^2J_{ab}$ = 12.1 Hz, 2H, CH_2Ph), 5.60 (br. d, *J* = 8.5 Hz 1H, NH), 7.26 (br. s, 1H, NH), 7.35 (s, 5H, Ph).– ^{13}C-NMR (100.62 MHz, $CDCl_3$): δ = 13.6 (CH_2CH_3), 18.6 (CH_2CH_3), 28.0 ($C(CH_3)_3$), 32.4 (OCH_2CH_2), 42.5 (Gly-CαH_2), 52.1 (d, $^1J_{C,P}$ = 148.4 Hz, CHP), 67.4 (CH_2Ph), 67.6 (OCH_2CH_2), 82.3 ($C(CH_3)_3$), 128.1, 128.2, 128.5 (H-bonded aromatic C), 136.1 (alkyl-bonded aromatic C), 156.0 (NHCOO), 164.9 (CONH), 168.0 ($COOC(CH_3)_3$).– MS (100 eV): *m/z* (%) = 515 (100) [M+H$^+$].– Anal. ($C_{24}H_{39}N_2O_8P$).

General Method for the Preparation of the Fully Protected Tripeptides (**5a–5d**)

A solution of *N*-[2-benzyloxycarbonylamino-2-(dialkoxyphosphinyl)-acetyl]-glycine *tert*-butyl ester (**4a–4d**, 7.5 mmol) in methanol (20 ml) was hydrogenated at 3 atm in the presence of palladium on charcoal (10 %; 0.4 g). After 2–4 h, hydrogenation was complete (checked by TLC.), the suspension was filtered and the filtrate was concentrated *in vacuo*. The unstable *N*-(2-amino-2-dialkoxyphosphinylacetyl)-glycine *tert*-butyl ester was immediately *N*-acylated by reaction with *N*-Benzyloxycarbonyl-L-glutamic acid α-benzyl ester (7.5 mmol) and dicyclohexylcarbodiimide (8.4 mmol) in acetonitrile (20 ml) as described above for the synthesis of *N*-[2-benzyloxycarbonylamino-2-(dialkoxyphosphinyl)-acetyl]-glycine *tert*-butyl ester (**4a–4d**). The crude products were purified by column chromatography on silica gel using ethyl acetate/*n*-hexane 7:3 to 9:1 (*V/V*) as eluent (column dimension: 500 × 30 mm) and by subsequent crystallisation from ethyl acetate.

N-[(N-Benzyloxycarbonyl-Oα-benzyl-(S)-glutamoyl)-(2RS)-(±)-2-amino-(dimethoxyphosphinyl)-acetyl]-glycine tert-butyl ester (**5a**)

Yield 3.2 g (63 %) colourless oil.– IR: ν = 3305 cm^{-1}, 3066, 3035, 2958, 1744, 1700, 1669, 1534, 1369, 1248.– ^{1}H-NMR (300.13 MHz, $CDCl_3$, diastereomeric ratio 52:48): δ = 1.45, 1.46 ppm (s, 9H, $C(CH_3)_3$), 1.90–2.10 (m, 2H, Glu-CβH_2), 2.22–2.39 (m, 2H, Glu-CγH_2), 3.74–3.83 (m, 6H, $POCH_3$), 3.92, 3.94 (d, *J* = 5.1 Hz, 2H, Gly-CαH_2), 4.36–4.55 (m, 1H, Glu-CαH), 5.09, 5.10 (s, 2H, $NHCO_2CH_2C_6H_5$ and 1H, CHP), 5.17 (br. s, 2H, $CHCO_2CH_2C_6H_5$), 5.69, 5.75 (br. d, *J* = 7.9 Hz 1H, $NHCO_2CH_2C_6H_5$), 6.68 (br. d, *J* = 7.3 Hz 1H, CONHCHP), 7.18 (br. t, *J* = 5.0 Hz, 1H, $CONHCH_2$), 7.34 (br. s, 10H, Ph).– ^{13}C-NMR (100.62 MHz, $CDCl_3$): δ = 28.0 ($C(CH_3)_3$), 28.2 (Glu-CβH_2), 31.9 (Glu-CγH_2), 42.5 (Gly-CαH_2), 49.8 (d, $^1J_{C,P}$ = 148.0 Hz, CHP), 53.5 (Glu-CαH) 53.8, 54.4, ($POCH_3$), 67.1, 67.3 (CH_2Ph), 82.3 ($C(CH_3)_3$), 128.2, 128.3, 128.5, 128.6 (H-bonded aromatic C), 135.2, 136.2 (alkyl-bonded aromatic C), 156.0 (NHCOO), 164.8, 165.0 (CONH), 168.1 ($COOC(CH_3)_3$), 171.8 (Glu-COO).– MS (100 eV): *m/z* (%) = 650 (24) [M+H$^+$], 559 (100).– Anal. ($C_{30}H_{40}N_3O_{11}P$).

