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Interaction of L-γ-glutamyl-α,β-dehydroamino acids with γ-glutamyl transpeptidase

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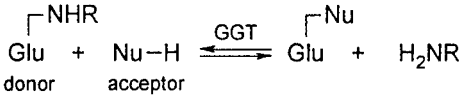
Three γ-glutamyl α,β-dehydroamino acids: L-γ-glutamyl-dehydroalanine, L-γ-glutamyl-(*Z*)-dehydrobutyrine and L-γ-glutamyl-(*E*)-dehydrobutyrine have been prepared as potential ligands (inhibitors or substrates) for γ-glutamyl transpeptidase (GGT). Both isomers of γ-glutamyl-dehydrobutyrines proved to be inhibitors of GGT, slightly better than the saturated analogue, L-γ-glutamyl-L-butyrine. However, their solvolysis catalysed by the enzyme is slower than that of the latter. L-γ-Glutamyl-(*E*)-dehydrobutyrine seems to be a more active compound in both enzymatic tests. L-γ-Glutamyl-dehydroalanine elicited only low inhibitory activity and, moreover, was unstable under conditions of the solvolysis test.

1. Introduction

γ-Glutamyl transpeptidase (GGT; EC 2.3.2.2), a widely distributed membrane-bound enzyme transfers the γ-glutamyl moiety from glutathione, its conjugates or glutamine (a donor) to some acceptors, which may be water or an amino acid (Scheme 1). The enzyme is of major importance in the γ-glutamyl cycle, a metabolic pathway that results in the degradation and the biosynthesis of glutathione, while amino acids may be transported through the membrane into the cell. So, γ-glutamyl transpeptidase functions as a glutathionase and moreover its overall physiological role is to contribute to the intracellular transfer of amino acids [1, 2]. Inhibitors of the enzyme are attracting attention in an effort to develop novel therapeutic drugs, first of all against neoplastic diseases [3, 4]. Substrate properties of some γ-glutamyl compounds are important for drug latentiation [5].

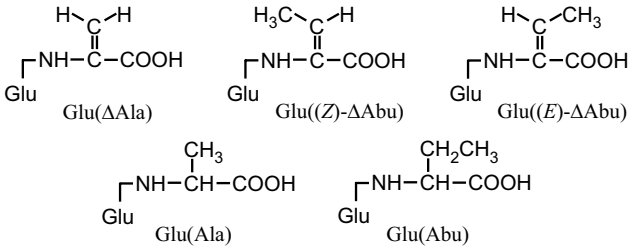
Scheme 1



R = e.g. $CH(CH_2SH)CONH–Gly$, $CH(CH_2SX)CONH–Gly$, H
Nu = e.g. OH, –Xaa, –Xaa–Yaa
aa = amino acid residue

Beside the known physiological substrates a large number of γ-glutamyl amino acids were examined as donors and many amino acids and peptides were studied as γ-glutamyl acceptors [6]. A few model substrates have been developed and L-γ-glutamyl-*p*-nitroanilide and glycylglycine were found to be among the best γ-glutamyl donors and acceptors, respectively [1]. A number of γ-glutamyl derivatives proved to be weak competitive inhibitors of the transpeptidation catalysed by GGT [6].

α,β-Dehydroamino acids are the natural variants of common amino acids. They have a double bond between the C^{α} and C^{β} atoms and thus chirality gets lost and (*Z*)/(*E*) isomerism appears. Both (*Z*) and (*E*)-forms occur in nature [7]. The prototypical molecule of the α,β-dehydroamino acid family is dehydroalanine (ΔAla), and the simplest (*Z*) and (*E*) representatives are (*Z*)-dehydrobutyrine [(*Z*)-ΔAbu] and (*E*)-dehydrobutyrine [(*E*)-ΔAbu], respectively. It seemed interesting whether L-γ-glutamyl α,β-dehydroamino acids, unsaturated analogues of L-γ-glutamyl amino acids, may interact with γ-glutamyl-transpeptidase. Therefore, we prepared L-γ-glutamyl-dehydroalanine [Glu(ΔAla)], L-γ-glutamyl-(*Z*)-dehydrobutyrine [Glu((*Z*)-ΔAbu)] and L-γ-glutamyl-(*E*)-dehydrobutyrine [Glu((*E*)-ΔAbu)], and assessed their behaviour against this enzyme in comparison with their saturated counterparts L-γ-glutamyl-L-alanine [Glu(Ala)] and L-γ-glutamyl-L-butyrine [Glu(Abu)].



