Peptide *p*-nitrophenylanilides containing (*E*)-dehydrophenylalanine—synthesis, structural studies and evaluation of their activity towards cathepsin C†

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Tetrapeptide *p*-nitroanilides containing (*E*)-dehydrophenylalanine were synthesized and evaluated as inhibitors and substrates of cathepsin C. Peptides containing a free, unblocked amino group appeared to be quite good substrates of the enzyme, whereas fully protected peptides acted as very weak inhibitors. Structural studies by means of NMR and CD, alongside with molecular modelling, have proved that these peptides are hydrolysed in one step by direct removal of *p*-nitroaniline from the tetrapeptide.

Introduction

Dehydroamino acids contribute in a catalytic role in the active sites of some yeast and bacterial enzymes,[1] as well as occuring in a variety of peptide antibiotics of bacterial origin, including the lantibiotics[2] (nisin, subtilin, epidermin, gallidermin) and more highly modified peptides.

Dehydroamino acid residues in peptides have been found to influence the main-chain and side-chain dramatically, due to the presence of the $C^{\alpha}{=}C^{\beta}$ double bond.[3] For example, dehydroalanine adopts a roughly planar conformation with *trans* orientation for the ψ and φ torsions and induces an inverse γ-turn in the preceding residue.[1] (*Z*)-Dehydrophenylalanine exerts a β-turn conformation in short peptides[4] and 3_{10}-helical conformation in the case of peptides with longer main-chain.[5–7] This suggests that dehydroamino acid residues exert a powerful conformational influence, independent on other constraints. Thus, introduction of dehydroamino acid residues into bioactive peptide sequences has become a useful tool to study structure–function relationships and to provide new analogues of enhanced activity.

Cathepsins form quite a large family of lysosomal proteases involved in many physiological functions in the human body. Elevated activity of these enzymes in serum or the extracellular matrix often signifies a number of gross pathological conditions. Cathepsin-mediated diseases include: Alzheimer's disease, numerous types of cancer, autoimmune related diseases such as arthritis and the accelerated breakdown of bone structure seen with osteoporosis. Cathepsin C (dipeptidyl dipeptidase I; EC 3.4.14.1) belongs to the papain family of proteases[8] and sequentially removes dipeptides from the free N-termini of proteins and peptides. It has a broad substrate specificity being able to hydrolyse out nearly every possible dipeptide unit, with the exception of those containing basic amino acids (Arg or Lys) at the N-terminal position or Pro on either side of the scissile bond. It is also quite unusual in that it requires the presence of halide ions for its activity. The main function of cathepsin C is protein degradation in lysosymes, but it is also found to participate in the activation in cytotoxic T lymphocytes and natural killer cells (granzymes A and B), mast cells (tryptase and chymase), and neutrophils (cathepsin G and elastase) by removing their N-terminal activation dipeptides.[9,10] Loss of function mutations in the cathepsin C gene result in periodontal disease and palmoplantar keratosis.[11]

Since dehydroamino acids are quite reactive and various thiol nucleophiles are known to add to their double bonds[9,12] we speculated that short dehydropeptide mimetics of artificial substrates of cathepsin C might act as alkylating inhibitors of the enzyme. Quite surprisingly, however, tri- and tetrapeptides containing ΔAla and Δ^{Z}Phe residues acted only as substrates of cathepsin C with activity comparable to their classic counterparts.[13] The results of structural and conformational investigations have shown that in majority of these peptides we have observed conformations similar to these found for model peptides.[14]

In order to understand better the observed phenomenon we have synthesized a set of analogous peptides (Fig. 1) containing Δ^{E}Phe in place of Δ^{Z}Phe and determined their conformations in solution, evaluated their influence on cathepsin C and modelled their interactions with the enzyme by means of quantum chemistry methods.

Results and discussion

Chemistry

The synthesis of dehydropeptide mimetics containing Δ^{E}Phe is not trivial and is still challenging. According to the literature,

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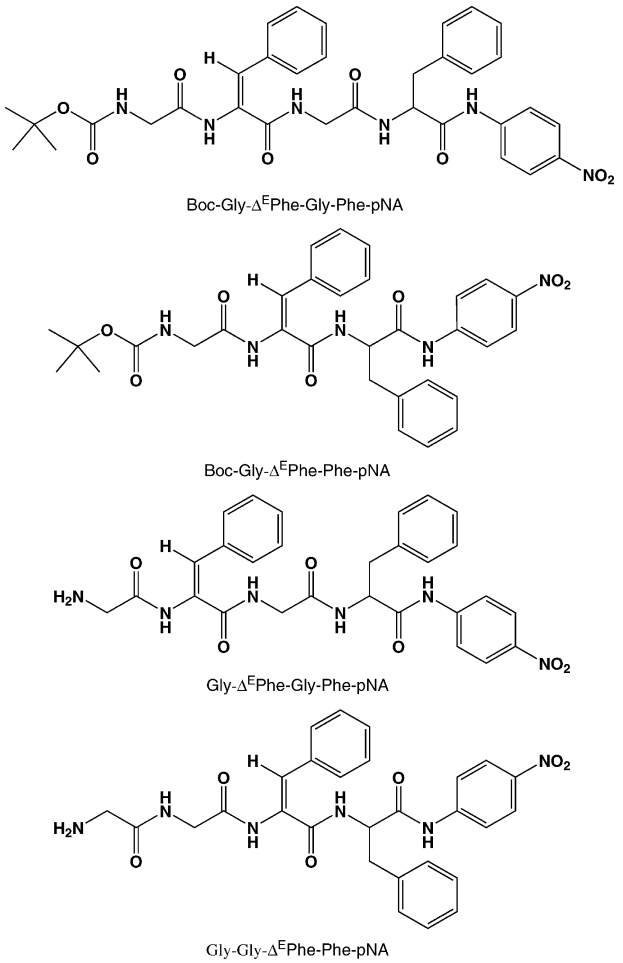
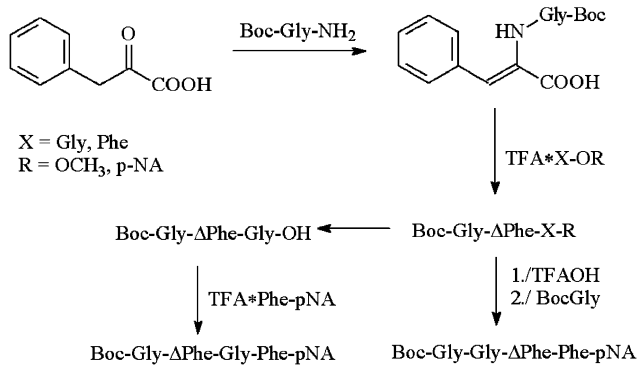


Fig. 1 Structures of investigated peptides.