N-[(N-Benzyloxycarbonyl-Oα-benzyl-(S)-glutamoyl)-(2RS)-(±)-2-amino-(diethoxyphosphinyl)-acetyl]-glycine tert-butyl ester (**5b**)

Yield 3.2 g (63 %) colourless powder.– mp 130 °C.– IR (KBr): ν = 3320 cm^{-1}, 3068, 3037, 2981, 2935, 1751, 1690, 1649, 1540, 1368, 1242.– ^{1}H-NMR (300.13 MHz, $CDCl_3$, diastereomeric ratio 50:50): δ = 1.24–1.33 ppm (m, 6H, OCH_2CH_3), 1.45, 1.46 (s, 9H, $C(CH_3)_3$), 1.90–2.10 (m, 2H, Glu-CβH_2), 2.25–2.39 (m, 2H, Glu-CγH_2), 3.90–3.95 (m, Gly-CαH_2), 4.17 ppm (m_c, 4H, OCH_2CH_3), 4.36–4.57 (m, 1H, Glu-CαH), 5.08 (s, 2H, $NHCO_2CH_2C_6H_5$ and 1H, CHP), 5.17 (s, 2H, $CHCO_2CH_2C_6H_5$), 5.71, 5.78 (br. d, *J* = 7.8Hz 1H, $NHCO_2CH_2C_6H_5$), 6.61, 6.67 (br. d, *J* = 8.6 Hz 1H, CONHCHP), 7.22 (br. t, *J* = 5.0 Hz, 1H, $CONHCH_2$), 7.34 (br. s, 10H, Ph).– ^{13}C-NMR (100.62 MHz, $CDCl_3$): δ = 16.3 (CH_2CH_3), 28.0 ($C(CH_3)_3$), 28.4 (Glu-CβH_2), 31.9 (Glu-CγH_2), 42.5 (Gly-CαH_2), 50.3 (d, $^1J_{C,P}$ = 148.4 Hz, CHP), 53.5 (Glu-CαH), 63.6 (d, $^2J_{C,P}$ = 5.9 Hz, CH_2CH_3), 64.1 (d, $^2J_{C,P}$ = 5.9 Hz, CH_2CH_3), 67.1, 67.3 (CH_2Ph), 82.2, 82.3 ($C(CH_3)_3$), 128.2, 128.3, 128.4, 128.5, 128.6 (H-bonded aromatic C), 135.3, 136.2 (alkyl-bonded aromatic C), 156.3 (NHCOO), 165.0, 165.1 (CONH), 168.1, 168.2 ($COOC(CH_3)_3$), 171.8 (Glu-COO).– MS (100 eV): *m/z* (%) = 678 (59) [M+H$^+$], 225 (100).– Anal. ($C_{32}H_{44}N_3O_{11}P$).