2. Investigations, results and discussion

The synthetic pathway followed for the preparation of the α,β-dehydropeptide molecules is depicted in Scheme 2. We performed the condensation of Cbz-glutamine with an α-oxo acid, with azeotropic water removal, isolated the products by CC and purified them by preparative HPLC. The Cbz-protection was removed with trifluoroacetic acid, peptides were liberated from trifluoroacetate anion and purified by ion chromatography. Table 1 lists the analytical data of the target compounds.

The inhibitory activity was determined for the reaction of L-γ-glutamyl-*p*-nitroanilide and glycylglycine by the spectrophotometric measurement of the *p*-nitroaniline released [1]. In the presence of maleate, γ-glutamyl transpeptidase catalyses the formation of γ-glutamyl hydroxamate from hydroxylamine and a number of γ-glutamyl compounds at relative rates which closely approximate those found for transpeptidation reactions with amino acids [8]. This provides the basis for a convenient spectrophotometric measurement of the coloured hydroxamate complex with the ferric ion. The amount of the resulting complex is a measure of the susceptibility of the γ-glutamyl compound to the cleavage by the enzyme, *e.g.* to so called solvolysis. In this test, *S*-methylglutathione is a reference γ-glutamyl donor whose activity is set at 100 [8].

In the present work, three L-γ-glutamyl α,β-dehydroamino acids: Glu(ΔAla), Glu((*Z*)-ΔAbu) and Glu((*E*)-ΔAbu) of

Scheme 2

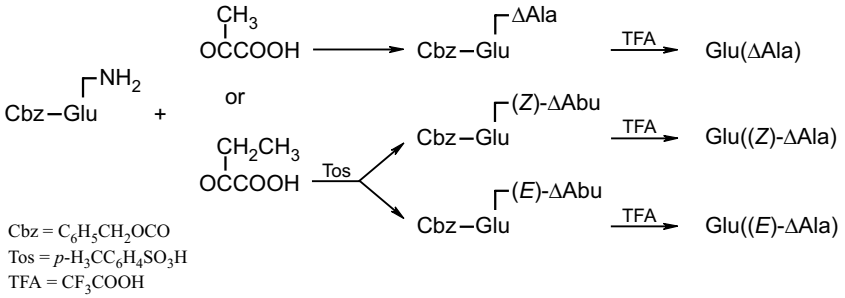


Table 1: Synthesized L-γ-glutamyl-α,β-dehydroamino acids

Peptide	M.p. (°C)	HPLC [a]		Formula Molecular mass C, H, N	TLC: R_f coefficient [b]			
		t_R (min)	Purity (%)		A	B	C	D
Glu(ΔAla)	164–166	5.10	98.0	$C_8H_{12}N_2O_5 \cdot H_2O$ 234.2	0.42	0.34	0.62	0.53
Glu((*Z*)-ΔAbu)	182–185	4.35	98.0	$C_9H_{14}N_2O_5 \cdot ½\ H_2O$ 239.2	0.38	0.36	0.59	0.52
Glu((*E*)-ΔAbu)	189–191	5.00	98.5	$C_9H_{14}N_2O_5 \cdot ½\ H_2O$ 239.2	0.38	0.36	0.59	0.52

[a]: 0.1% TFA/methanol (95 : 5) [b]: A: chloroform/methanol.conc. ammonia (6 : 5 : 2), B: butanol/acetic acid/ethyl acetate/water (1 : 1 : 1 : 1), C: butanone/pyridine/acetic acid/water (14 : 3 : 6 : 15), D: ethanol/water (4 : 1)

purity ≥98% have been synthesised. They constitute a novel class of α,β-dehydropeptides, viz. are α,β-dehydroisopeptides, and were obtained via the condensation of Cbz-glutamine with an α-oxo acid. This simple method of synthesis that gives good results for α-amino acyl α,β-dehydroamino acids [9, 10] is not quite satisfying for γ-glutamyl α,β-dehydroamino acids. The formation of the latter would require a rather novel approach in the future as the dominant reaction that we have found during the condensation is the intramolecular dehydration of the N^{α}-protected glutamine which leads to the N^{α}-protected glutarimide.