the only way of their synthesis relies on photoisomerization of Δ^ZPhe to the desirable (*E*)-isomer.[8] The strategy applied in this work is briefly illustrated in Scheme 1. Dehydropeptides were obtained by a standard solution method using a mixed anhydride procedure. The starting substrate, fully protected dehydrotripeptide-Boc-Gly-Δ^EPhe-Gly-OMe, was obtained according to the literature as a mixture containing nearly equivalent quantities of both Δ^ZPhe and Δ^EPhe isomers.[15] Successful resolution of these isomeric dehydrodipeptides by column chromatography followed by deprotection of either N- or C-termini under mild conditions enabled to elongate the peptide chain and finally to obtain the desired dehydrotetrapeptides shown in Fig. 1. It is of note that Δ^EPhe is far less thermodynamically stable than its (*Z*) counterpart and it has a tendency to isomerise under the reaction conditions. Thus upon the condensation step leading to Boc-Gly-Δ^EPhe-Phe-*p*NA the product was contaminated with Boc-Gly-Δ^ZPhe-Phe-*p*NA. Resolution of these peptide isomers was achieved by column chromatography and fractional crystallization.

Structural and conformational studies

NMR and CD studies were undertaken in order to compare structures and conformational preferences of dehydropeptides



Scheme 1 Synthesis of investigated peptides.

shown in Fig. 1 with those exhibited by their counterparts containing either Δ^ZPhe or Phe in the place of Δ^EPhe.

CD spectroscopy. CD measurements were carried out in two solvents (methanol and acetonitrile) for the region down to 200 nm because at shorter wavelengths there are overlapping contributions of the peptide, aromatic and unsaturated chromophores which makes the analysis very complex. In the near-UV region Cotton effects associated with the (*E*)-dehydrophenylalanine and *p*-nitroanilide groups were observed. They are strongly dependent on the peptide conformation and therefore very useful in this type of conformational studies. The obtained CD spectra are presented in Fig. 2. In the case of methanolic solutions of Boc-Gly-Δ^EPhe-Phe-*p*NA and Gly-Δ^EPhe-Gly-Phe-*p*NA a very strong and large positive band at 280 nm and negative band at 309 nm were observed with spectra of both compounds being very similar. This suggests that they adopt very similar and quite rigid conformations in methanol. Quite oppositely, in the case of Gly-Gly-Δ^EPhe-Phe-*p*NA CD bands of very low intensity were observed, which suggests that conformation of this peptide in methanol is completely unordered and that (*E*)-dehydrophenylalanine most likely exerts no influence on peptide chain conformation. When using acetonitrile as a solvent quite similar spectra have been obtained for all three studied compounds. In every case a strong positive band in the range 280–290 nm and negative band at 310 nm were observed, which suggests that peptides adopt ordered conformations in this solvent.

NMR spectroscopy. In order to find some evidence of the presence of intramolecular hydrogen bonds in the studied peptides we have performed measurements of the dependence of chemical shifts of their amide protons on solvent polarity. The experiments have been made in chloroform and in mixtures of $CDCl_3$–DMSO of various concentration. The obtained results are presented in Tables 1 and 2.

Generally, if an amide proton is involved in creation of hydrogen bonding its chemical shift is independent of the polarity of the solvent and would remain at the same position invarient of the concentration of DMSO in chloroform. The obtained results suggest that in this group of peptides there are no observed strong hydrogen bonds—in all cases chemical shifts of amide protons increase with the increase of the solvent polarity. However, the problems with solubility of the studied compounds and observed overlapping of amide

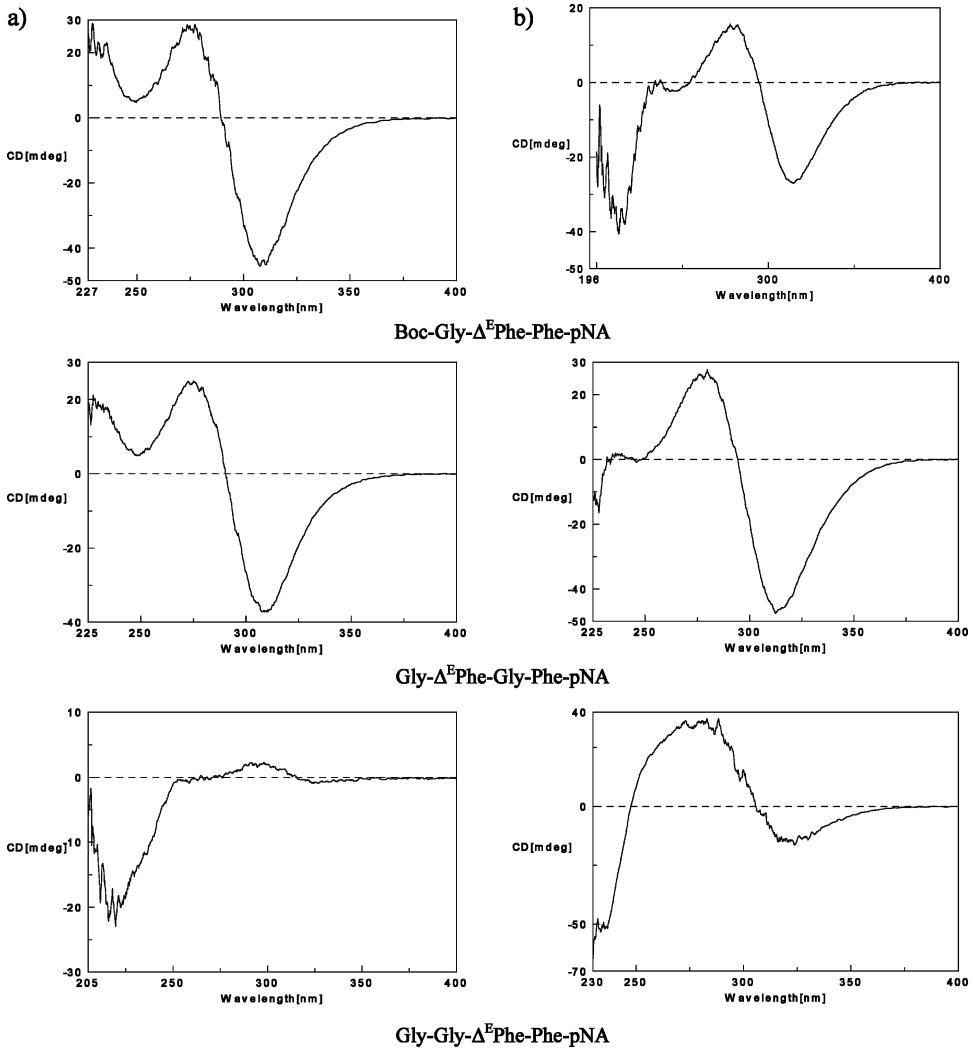


Fig. 2 CD spectra of peptides obtained in (a) methanol and (b) acetonitrile solution.

signals with signals derived from aromatic protons made this experiment not easy to interpret and therefore of limited usefulness.