N-[(N-Benzyloxycarbonyl-Oα-benzyl-(S)-glutamoyl)-(2RS)-(±)-2-amino-(diisopropoxyphosphinyl)-acetyl]-glycine tert-butyl ester (**5c**)

Yield 3.75g (71 %) colourless powder.– mp 125 °C.– IR (KBr): ν = 3317 cm^{-1}, 3037, 2977, 2938, 1739, 1687, 1642, 1534, 1456, 1387, 1263.– ^{1}H-NMR (300.13 MHz, $CDCl_3$, diastereomeric ratio 54:46): δ = 1.25–1.35 ppm (m, 12H, $CHCH_3$), 1.45, 1.46 (s, 9H, $C(CH_3)_3$), 1.97 (m_c, 2H, Glu-CβH_2), 2.23–2.39 (m, 2H, Glu-CγH_2), 3.92 (d, *J* = 5.0 Hz, 2H, Gly-CαH_2), 4.43, 4.52 (m_c, 1H, Glu-CαH), 4.75 ppm (m_c, 2H, $CHCH_3$), 5.08, 5.10 (s, 2H, $NHCO_2CH_2C_6H_5$ and 1H, CHP), 5.17 (s, 2H, $CHCO_2CH_2C_6H_5$), 5.71, 5.81 (br. d, *J* = 8.2 Hz 1H, $NHCO_2CH_2C_6H_5$), 6.43, 6.76 (br. d, *J* = 9.0 Hz 1H, CONHCHP), 7.24 (br. t, *J* = 4.0 Hz, 1H, $CONHCH_2$), 7.34 (br. s, 10H, Ph).– ^{13}C-NMR (100.62 MHz, $CDCl_3$): δ = 23.7, 24.0, ($CHCH_3$), 28.0 ($C(CH_3)_3$), 28.5 (Glu-CβH_2), 32.1 (Glu-CH_2), 42.5 (Gly-CαH_2), 50.8 (d, $^1J_{C,P}$ = 142.4 Hz, CHP), 53.4, 53.6 (Glu-CαH), 67.0, 67.3 (CH_2Ph), 73.1 (d, $^2J_{C,P}$ = 5.1 Hz, $CHCH_3$), 82.1, 82.2 ($C(CH_3)_3$), 128.1, 128.3, 128.4, 128.5, 128.6 (H-bonded aromatic C), 135.3, 136.2 (alkyl-bonded aromatic C), 156.3 (NHCOO), 165.1 (CONH), 168.1 ($COOC(CH_3)_3$), 171.8 (Glu-COO).– MS (100 eV): *m/z* (%) = 706 (100) [M+H$^+$].– Anal. ($C_{34}H_{48}N_3O_{11}P$).

N-[(N-Benzyloxycarbonyl-Oα-benzyl-(S)-glutamoyl)-(2RS)-(±)-2-amino-(di-n-butoxyphosphinyl)-acetyl]-glycine tert-butyl ester (**5d**)

Yield 2.9 g (53 %) colourless crystals.– mp 63 °C.– IR (KBr): ν = 3324 cm^{-1}, 3066, 3036, 2960, 2934, 1748, 1700, 1654, 1540, 1368, 1274.– ^{1}H-NMR (300.13 MHz, $CDCl_3$, diastereomeric ratio 50:50): δ = 0.88–0.93 ppm (m, 6H, CH_2CH_3), 1.32–1.40 (m, 4H, CH_2CH_3), 1.45, 1.46 (s, 9H, $C(CH_3)_3$), 1.60–1.66 (m, 4H, OCH_2CH_2), 1.90–2.06 (m, 2H, Glu-CβH_2), 2.27–2.39 (m, 2H, Glu-CγH_2), 3.90, 3.92 (d, *J* = 4.9 Hz, 2H, Gly-CαH_2), 4.10 ppm (m_c, 4H, OCH_2CH_2), 4.35–4.57 (m, 1H, Glu-CαH), 5.09, 5.10 (s, 2H, $NHCO_2CH_2C_6H_5$ and 1H, CHP), 5.17 (s, 2H, $CHCO_2CH_2C_6H_5$), 5.75, 5.81