L-γ-Glutamyl-(*E*)-dehydrobutyrine and its (*Z*)-isomer proved weak, competitive inhibitors for GGT in the transpeptidation between L-γ-glutamyl-*p*-nitroanilide and glycylglycine. K_i values were 1.2 mM and 2.9 mM, respectively, while the corresponding value of the saturated analogue, L-γ-glutamyl-L-butyrine is reported to be 3.4 mM [6]. For L-γ-glutamyl-L-alanine we determined K_i as equal to 1.0 mM. The inhibitory activity of L-γ-glutamyl-dehydroalanine was lower: the inhibition by the former and latter peptide amounted to 19% and 3%, respectively, after 10 min, under the same conditions of the Tate-Meister test [1] (K_i for this reaction has not been determined). The inhibitory potency of γ-glutamyl-α,β-dehydroamino acids turned out to be of the same order of magnitude as that of the saturated counterparts. It represents also the same order of magnitude as that of the glutamine derivatives with an unsaturated *N*-γ-substituent: cyjanomethyl or allyl, or with the "pseudounsaturated" one, cyclopropylmethyl. These derivatives elicit *in vitro* some promising antiplasmodium activities that are attributed to the GGT inhibition [11].

Table 2 gives the data obtained on the susceptibility of L-γ-glutamyl-(*Z*)- and -(*E*)-dehydrobutyrines to solvolysis. L-γ-Glutamyl-dehydroalanine was unstable under the test conditions, which may be related to the chemical reactivity of the dehydroalanine residue with supernucleophiles [12, 13]. L-γ-Glutamyl-butyrines seem to be cleaved by γ-glutamyl transpeptidase rather readily. Other L-γ-glutamyl amino acids without the C^{α} hydrogen atom as L-γ-glutamyl-isobutyric acid [14] and its-like L-γ-glutamyl-α,α-dialkylamino acids [5] show only weak activity, if any, which is probably caused by steric hindrance. The obtained result is consistent with the little donor specificity found in the hydrolysis reaction, when the structure of γ-glutamyl adduct proved to be irrelevant for the enzyme action provided a free γ-peptide bond proton is present. It is suggested that the more acidic is this proton, the more active is the compound [15]. The acidity of the relevant proton of L-γ-glutamyl-(*Z*)- and (*E*)-dehydrobutyrines and the saturated analogue is now under investigation in our laboratory [16].

Table 2: Solvolysis of γ-glutamyl compounds catalysed by γ-glutamyl transpeptidase

Compound	Relative activity [a]
S-Methylglutathione	100 [b]
L-γ-glutamyl-L-butyrine	95 [5]
L-γ-glutamyl-(*E*)-dehydrobutyrine	~50 [c]
L-γ-glutamyl-(*Z*)-dehydrobutyrine	~43 [c]

[a] Each value represents the average of triplicate determination
[b] Specific activity = 1.5 μmol of L-γ-glutamyl hydroxamate formed min^{-1} (mg of protein)$^{-1}$
[c] Estimated. α-Oxo butyric acid resulting from the decomposition of α,β-dehydrobutyrines forms also the coloured complex with hydroxylamine and the ferric ion. Calculation were performed assuming the ratio of the formed L-γ-glutamyl hydroxamate and α-oxo butyric acid as 1 : 1

The capability of a drug molecule of being a GGT substrate provides the potential for administration of this molecule as the latent drug. The parent drug is liberated in a tissue that has abundant GGT [5]. The substrate activity of γ-glutamyl-α,β-dehydroamino acids promises a possibility for the latentiation in the compounds of the α,β-dehydro-

peptide type, of an *N*-protected peptide drug. The released, on the enzymatic cleavage, α,β-dehydroamino acyl would be processed *in vivo* into the α-oxo acyl [17] protecting the peptide N-terminus.

In conclusion, this preliminary investigation showed that diastereomeric L-γ-glutamyl-(*Z*)- and -(*E*)-dehydrobutyrines are GGT inhibitors, slightly better than their saturated analogue, L-γ-glutamyl-L-butyrine, but they are not so readily solvolysed by this enzyme. L-γ-Glutamyl-(*E*)-dehydrobutyrine seems to be the more active compound in both enzymatic tests. L-γ-Glutamyl-dehydroalanine elicits only low inhibitory activity as compared to that of L-γ-glutamyl-L-alanine and, unfortunately, does not survive under the conditions of the solvolysis test.