It is well known that an increase of temperature results in changing values of chemical shifts of amide protons. The lack of such a change suggests that the amide proton is involved in creation of hydrogen bonding. The main parameter indicating the presence of a hydrogen bond is the value of $d\delta/dT$ [ppm K^{-1}]. It is well established that the existence of a hydrogen bond is reflected in the value of this coefficient being lower than 0.004 ppm K^{-1}. Observed chemical shifts of amide protons *vs.* temperature recorded in DMSO solutions are shown in Table 3.

Table 1 Dependence of solvent polarity on amide proton chemical shifts for Gly-Δ^{E}Phe-Gly-Phe-*p*NA

	δ (ppm)			
% DMSO in $CDCl_3$	NH *p*NA	NH Δ^{E}Phe	NH Gly[3]	NH Gly[1]
$CDCl_3$	—	—	—	—
5	—	—	—	—
10	—	—	—	—
15	10.50	9.95	8.13	—
20	10.49	9.97	8.42	7.79
30	10.64	9.96	—[a]	—[a]
40	10.75	9.94	—[a]	8.59
50	10.75	10.30	—[a]	8.59

[a] Overlapping with signals of aromatic protons.

Table 2 Dependence of solvent polarity on amide protons chemical shifts for Boc-Gly-Δ^{E}Phe-Gly-Phe-*p*NA

	δ (ppm)				
% DMSO in $CDCl_3$	NH Δ^{E}Phe	NH *p*NA	NH Gly[3]	NH Phe	NH Gly[1]
$CDCl_3$	9.02	8.66	—	—	—
5	9.83	9.31	—	—	5.68
10	9.99	9.41	8.39	8.27	5.97
15	10.09	9.47	8.61	8.32	6.18
20	10.16	9.54	8.74	8.34	6.37
30	10.22	9.68	8.84	8.36	6.63
40	10.23	9.76	8.89	8.36	6.78
50	10.22	9.85	8.90	8.39	6.87

Based on the data presented in Table 3 it might be assumed that in solutions of Gly-Δ^EPhe-Gly-Phe-*p*NA the only hydrogen bonding is formed by N-terminal glycine, because in the case of NHGly(1) the observed $d\delta/dT$ was equal to 0.002 ppm K^{-1} and thus it is lower than the indicative value of 0.004. Far more interesting results were obtained for Gly-Gly-Δ^EPhe-Phe-*p*NA where recorded values of $d\delta/dT$ clearly indicate involvement of two amide moieties in hydrogen bonding. These are amide groups derived from: *p*-nitroanilide ($d\delta/dT$ = 0.0035) and N-terminal glycine ($d\delta/dT$ = 0.0018 ppm K^{-1}). These results were compared with those obtained for the substrate of both tetrapeptides, namely Boc-Gly-Δ^EPhe-Phe-*p*NA tripeptide. In this case only one amide group, derived from the nitroanilide part of the molecule, is involved in hydrogen bond formation (for NH*p*NA the observed $d\delta/dT$ was 0.0036 ppm K^{-1}). The results, although limited to only three examples, enable to notice that elongation of the peptide chain towards N-termini resulted in a more ordered structure of the molecule. Anyway, comparison of the abilities of hydrogen bond formation by dehydrotetrapeptides containing Δ^EPhe, Δ^ZPhe and Phe have shown, that the peptides contain-

Table 3 Temperature dependence on chemical shifts of amide protons in Gly-Δ^EPhe-Gly-Phe-*p*NA

	$d\delta/dT$ (ppm K^{-1})		
	G-Δ^EF-G-F-*p*NA	G-G-Δ^EF-F-*p*NA	Boc-G-Δ^EF-F-*p*NA
NH *p*NA	0.0057	0.0035	0.0036
NH Δ^EPhe	0.0052	0.0071	0.0101
NH Phe	0.0080	0.0104	0.0122
NH Gly(1)	0.0020	0.0018	0.0114
NH Gly	0.0078	0.0050	—

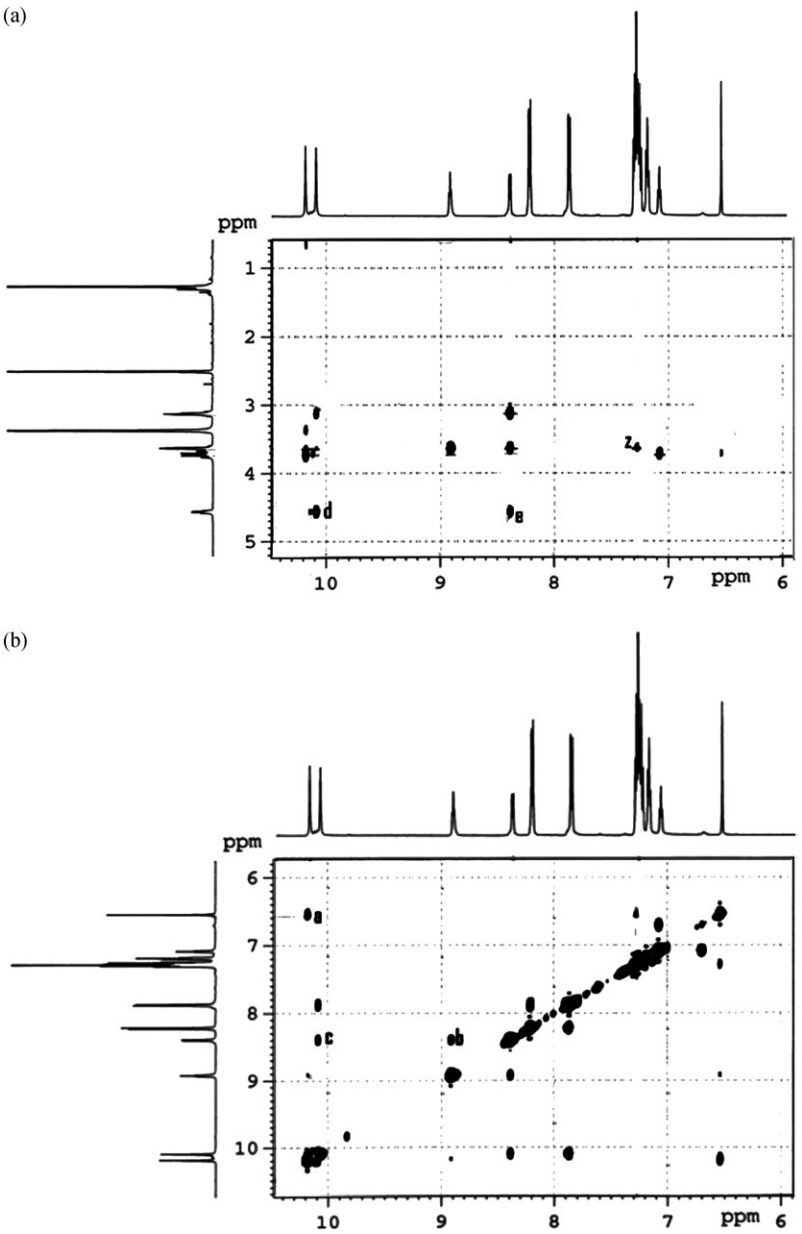


Fig. 3 Two-dimensional ROESY spectra of Boc-Gly-Δ^EPhe-Gly-Phe-*p*NA in DMSO.

ing the (*Z*) isomer of dehydrophenylalanine more closely resemble those containing phenylalanine than do peptides of Δ^{E}Phe.