(br. d, J = 8.2 Hz 1H, N*H*CO$_2$CH$_2$C$_6$H$_5$), 6.63, 6.74 (br. d, J = 9.0 Hz 1H, CON*H*CHP), 7.21 (br. t, J = 4.5 Hz, 1H, CON*H*CH$_2$), 7.34 (br. s, 10H, Ph).– ^{13}C-NMR (100.62 MHz, $CDCl_3$): δ = 13.6 (CH_2CH_3), 18.6 (CH_2CH_3), 28.0 (C(CH_3)$_3$), 28.6 (Glu-CβH$_2$), 32.0 (Glu-CγH$_2$), 32.4 (OCH$_2$CH$_2$), 42.4, 42.5 (Gly-CαH$_2$), 50.1 (d, $^1J_{C,P}$ = 149.2 Hz, CHP), 53.6 (Glu-CαH), 63.6 (d, $^2J_{C,P}$ = 5.9 Hz, CH_2CH_3), 64.1 (d, $^2J_{C,P}$ = 5.9 Hz, CH_2CH_3), 67.1, 67.3 (CH$_2$Ph), 67.7 (d, $^2J_{C,P}$ = 5.1 Hz, OCH$_2$CH$_2$), 82.2, 82.3 (C(CH_3)$_3$), 128.1, 128.3, 128.5, 128.6 (H-bonded aromatic C), 135.2, 136.2 (alkyl-bonded aromatic C), 156.2, 156.3 (NHCOO), 164.9, 165.0 (CONH), 168.0, 168.1 (COOC(CH_3)$_3$), 171.8 (Glu-COO).– MS (100 eV): *m/z* (%) = 743 (9) [M+H$^+$], 225 (100).– Anal. ($C_{36}H_{52}N_3O_{11}P$).

*General Method for the Preparation of (S)-γ-glutamoyl-(2RS)-2-amino-(dialkoxyphosphinyl)-acetyl]-glycine tert-butyl ester (**6a–6b**)*

A solution of *N*-[(*N*-benzyloxycarbonyl-*O*α-benzyl-(S)-glutamoyl)-(2RS)-(±)-2-amino-(di-*n*-alkoxyphosphinyl)-acetyl]-glycine *tert*-butyl ester (**5a–5b**) (2.0 mmol) in methanol (20 ml) was hydrogenated at 3 atm and ambient temp. in the presence of palladium on charcoal (10 %; 0.4 g). After 2–4 h, hydrogenation was complete (checked by TLC.), the suspension was filtered and the filtrate was concentrated *in vacuo*. Ethyl acetate (20 ml) was added to the residue and the suspension was extracted with 20 ml 1N aqueous solution of formate. The aqueous fraction was washed two times with diethyl ether reduced to 5 ml *in vacuo* and freeze-dried. The products were checked by HPLC (method A and B). No UV-active compound nor ninhydrin detectable impurities (> 3%) were detected.

*(S)-γ-Glutamoyl-(2RS)-(±)-2-amino-(dimethoxyphosphinyl)-acetyl-glycine tert-butyl ester monoformate salt (**6a**)*

Yield 0.69 g (73 %) colourless amorphous powder.– IR (KBr): ν = 3110 cm^{-1}, 3005, 1748, 1682, 1552, 1418, 1240.– ^{1}H-NMR (400.13 MHz, [D$_6$]DMSO, diastereomeric ratio 52:48): δ = 1.40 (s, 9H, C(CH_3)$_3$), 1.96 (m$_c$, 2H, Glu-CβH$_2$), 2.46–2.53 (m, 2H, Glu-CγH$_2$), 3.34–3.38 (m, 1H, Glu-CαH), 3.78 (d, J = 6.1 Hz, 2H, Gly-CαH$_2$), 3.75–3.85 (m, 6H, POCH_3), 5.09, 5.11 (dd, J_1 = 9.6 Hz, J_2 = 21.6 Hz, 1H, CHP), 8.22 (s, 1H, HCOOH), 8.54 (m$_c$, 1H, NH), 8.65–8.75 (m, NH, OH).– ^{13}C-NMR (100.62 MHz, [D$_6$]DMSO): δ = 27.3 (Glu-CβH$_2$), 27.9 (C(CH_3)$_3$), 31.7 (Glu-CγH$_2$), 42.2 (Gly-CαH$_2$), 50.1 (d, $^1J_{C,P}$ = 146.7 Hz, CHP), 53.5 (Glu-CαH), 53.1, 53.8 (POCH$_3$), 81.3 (C(CH_3)$_3$),), 163.7 (HCOOH), 164.8, 165.0, 170.5, 171.4 (CONH, COO), 168.3 (COOC(CH_3)$_3$).– MS (100 eV): *m/z* (%) = 426 (60) [M+H$^+$].