3. Experimental

3.1. Synthesis of the compounds

3.1.1. General

Cbz-Glu(NH_2) (m.p. 135–137 °C; $[\alpha]_D^{20} = 7.0°$, c = 2, EtOH) and pyruvic acid came from Fluka. The latter was distilled twice, directly before use (b.p. 45–46 °C at 2 torr; m.p. 11 °C). α-Oxo butyric acid was prepared according to Kozłowski et al. [20]. Purified solvents were stored over molecular sieves. Organic solutions were dried over anh. Na_2SO_4. The solvents from reaction mixtures and CC fractions were removed with a vacuum rotary evaporator at a bath temperature not exceeding 30 °C. Reactions were monitored and the homogeneity of the products was checked using silica gel plates (Merck # 5553). Spots were visualised with fluorescein-bromine, tolidine-chlorine and/or ninhydrin. Analytical and preparative HPLC were performed on a Beckman "System Gold" chromatograph with a Model 168 diode array detector operating at 210 nm. Analytical runs were done using an Alltech Alltima C_{18}, 5 µm, 150 × 4.6 mm column, a 5 µl loop and a flow rate of 1 ml/min. A Separon SGX, C18, 10 µm, 250 × 22 mm column (Czech Republic), a 400 µl loop and flow rate 16 ml/min were applied for preparative runs. The m.p.'s were determined on a Boëtius heating block and are uncorrected. The specific optical rotations were measured in MeOH (c = 1) with a polarimeter Polamat A (Carl Zeiss). ^{1}H NMR spectra were recorded with a 100 MHz Tesla BS567 spectrometer using internal tetramethylsilane. Elemental analyses were performed on a Perkin-Elmer analyser and the results, indicated by symbols C, H, N, were within ± 0.4% of the theoretical values.

3.1.2. L-γ-Glutamyl-L-alanine

The compound was obtained from Cbz-Glu and Ala-OtBu by means of the mixed anhydride with ClCOOiBu. The tBu ester was removed with TFA, and Cbz-protection with H_2/Pd. The peptide analytical data are consistent with the literature data [21].

3.1.3. N^{α}-Benzyloxycarbonyl-L-γ-glutamyl-dehydroalanine

Cbz-Glu(NH_2) (2.90 g, 10 mmol) and pyruvic acid (2.35 g, 25 mmol) in chloroform:1,2-dimethoxyethane (14 ml : 11 ml) were refluxed with azeotropic water removal at bath temperature 95 °C for 7 h. Solvents were evaporated, the residue was dissolved in ethyl acetate (50 ml) and extracted with water (3 × 10 ml). The organic phase was evaporated and the residue was dissolved in methanol (5 ml), applied to a Dowex 2 × 8 column in the acetate form, and eluted with methanol (250 ml) and successively with a 1, 2, 5 and 10% methanolic formic acid (150 ml each). Cbz-Glu(ΔAla) was in the fractions with 5 and 10% acid. Hydroquinone (10 mg) was added and solvents were evaporated. The obtained oil (1.20 g) was dissolved in methanol (2 ml) and subjected to the preparative HPLC with 0.1% TFA : MeOH (75 : 25) to furnish Cbz-Glu(ΔAla) (0.365 g; 10.4% yield) as a hygroscopic foam; HPLC: 0.1% TFA : CH_3CN (70 : 30), t_R 7.22 min, 95% purity; $[\alpha]_D^{20} = -1.1°$; ^{1}N NMR (($CD_3)_2SO$, ppm ⟨Hz⟩) 1.79–2.05 (m, 4 H, $^{\beta}CH_2CH_2^{\gamma}$), 4.01 (m, 1 H, $C^{\alpha}H$), 5.07 (s, 2 H, PhCH_2), 5.67 (s, 1 H, =CH_2(*Z*)), 6.24 (s, 1 H, =CH_2(*E*)), 7.36 (s, 5 H, C_6H_5), 7.55 (d ⟨7⟩, 1 H, OCON*H*), 9.06 (s, 1 H, CON*H*C=CH_2). Cbz-Glu(ΔAla) is unstable and after seven days' storage sets in to decompose yielding Cbz-Glu(NH_2) and other compounds.

3.2. N^{α}-Benzyloxycarbonyl-L-γ-glutamyl-(Z)- and (E)-dehydrobutyrine

Cbz-Glu(NH_2) (4.35 g, 15 mmol), α-oxo butyric acid (2.04 g, 16.5 mmol) and *p*-toluenesulfonic acid hydrate (0.19 g, 1 mmol) in chloroform : 1,2-dimethoxyethane (14 ml : 11 ml) were refluxed as above. Solvents were evaporated, the residue was dissolved in ethyl acetate (60 ml) and extracted with water (3 × 10 ml). The organic phase was evaporated, the residue was dissolved in MeOH (5 ml), applied to a Dowex 2 × 8 column in the acetate form and eluted with methanol (250 ml) and successively with a 1, 2, 5 and 10% methanolic acetic acid (100 ml each). Cbz-Glu(ΔAbu) was in the fractions with 5 and 10% acid, and they were evaporated. The obtained oil (2.28 g) was dissolved in methanol (11.5 ml) and subjected to the preparative HPLC with 0.1% TFA : MeOH (70 : 30). The separated isomers (*Z*) and (*E*) were crystallised from acetone/petroleum ether to give 0.624 g (11% yield) and 0.227 g (4% yield), respectively.