The most useful information about conformational preferences of investigated peptides had been obtained from NOESY and ROESY experiments. Two-dimensional spectra of Boc-Gly-Δ^{E}Phe-Gly-Phe-*p*NA, for which the most interesting results were obtained, are shown in Fig. 3. Analysis of nondiagonal signals recorded in DMSO allowed the following contacts to be detected: (a) NH Δ^{E}Phe ↔ $C^{\beta}H$ Δ^{E}Phe, (b) NH Gly[3] ↔ NH Phe, (c) NH *p*NA ↔ NH Phe, (d) NH *p*NA ↔ $C^{\alpha}H$ Phe, (e) NH Phe ↔ $C^{\alpha}H$ Phe, (f) CH_3 Boc ↔ $C^{\beta}H_2$ Phe. Based on these data (especially the contact between CH_3 Boc ↔ $C^{\beta}H_2$ which suggests turned conformation of peptide chain) the conformation of this dehydropeptide was proposed (Fig. 4). The most significant, from a conformational point of view, are contacts between amide protons of neighbouring amino acid residues (b and c), which clearly indicate the existence of a turned conformation. These results, reinforced by molecular modeling, also confirm the possibility of formation of intramolecular hydrogen bonds between hydrogen of *p*-nitroanilide and carboxylate oxygen of glycine(3), and thus in some manner confirm the results from temperature studies.

Enzymatic studies

All four dehydrotetrapeptides were evaluated as substrates and inhibitors of cathepsin C. Those which possess an unblocked N-terminal moiety (studied as trifluoroacetates) exerted quite good substrate activity, whereas Boc-peptides acted as very weak inhibitors of the enzyme causing around 5% inhibition of its activity. In Table 4, the substrate activities of the studied peptides are compared with those determined earlier[13] for their counterparts containing Δ^{Z}Phe and Phe and additionally with that obtained for the standard artificial substrate of the enzyme—*p*-nitroanilide of glycylphenylalanine.

As seen from Table 4 affinities of the dehydropeptides containing Δ^{E}Phe towards cathepsin C are significantly higher (which is represented by lower K_M values) than those determined for Δ^{Z}Phe-containing dehydrotetrapeptides, as well as those found for the model tetrapeptides. As expected, they are an order of magnitude more weakly bound than the standard substrate—*p*-nitroanilide of glycylphenylalanine. There is no possibility to draw any meaningful relationship between the structure of the peptide and the rate of its hydrolysis represented by the V_{max} value (the higher the V_{max} value, the better is the substrate).

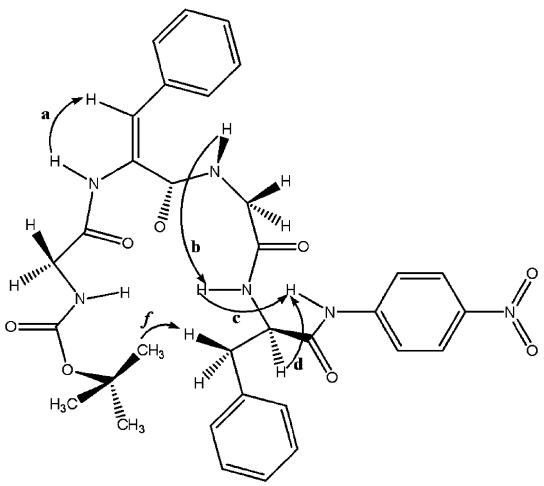


Fig. 4 Schematic conformation of Boc-Gly-Δ^{E}Phe-Gly-Phe-*p*NA in DMSO solution.

Table 4 Substrate activity of dehydrotetrapeptides towards cathepsin C

Peptide	Michaelis constant, K_M/mM	V_{max}/μmol min^{-1}
Gly-Δ^{E}Phe-Gly-Phe-*p*NA	3.2	0.008
Gly-Δ^{Z}Phe-Gly-Phe-*p*NA	7.8	0.224
Gly-Phe-Gly-Phe-*p*NA	22.5	0.075
Gly-Gly-Δ^{E}Phe-Phe-*p*NA	4.3	0.060
Gly-Gly-Δ^{Z}Phe-Phe-*p*NA	7.8	0.003
Gly-Gly-Phe-Phe-*p*NA	Insoluble in water	
Gly-Phe-*p*NA	0.23	0.014

Molecular modelling

The affinity of inhibitors to proteins is determined by the intermolecular interactions in the ligand–receptor system. The understanding of the physical nature of these interactions is vital for understanding the molecular assembly of the enzyme–inhibitor complex and for understanding the mode of inhibitory action.

Optimal conformations of the inhibitory dehydrotetrapeptides were obtained by using Gaussian03 at the HF/6-31g (d,p) level[16] in the gas phase and they were docked to the active site of cathepsin C available from Protein Data Bank EC 3.4.14.1[17] by using the AutoDock program.[18,19] We chose these structures for the docking process, because they were in good agreement with the structures predicted from the NMR experiment. Moreover, use of AutoDock to the docking process required good assignment of charge, which we obtained from the *ab initio* calculations with the Merz–Singh–Kollman scheme.[20] It was possible to use these structures, because in the next step the complexes of dehydropeptides with enzyme were optimized using Accelrys's DISCOVER program with the cff97 force field. After the docking process, for each peptide we obtained a variety of structures, where each showed a different binding mode. In the next stage we selected the most probable docked structures,