*(S)-γ-Glutamoyl-(2RS)-(±)-2-amino-(diethoxyphosphinyl)-acetyl-glycine tert-butyl ester monoformate salt (**6b**)*

Yield 0.81 g (81 %) colourless amorphous powder.– IR (KBr): ν = 3115 cm^{-1}, 3002, 1745, 1654, 1502, 1353, 1249.– ^{1}H-NMR (400.13 MHz, [D$_6$]DMSO, diastereometric ratio 50:50): δ = 1.21 ppm (br. t, J = 5.2 Hz, 6H, CH_2CH_3), 1.40 (s, 9H, C(CH_3)$_3$), 1.86 (m$_c$, 2H, Glu-CβH$_2$), 2.37–2.42 (m, 2H, Glu-CγH$_2$), 3.32–3.35 (m, 1H, Glu-CαH), 3.73, 3.74 (d, J = 5.9 Hz, 2H, Gly-CαH$_2$), 4.04 ppm (m$_c$, 4H, CH_2CH_3), 5.09, 5.11 (dd, J_1 = 9.6 Hz, J_2 = 21.6 Hz, 1H, CHP), 8.22 (s, 1H, HCOOH), 8.54 (m$_c$, 1H, NH), 8.65–8.75 (m, NH, OH).– ^{13}C-NMR (100.62 MHz, [D$_6$]DMSO): δ = 16.28, 16.33 (CH_2CH_3), 27.2 (Glu-CβH$_2$), 27.9 (C(CH_3)$_3$), 31.5, 31.6 (Glu-CγH$_2$), 41.9 (Gly-CH_2), 50.5 (d, $^1J_{C,P}$ = 145.8 Hz, CHP), 53.5 (Glu-CαH), 62.9 (d, $^2J_{C,P}$ = 6.8 Hz, CH_2CH_3), 80.9 (C(CH_3)$_3$), 163.7 (HCOOH), 165.9, 170.9, 171.7 (CONH, COO), 168.5 (COOC(CH_3)$_3$).– MS (100 eV): *m/z* (%) = 454 (100) [M+H$^+$].

*(S)-γ-Glutamoyl-(2RS)-(±)-2-amino-(diisopropoxyphosphinyl)-acetyl-glycine tert-butyl ester monoformiate salt (**6c**)*

Yield 0.91 g (86 %) colourless amorphous powder.– mp 125 °C.– IR (KBr): ν = 2992 cm^{-1}, 2940, 1745, 1648, 1550, 1395, 1254.– ^{1}H-NMR (400.13 MHz, [D$_6$]DMSO, diastereomeric ratio 51:49): δ = 1.19–1.26 ppm (m, 12H, CHCH_3), 1.40 (s, 9H, C(CH_3)$_3$), 1.86 (m$_c$, 2H, Glu-CβH$_2$), 2.32–2.44 (m, 2H, Glu-CγH$_2$), 3.22, 3.26 (t, J = 6.8 Hz, 1H, Glu-CαH), 3.71, 3.73 (d, J = 5.7 Hz, 2H, Gly-CαH$_2$), 4.61 ppm (m$_c$, 2H, $CHCH_3$), 5.00, 5.02 (dd, J_1 = 9.1 Hz, J_2 = 22.4 Hz, 1H, CHP), 8.30 (s, 1H, HCOOH), 8.48 (m$_c$, 1H, NH), 8.64 (m$_c$, NH, OH).– ^{13}C-NMR (100.62 MHz, [D$_6$]DMSO): δ = 23.5, 23.6, (d, $^2J_{C,P}$ = 5.6 Hz, $CHCH_3$), 27.9 (C(CH_3)$_3$), 28.4 (Glu-CβH$_2$), 32.0 (Glu-CγH$_2$), 41.9 (Gly-CαH$_2$), 51.1 (d, $^1J_{C,P}$ = 146.2 Hz, CHP), 53.4, 53.6 (Glu-CαH), 71.4 ($CHCH_3$), 82.0 (C(CH_3)$_3$), 163.5 (HCOOH), 165.8, 170.9, 171.6 (CONH, COO), 168.6 (COOC(CH_3)$_3$).– MS (100 eV): *m/z* (%) = 482 (100) [M+H$^+$].