Cbz-Glu((*Z*)-ΔAbu); m.p. 193–195 °C; $[\alpha]_D^{20} = -4.5°$; HPLC: 0.1% TFA : CH_3CN (70 : 30), t_R 4.00 min, 98.5% purity; ^{1}N NMR ($CDCl_3$ + $(CD_3)_2SO$, ppm ⟨Hz⟩) 1.61 (d ⟨7⟩, 3 H, =CHCH_3), 1.79–2.05 (m, 4 H, $^{\beta}CH_2CH_2^{\gamma}$), 4.05 (m, 1 H, $C^{\alpha}H$), 5.06 (s, 2 H, PhCH_2), 6.46 (q ⟨7⟩, 1 H, =$CH$$CH_3$), 7.34 (s, 5 H, C_6H_5), 7.42 (d ⟨7⟩, 1 H, OCON*H*), 8.93 (s, 1 H, CON*H*C=CH).

Cbz-Glu((*E*)-ΔAbu); m.p. 173–175 °C; $[\alpha]_D^{20} = -5.0°$; HPLC: 0.1% TFA : CH_3CN (70 : 30), t_R 4.78 min, 99.0% purity; ^{1}N NMR ($CDCl_3$ + $(CD_3)_2SO$, ppm ⟨Hz⟩) 1.84 (d ⟨7⟩, 3 H, =CHCH_3), 1.79–2.05 (m, 4 H, $^{\beta}CH_2CH_2^{\gamma}$), 4.05 (m, 1 H, $C^{\alpha}H$), 5.06 (s, 2 H, PhCH_2), 5.95 (q ⟨7⟩, 1 H, =$CH$$CH_3$), 7.34 (s, 5 H, C_6H_5), 7.42 (d ⟨7⟩, 1 H, OCON*H*), 8.98 (s, 1 H, CON*H*C=CH).

3.3. L-γ-Glutamyl-dehydroalanine and L-γ-glutamyl-(Z)- and -(E)-dehydrobutyrine

0.5 mmol of Cbz-Glu(ΔAla) (0.175 g) or Cbz-Glu((*Z*)-ΔAbu) or Cbz-Glu((*E*)-ΔAbu) (0.182 g) in anh. TFA (10 ml) was left standing in the dark for 5 days and then the acid evaporated. Ether was added (20 ml) and evaporated. The operations were repeated three times. The (*E*)-compound was crystallised from acetone/petroleum ether. Two remainders were crude. These trifluoroacetates were dissolved in water (2 ml) and loaded on a Dowex WGR column in the acetate form (Serva 41450, a 5 ml bed). Glu(ΔAla) was eluted with 5% acetic acid and both Glu(ΔAbu) isomers were eluted with water alone. Solvents were evaporated the residue was dissolved in water (2 ml) and precipitated with ethanol. Yield of Glu(ΔAla), Glu((*Z*)-ΔAbu) and Glu((*E*)-ΔAbu) amounted to 10%, 60 and 65%, respectively. Analytical data of the compounds are given in Table 1.

3.4. Enzymatic investigations

3.4.1. General

γ-Glutamyl transpeptidase (type 1; 6 µ/mg), L-γ-glutamyl-*p*-nitroanilide, glycylglycine, *S*-methylglutathione, sodium maleate, hydroxylamine hydrochloride and $MgCl_2$ came from Sigma. Ferric chloride reagent was prepared according to Pamilijans et al. [22]. Spectrophotometric measurements were performed on an UV-VIS Specord M40 at 410 nm for L-γ-glutamyl-*p*-nitroanilide and at 535 nm for L-γ-glutamyl hydroxamate.

3.4.2. Inhibitory activity

The enzyme assay was conducted as described by Tata and Meister [1]. The concentrations of potential inhibitors, L-γ-glutamyl-*p*-nitroanilide ($K_M = 0.9$ mM) and glycylglycine were, 1, 0.5–2 and 20 mM, respectively. The apparent K_i values were derived from Lineweaver-Burk plots.

3.4.3. Solvolysis

The enzyme assay was performed according to Magnan et al. [5] with the modifications as follows. The final volume (1 ml) contained Tris-HCl buffer (0.12 M; pH 8.0), a γ-glutamyl compound (10 mM), hydroxylamine hydrochloride (200 mM), sodium maleate (50 mM), $MgCl_2$ (0.7 mM) and GGT (1 u/ml). After incubation at 37 °C for 30 min, ferric chloride reagent (1.5 ml) was added.

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