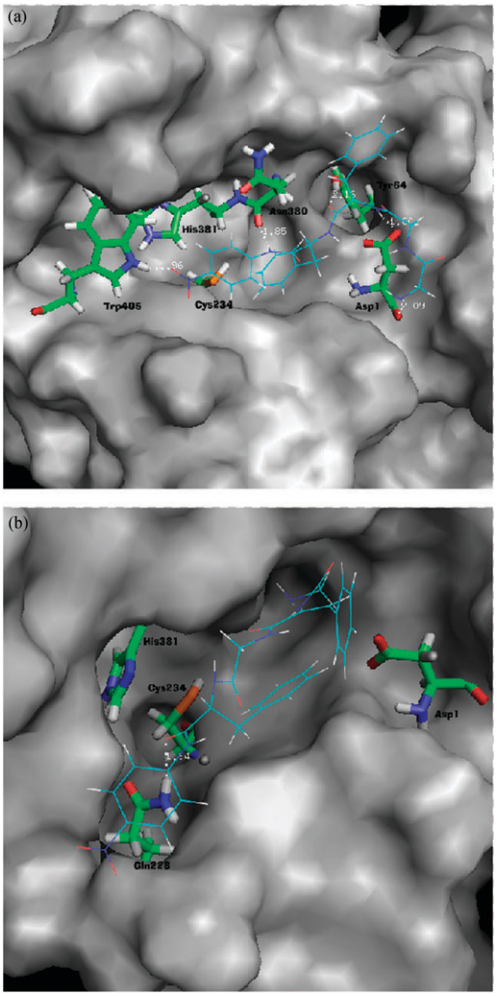


Fig. 5 Binding of Gly-Gly-Δ^EPhe-Phe-*p*NA (top) and of Gly-Δ^EPhe-Gly-Phe-*p*NA (bottom) in the active site of cathepsin C with shown intermolecular hydrogen bonds (white dashed lines).

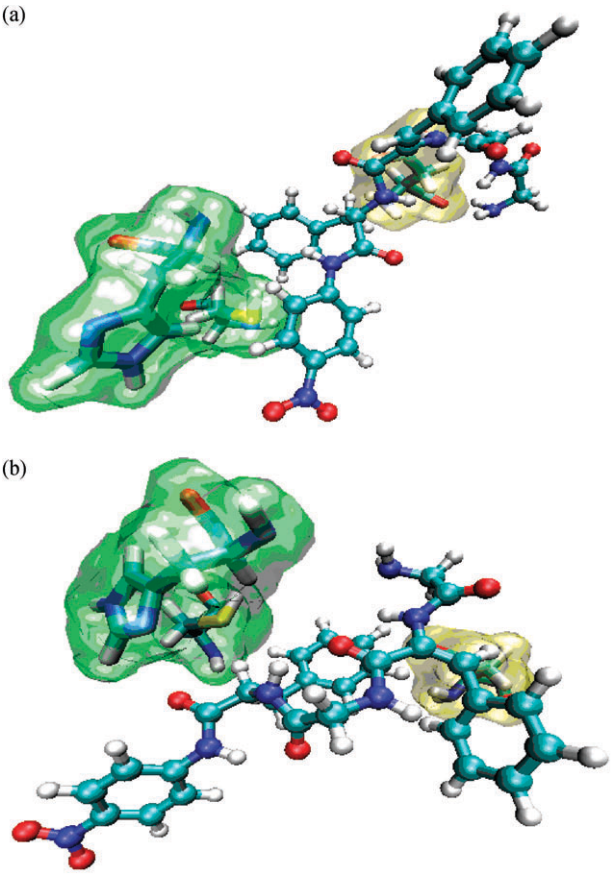


Fig. 6 Binding of Gly-Gly-Δ^EPhe-Phe-*p*NA (top) and Gly-Δ^EPhe-Gly-Phe-*p*NA (bottom) in the active site of cathepsin C.

and from these, after optimisation, we chose those which had the best ligand–enzyme interaction and thus the lowest interaction energies. As seen from Fig. 5 the docked peptides are stabilised by intermolecular hydrogen bonds. The arrangement of Gly-Gly-Δ^EPhe-Phe-*p*NA (Fig. 5, top) is stabilised by five intermolecular hydrogen bonds and the interaction energy is equal to -115.8 kcal mol^{-1}, while the arrangement of Gly-Δ^EPhe-Gly-*p*NA (Fig. 5, bottom) is stabilised by only one intermolecular hydrogen bond and the interaction energy is equal to -84.17 kcal mol^{-1}. As is shown in Fig. 6 both peptides are able to bind to the active site in such a manner that allows direct hydrolysis of *p*-nitrophenylamide if considering both placement of the active site residues and proximity of the hydrolysed amide bond to the thiol fragment of active site cysteine 234. However, the binding modes differ substantially between these two peptides. As seen from Fig. 6, Gly-Gly-Δ^EPhe-Phe-*p*NA is submerged into the binding cavity of cathepsin much more deeply than is Gly-Δ^EPhe-Gly-Phe-*p*NA. Thus, although both compounds are bound with nearly identical affinity the hydrolysis of Gly-Gly-Δ^EPhe-Phe-*p*NA requires its removal from this cavity in order to reach proper geometry for reaction. This reasoning finds some confirmation when the dependence of rate of *p*-nitroaniline formation *vs.* time (Fig. 7), which exhibits some lag-phase and non-typical course (all the other curves are linear), is analysed.

Molecular modelling also enabled to propose the origin of the observed very low inhibitory activity of totally protected dehydrotetrapeptides. As shown in Fig. 8 for a representative example (Boc-Gly-Δ^EPhe-Gly-Phe-*p*NA) these peptides adhere to the surface of the enzyme and form a "cap" covering the entrance to its active site.

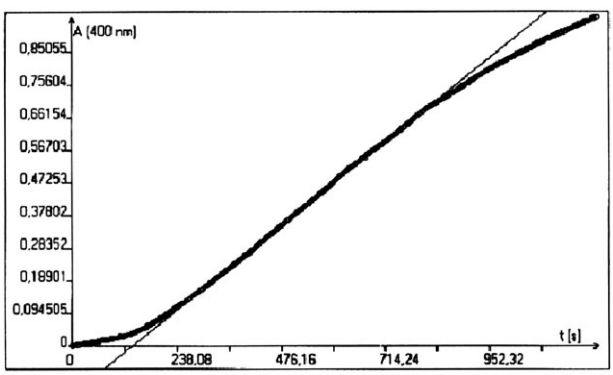


Fig. 7 Dependence of concentration of *p*-nitrophenylaniline *vs.* time upon hydrolysis of Gly-Gly-Δ^EPhe-Phe-*p*NA by cathepsin C.

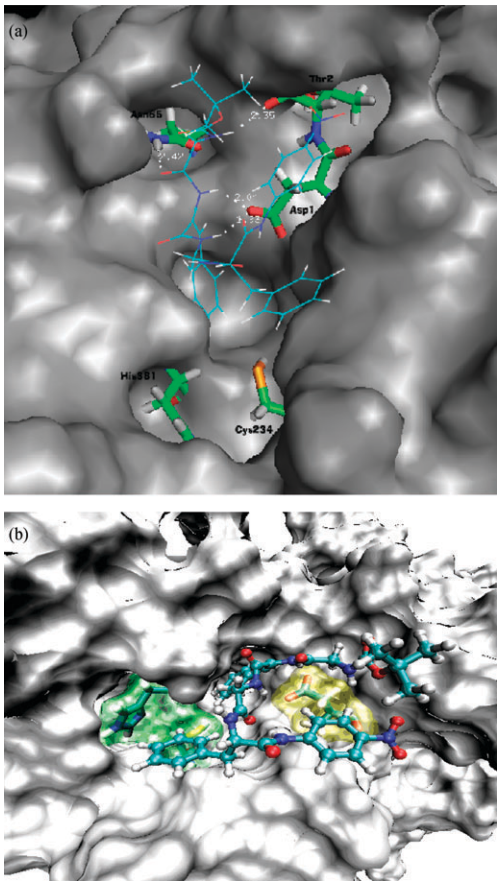


Fig. 8 Binding of Boc-Gly-Δ^{E}Phe-Gly-Phe-*p*NA over the active site of cathepsin C; the upper panel shows the intermolecular hydrogen bonds.