*(S)-γ-Glutamoyl-(2RS)-(±)-2-amino-(di-n-butoxyphosphinyl)-acetyl-glycine tert-butyl ester monoformiate salt (**6d**)*

Yield 0.84 g (78 %) colourless amorphous powder.– IR (KBr): ν = 2956 cm^{-1}, 1744, 1679, 1567, 1373, 1241.– ^{1}H-NMR (400.13 MHz, [D$_6$]DMSO, diastereomeric ratio 51:49): δ = 0.87 ppm (br. t, J = 7.4 Hz, 6H, CH_2CH_3), 1.31 (m$_c$, 4H, CH_2CH_3), 1.40 (s, 9H, C(CH_3)$_3$), 1.55 (m$_c$, 4H, OCH$_2$CH$_2$), 1.88 (m$_c$, 2H, Glu-CβH$_2$), 2.39 (m$_c$, 2H, Glu-CγH$_2$), 3.36–3.45 (m, 1H, Glu-CαH), 3.73, 3.75 (d, J = 5.8Hz, 2H, Gly-CαH$_2$), 3.99 ppm (m$_c$, 4H, OCH_2CH$_2$), 5.07, 5.10 (dd, J_1 = 9.3 Hz, J_2 = 21.3 Hz, 1H, CHP), 8.21 (s, 1H, HCOOH), 8.52 (m$_c$, 1H, NH), 8.62–8.70 (m, NH, OH).– ^{13}C-NMR (100.62 MHz, [D$_6$]DMSO): δ = 13.6 (CH_2CH_3), 18.3 (CH_2CH_3), 27.1 (Glu-CβH$_2$), 27.8 (C(CH_3)$_3$), 31.4 (Glu-CγH$_2$), 32.1 (OCH$_2$CH$_2$), 41.9 (Gly-CαH$_2$), 50.5 (d, $^1J_{C,P}$ = 147.5 Hz, CHP), 53.0 (Glu-CαH), 66.4 (d, $^2J_{C,P}$ = 6.8 Hz, CH_2CH_3), 80.9 (C(CH_3)$_3$), 163.6 (HCOOH), 165.9, 171.5, 171.6 (CONH, COO), 168.5 (COOC(CH_3)$_3$).– MS (100 eV): *m/z* (%) = 510 (100) [M+H$^+$].

*General Method for the Preparation of (S)-γ-glutamoyl-(2RS)-2-amino-(dialkoxyphosphinyl)-acetyl-glycines (**1a–1d**)*

(S)-γ-Glutamoyl-(2RS)-2-amino-(dialkoxyphosphinyl)-acetyl]-glycine tert-butyl ester (**6a–6d**) (0.1 mmol) were dissolved in ice cold 0.25 N HBr in acetic acid (0.5 ml). The reaction was usually complete after 10–15 h at 4 °C (checked by HPLC, method B). The solvent and excess of reagent was removed with a stream of nitrogen at 0 °C. After evaporation over night (0.05 Torr), the residue was dissolved in 100 µl DMSO for inhibition studies or [D$_6$]DMSO for NMR analysis. The products were checked (method A and B) and quantified (method B) by HPLC. UV-active compounds or ninhydrin detectable impurities were generally below 5%.