The position of the ligand is stabilised by four intermolecular hydrogen bonds (Fig. 8, top) and the interaction energy is equal to -110.5 kcal mol^{-1}.

Therefore the activity of the studied dehydrotetrapeptides does not result from similarity to peptidyl substrates of cathepsin C, for which the free, unblocked N-terminal amino group is required, but from simple adhesion to the part of the enzyme forming an entrance to its active site. This adhesion is governed mainly by weak van der Waals forces, which implies weak inhibitory activity of the studied compounds.

Conclusions

The comparison of results obtained for peptide *p*-nitrophenylanilides have showed that this group of compounds adopts a more ordered conformation then their saturated counterparts and analogues which contain ΔAla or Δ^{Z}Phe residues. Moreover, peptides which contain a free, unblocked amino group appeared to be quite good substrates of cathepsin C, whereas fully protected peptides acted as very week inhibitors. The correlation of spectroscopic data (NMR and CD) with molecular modelling results suggested that these peptides are hydrolysed in one step by direct removal of *p*-nitroaniline from the peptide chain.

Experimental

General

Materials were obtained from commercial suppliers (Sigma-Aldrich, Fluka, Merck) and used without purification unless otherwise stated. Column chromatography was performed on silica gel H60 (70–230 mesh). NMR spectra were recorded on Bruker Avance DRX300 and Bruker AMX600 instruments in $CDCl_3$, deuterated DMSO and $CDCl_3$–DMSO mixtures and chemical shifts are given relative to $SiMe_4$. In all cases 15 mM peptide solutions were prepared. CD spectra were recorded on a Jasco J-715 spectropolarimeter at room temperature. Spectra were measured in methanol and acetonitrile with concentrations of the solutions being 0.15–0.25 mg ml^{-1}; a pathlength of 1 mm was used.

Syntheses

Boc-Gly-Δ^{E}Phe-Gly-OMe. This was obtained similarly as described in the literature using the mixed carboxylic acid–carbonic anhydride approach. Resolution of (*Z*) and (*E*) isomers was achieved by column chromatography using silica gel H60 (Merck) and solutions of ethyl acetate and benzene of varying concentration (from 5 to 60% v/v of EtOAc) as eluent. The desired product was precipitated from benzene–ethyl actetate (4 : 1 v/v) with hexane and was obtained as a white powder. Yield 34%; mp 133.5–135 °C. Isolated Boc-Gly-Δ^{Z}Phe-Phe-OMe was purified by precipitation with hexane from its ethyl acetate solution. Yield 38%, mp 168–170 °C. $C_{19}H_{25}N_3O_6$ (391.41): calc.: C 58.39, H 6.44, N 10.73; found: C 58.12, H 6.66, N 10.65%.

Boc-Gly-Δ^{E}Phe-Phe-*p*NA. This was obtained starting from Boc-Gly-ΔPhe^{E}OH prepared according to the literature procedure.[21] Thus, triethylamine (1.39 mL, 10.0 mmol) was added to a stirred solution of Boc-Gly-Δ^{E}PheOH (1.60 g, 5.0 mmol) in tetrahydrofuran (12 mL) and the solution was cooled to -10 °C. Then isobutylchloroformate (0.654 mL, 5.0 mmol) was added and the mixture left for 1.5 min. Finally, L-phenylalanine *p*-nitrophenylanilide trifluoroacetate (1.997 g, 5.0 mmol) was added to this mixture and the reaction was carried out for 24 h at room temperature. The formed precipitate was filtered off and solvent was removed under reduced pressure. The resulting oil was dissolved in ethyl acetate (25 mL) and washed successively with 2 M hydrochloric acid (2 × 10 mL), saturated potassium bicarbonate (2 × 10 mL) and brine (10 mL). The organic layer was dried over anhydrous $MgSO_4$, drying agent was removed by filtration and solvent was evaporated *in vacuo*. This procedure provided the mixture of (*Z*) and (*E*) isomers, which were resolved by means of column chromatography using silica gel H60 (Merck) and a gradient of ethyl acetate in benzene as eluent (from 15 to 45% of ethyl acetate). The desired product was slowly precipitated with hexane from a mixture of methanol and diethyl ether (15 : 1 v/v). Yield: 0.520 g (18%), mp 201–203 °C. Unfortunately, the (*Z*) isomer appeared to be the main product of reaction. It was purified by precipitation with hexane from an ethyl actetate–methanol mixture (4 : 1 v/v). Yield: 1.54 g (52%), mp 198.5–201.5 °C.

Elemental analysis: calc. for $C_{31}H_{33}O_7N_5$ (587.5): C 63.36, H 5.66; found: C 63.49, H 5.61%. ^{1}H NMR (DMSO): 1.38 (s, 9H, CH_3 Boc), 3.09 + 2.87 (m, 2H, $C^{\beta}H_2$ Phe), 3.86 + 3.73 (m, 2H, C^{α} H_2Gly), 4.76 (q, 1H, C^{α}H Phe), 6.47 (s, 1H, C^{β}H Δ^EPhe), 7.28 + 7.02 (m, aromatic rings + NH Gly), 8.08 + 8.22 (2 × d, 4H, aromatic ring of *p*NA), 8.74 (d, 1H, NH Phe), 10.19 (s, 1H, NH Δ^EPhe), 10.21 (s, 1H, NH *p*NA); ^{13}C NMR (DMSO): 28.2 (CH_3 Boc), 36.4 (C^{β}Phe), 43.0 (C^{α}Gly), 55.1 (C^{α}Phe), 78.1 (C Boc), 117.9 ($C^{\beta}\Delta^E$Phe), 119.3 + 124.6 (aromatic ring of *p*NA), 123.0 + 126.9 (aromatic rings), 133.8 ($C^{\alpha}\Delta^E$Phe), 137.4 ($C^{\gamma}\Delta^E$Phe), 144.6 + 142.1 (aromatic ring of *p*NA), 155.7 (CO Boc), 164.4 (CO Δ^EPhe), 169.0 (CO Gly), 170.2 (CO Phe).

Boc-Gly-Δ^EPhe-Gly-Phe-*p*NA. This was obtained by alkaline deprotection of Boc-Gly-Δ^EPhe-Gly-OMe followed by coupling with Phe-*p*NA trifluoroacetate. Thus, 1 M sodium hydroxide solution (1.1 mL) was added to a solution of Boc-Gly-Δ^EPhe-Gly-OMe (0.380 g, 1.0 mmol) in a mixture of 2.0 mL MeOH and 1.0 mL water. The reaction mixture was stirred at room temperature for 2 h and then acidified with 1 M hydrochloric acid to pH 4. To the resulting solution, brine (50 mL) was added, and the aqueous phase extracted with ethyl acetate (5 × 5 mL). Combined organic layers were then washed successively with 0.5 M hydrochloric acid (2 × 3 mL) and brine (2 × 3 mL). The organic layer was dried over anhydrous $MgSO_4$ (1 h only!) and the drying agent was filtered off. The product was precipitated with hexane from an ethyl acetate–methanol mixture (29 : 1 v/v). Yield: 0.744 g (98%), mp 127–130 °C.

This product was used directly for *p*-nitrophenylanilide preparation. Thus, TBTU (0.337 g; 1.05 mM) was added to a stirred solution of Boc-Gly-Δ^EPhe-Gly-OH (0.377 g, 1.0 mM), trifluoroacetate of L-phenylalanine *p*-nitroanilide (0.399 g, 1.0 mmol) and triethylamine (0.286 mL, 2.05 mmol) in acetonitrile (4.0 mL). The reaction was carried out for 28 h at room temperature. Then the solvent was removed under reduced pressure and resulting oil dissolved in ethyl acetate (60 mL). The organic layer was washed successively with: 2 M hydrochloric acid (3 × 3 mL), saturated potassium bicarbonate (3 × 3 mL) and brine (3 mL) and dried over anhydrous $MgSO_4$; drying agent was removed by filtration and solvent was evaporated under reduced pressure. Pure product was precipitated from isopropanol with hexane. Yield: 0.527 g (82%). Elemental analysis: calc. for $C_{33}H_{36}O_8N_6$ (664.6): C 61.48, H 5.63; found: C 61.60, H 5.42%.

^{1}H NMR (DMSO): 1.27 (s, 9H, CH_3 Boc), 3.12 + 3.14 (m, 2H, $C^{\beta}H_2$ Phe), 3.63 (d, 2H, C^{α} H_2Gly(3)), 3.72 (t, 2H, C^{α} H_2Gly(1)), 4.76 (m, 1H, C^{α}H Phe), 6.55 (s, 1H, C^{β}H Δ^EPhe), 7.10 (t, 1H, NH Gly(1)), 7.32 + 7.18 (m, 10H, aromatic rings), 7.88 + 8.23 (2 × d, 4H, aromatic ring of *p*NA), 8.40 (d, 1H, NH Phe), 8.93 (s, 1H, NH Gly(3)), 10.10 (s, 1H, NH *p*NA), 10.20 (s, 1H, NH Δ^EPhe); ^{13}C NMR (DMSO): 28.0 (CH_3 Boc), 36.8 (C^{β}Phe), 42.9 (C^{α}Gly(3)), 43.1 (C^{α}Gly(1)), 55.9 (C^{α}Phe), 78.0 (C Boc), 117.8 ($C^{\beta}\Delta^E$Phe), 119.1 + 124.8 (aromatic ring of *p*NA), 126.5 + 129.2 (aromatic rings), 131.5 ($C^{\alpha}\Delta^E$Phe), 137.6 + 134.2 (aromatic rings), 144.8 + 142.5 (aromatic ring of *p*NA), 155.8 (CO Boc), 166.0 (CO Δ^EPhe), 168.8 (CO Gly(3)), 169.0 (CO Gly(1)), 170.0 (CO Phe).

Boc-Gly-Gly-Δ^EPhe-Phe-*p*NA. This was obtained by two-step procedure, namely removal of Boc from Boc-Gly-Δ^EPhe-Phe-*p*NA followed by acylation of the product with BocGly. Thus, Boc-Gly-Δ^EPhe-Phe-*p*NA (0.584 g, 1.0 mmol) was dissolved in anhydrous trifluoroacetic acid (3.0 mL) and stirred at room temperature for 30 min. Then dichloromethane (40 mL) was added and the volatile components of reaction mixture removed *in vacuo*. The glassy product was purified by dissolving in diethyl ether (20 mL) and evaporation of volatiles. This procedure was repeated three times. The resulting trifluoroacetate was dissolved in isopropanol and precipitated with hexane. Yield: 0.601 g (~100%).

TBTU (0.337 g, 1.05 mmol) was added to a stirred solution of Boc-Gly (0.176 g, 1.0 mmol), crude trifluoroacetate of Gly-Δ^EPhe-Phe-*p*NA (0.601 g, 1.0 mmol) and triethylamine (0.286 mL, 2.05 mM) in acetonitrile (4.0 mL) and the reaction was carried out for 24 h at room temperature. Then solvent was removed under reduced pressure and the resulting oil dissolved in ethyl acetate (60 mL). The organic layer was washed successively with: 2 M hydrochloric acid (4 × 3 mL), saturated potassium bicarbonate (4 × 3 mL) and brine (3 mL) and dried over anhydrous $MgSO_4$. The drying agent was removed by filtration and solvent evaporated *in vacuo*. The oily product was precipitated from isopropanol with hexane. Yield: 0.620 g (96%). Elemental analysis: calc. for $C_{33}H_{36}O_8N_6$ (664.6): C 61.48, H 5.63; found: C 61.32, H 5.57%.

^{1}H NMR (DMSO): 1.36 (s, 9H, CH_3 Boc), 3.10 + 2.9 (m, 2H, $C^{\beta}H_2$ Phe), 3.67 (d, 2H, C^{α} H_2Gly(1)), 3.94 (d, 2H, C^{α} H_2Gly(2)), 4.72 (q, 1H, C^{α}H Phe), 6.49 (s, 1H, C^{β}H Δ^EPhe), 7.15 (m, 10H, aromatic rings), 7.92 + 8.19 (m, 5H, aromatic ring of *p*NA + NH Gly(1)), 8.63 (d, 1H, NH Phe), 8.34 (d, 1H, NH Gly(2)), 10.02 (s, 1H, NH Δ^EPhe); 10.14 (s, 1H, NH *p*NA); ^{13}C NMR (DMSO): 28.16 (CH_3 Boc), 36.4 (C^{β}Phe), 41.6 (C^{α}Gly(1)), 43.2 (C^{α}Gly(2)), 55.2 (C^{α}Phe), 78.18 (C Boc), 119.11 ($C^{\beta}\Delta^E$Phe), 119.3 + 124.9 (aromatic ring of *p*NA), 126.5 + 129.1 (aromatic rings), 129.1 ($C^{\alpha}\Delta^E$Phe), 134.1 (aromatic ring of Δ^EPhe), 137.45 (C^{γ}Phe), 142.5 + 144.7 (aromatic ring of *p*NA), 164.4 (CO Δ^EPhe), 168.5 (CO Gly(1)), 169.9 (CO Gly(2)), 170.4 (CO Phe), 170.99 (CO Boc).