*(S)-γ-Glutamoyl-(2RS)-(±)-2-amino-(dimethoxyphosphinyl)-acetyl-glycine hydrobromide (**1a**)*

Yield 0.047 g (92 %) pale brown solid.– ^{1}H-NMR (400.13 MHz, [D$_6$]DMSO, diastereomeric ratio 52:48): δ = 1.96 (m$_c$, 2H, Glu-CβH$_2$), 2.46–2.53 (m, 2H, Glu-CγH$_2$), 3.34–3.38 (m, 1H, Glu-CαH), 3.78 (d, J = 6.1 Hz, 2H, Gly-CαH$_2$), 3.75–3.85 (m, 6H, POCH_3), 4.84 (dd, J_1 = 9.6 Hz, J_2 = 21.6 Hz, 1H, CHP), 8.54 (m$_c$, 1H, NH), 8.65–8.75 (m, NH, OH).– MS (100 eV): *m/z* (%) = 370 (31) [M+H$^+$].

*(S)-γ-Glutamoyl-(2RS)-(±)-2-amino-(diethoxyphosphinyl)-acetyl-glycine hydrobromide (**1b**)*

Yield 0.050 g (94 %) pale yellow solid.– ^{1}H-NMR (400.13 MHz, [D$_6$]DMSO, diastereomeric ratio 50:50): δ = 1.21 ppm (t, J = 7.0 Hz, 6H, CH_2CH_3), 1.98 (m$_c$, 2H, Glu-CβH$_2$), 2.44 (m$_c$, 2H, Glu-CγH$_2$), 3.79, 3.81 (d, J = 5.4 Hz, 2H, Gly-CH_2), 3.93 (m$_c$, 1H, Glu-CαH), 4.04, 4.06 ppm (q, J = 5.4 Hz, 4H, CH_2CH_3), 5.11 (dd, J_1 = 8.7 Hz, J_2 = 21.3 Hz, 1H, CHP), 8.12–8.32 (m, NH, OH), 8.38 (m$_c$, 1H, NH), 8.54 (br. d, J = 9.2 Hz, 1H, NH).– MS (100 eV): *m/z* (%) = 398 (45) [M+H$^+$].

*(S)-γ-Glutamoyl-(2RS)-(±)-2-amino-(diisopropoxyphosphinyl)-acetyl-glycine hydrobromide (**1c**)*

Yield 0.055 g (97 %) pale yellow solid.– ^{1}H-NMR (400.13 MHz, [D$_6$]DMSO, diastereomeric ratio 51:49): δ = 1.20–1.27 ppm (m, 12H, CHCH_3), 2.00 (m$_c$, 2H, Glu-CβH$_2$), 2.50 (m$_c$, 2H, Glu-CγH$_2$), 3.77–3.82 (m, 2H, Gly-CαH$_2$), 3.92 (m$_c$, 1H, Glu-CαH), 4.58–4.64 ppm (m, 2H, $CHCH_3$), 5.08 (dd, J_1 = 9.5 Hz, J_2 = 21.8 Hz, 1H, CHP), 8.26 (m, NH, OH), 8.45 (br. d, J = 9.2 Hz, 1H, NH). MS (100 eV): *m/z* (%) = 426 (44) [M+H$^+$].

*(S)-γ-Glutamoyl-(2RS)-(±)-2-amino-(di-n-butoxyphosphinyl)-acetyl-glycine hydrobromide (***1d***)*

Yield 0.054 g (91 %) pale yellow solid.– ^{1}H-NMR (400.13 MHz, [D$_6$]DMSO, diastereomeric ratio 54:46): δ = 0.87 ppm (t, *J* = 7.3 Hz, 6H, CH_2CH_3), 1.27–1.35 (m, 4H, CH_2CH_3), 1.52–1.57 (m, 4H, OCH_2CH_2), 1.99 (m_c, 2H, Glu-CβH_2), 2.41 (m_c, 2H, Glu-CγH_2), 3.77–3.81 (m, 2H, Gly-CαH_2), 3.96–4.02 ppm (m, 5H, OCH_2CH_2 and Glu-CαH), 5.15 (d, J_1 = 9.3 Hz, J_2 = 21.4 Hz, 1H, CHP), 8.25 (m_c, NH, OH), 8.37–8.39 (m, 1H, NH), 8.54 (br. d, *J* = 9.3 Hz, 1H, NH).– MS (100 eV): *m/z* (%) = 454 (56) [M+H$^+$].

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