Gly-Δ^EPhe-Gly-Phe-*p*NA. Boc-protected dehydrotetrapeptide *p*-nitroanilide (0.5 mmol) was dissolved in anhydrous trifluoroacetic acid (1.5 mL) and stirred at room temperature for 30 min. Then dichloromethane (20 mL) was added and volatile components of reaction mixture removed under reduced pressure. The obtained solid substance was purified by dissolving in diethyl ether (10 mL) and evaporation. This step was repeated three times, the resulting trifluoroacetate was dissolved in isopropanol (1 mL) and pure product precipitated by slow addition of hexane. Yield: 0.319 g (97%). Elemental analysis: calc. for $C_{28}H_{37}O_6N_6$ (553.52): C 60.7, H 6.73; found: C 50.96, H 6.75%.

^{1}H NMR (DMSO): 3.15 + 3.93 (m, 2H, $C^{\beta}H_2$ Phe), 3.68 + 3.82 (d, 4H, C^{α} H_2Gly(1) and Gly(3)), 4.68 (t, 1H, C^{α}H Phe), 6.73 (s, 1H, C^{β}H Δ^EPhe), 7.15 + 7.34 (m, 10H, aromatic rings), 7.82 + 8.20 (2 × d, 4H, aromatic ring of *p*NA), 8.04 (s, 2H, NH_2 Gly(1)), 8.34 (d, 1H, NH Phe), 8.54 (t, 1H, NH Gly(3)) 10.23 (s, 1H, NH Δ^EPhe); 10.53 (s, 1H, NH *p*NA); ^{13}C NMR (DMSO): 37.3 (C^{β}Phe), 40.7 (C^{α}Gly(3)), 43.8

(C^{α}Gly(1)), 55.2 (C^{α}Phe), 118.7 ($C^{\beta}\Delta^{E}$Phe), 119.0 + 124.8 (aromatic ring of *p*NA), 129.1 + 126.4 (aromatic rings), 130.8 ($C^{\alpha}\Delta^{E}$Phe), 137.2 + 134.0 (aromatic rings), 144.7 + 142.4 (aromatic ring of *p*NA), 162.54 (CO Δ^{E}Phe), 164.9 (CO Gly(3)), 168.4 (CO Gly(1)), 170.9 (CO Phe).

Gly-Gly-Δ^{E}Phe-Phe-*p*NA. This was obtained in identical manner as described above. Yield: 0.323 g (98%). Elemental analysis: calc. for $C_{28}H_{37}O_6N_6$ (553.52): C 60.7, H 6.73; found: C 50.98, H 6.62%.

^{1}H NMR (DMSO): 3.15 + 2.9 (m, 2H, $C^{\beta}H_2$ Phe), 3.67 (s, 2H, C^{α} H_2Gly(1)), 4.04 (d, 2H, C^{α} H_2Gly(2)), 4.77 (q, 1H, C^{α}H Phe), 6.55 (s, 1H, C^{β}H Δ^{E}Phe), 7.05 + 7.30 (m, 10H, aromatic rings), 7.97 + 8.25 (2 × d, 4H, aromatic ring of *p*NA), 8.14 (s, 2H, NH_2 Gly(1)), 8.72 (d, 1H, NH Phe), 8.86 (t, 1H, NH Gly(2)) 10.24 (s, 1H, NH Δ^{E}Phe); 10.26 (s, 1H, NH *p*NA); ^{13}C NMR (DMSO): 36.6 (C^{β}Phe), 40.3 (C^{α}Gly(1)), 42.2 (C^{α}Gly(2)), 55.3 (C^{α}Phe), 118.6 ($C^{\beta}\Delta^{E}$Phe), 119.3 + 124.9 (aromatic ring of *p*NA), 129.2 + 126.5 (aromatic rings), 131.4 ($C^{\alpha}\Delta^{E}$Phe), 134.2 (aromatic ring of Δ^{E}Phe), 137.4 (C^{γ}Phe), 144.9 + 142.4 (aromatic ring of *p*NA), 164.5 CO (Δ^{E}Phe), 166.6 (CO Gly(1)), 167.9 (CO Gly(2)), 170.4 (CO Phe).

Assay of the enzymatic activity

Cathepsin C was isolated according to the described procedure.[22] Enzymatic reaction was assayed at 37 °C in acetate buffer (pH 5) containing NaCl (10 mM final concentration) and 2-mercaptoethanol (5 mM final concentration). The assay mixture contained dehydrotetrapeptide (1–8 mM final concentration) and the course of reaction was monitored by following the change in absorbance at 400 nm. Michaelis constants (K_M) and maximal velocities (V_{max}) were obtained using the computer program kindly provided by Dr J. Hurek (University of Opole).

Molecular modelling

The structures of the studied dehydropeptides were optimized in program Gaussian03 at the HF/6-31g (d,p) level[16] in the gas phase using the Merz–Singh–Kollman scheme[20] in the determination of the atomic charges. The calculation of the docking process were performed using the AutoDock program.[18] The starting geometry of the dehydropeptides was taken from the *ab initio* calculations, and also we assigned the charges using charges from the *ab initio* calculation. The structure of cathepsin C was taken from the structure of human dipeptidyl peptidase I deposited EC 3.4.14. in the Protein Data Bank.[17] During the docking process the main chain of the dehydropeptide was fixed, whereas side chains and the terminal groups (–NH_2, –*p*NA, –Boc) were left as flexible. The coordinates of the SH proton from the Cys234 were taken as a grid center in the docking process. Several possible structures ligand–enzyme complexes for each dehydropeptide were obtained in this manner, which were grouped in clusters. For the next stage we chose structures from the most possible clusters. In the next step the selected complex compounds were optimized using Accelrys's DISCOVER program with the cff97 force field, at neutral pH and considering a 10 Å water layer. In the first step all heavy atoms were frozen and a steep descent algorithm with maximum derivative equal to 0.1 was used. In the next steps the side chain atoms from active side were unfrozen and all atoms of the dehydropeptide were analysed using the conjugate gradient algorithm and tether constraints on the unfrozen heavy atoms, which was decreased from a value of 200 to 20 kcal $Å^{-2}$, also the maximum derivative was decreased from a value of 30 to 1 kcal $Å^{-1}$. In the last step we optimized the complex without any force constant using a conjugate gradient algorithm with the maximum derivative equal to 0.1 and then 0.01 kcal $Å^{-1}$. To decide which arrangements were the best for each dehydropeptide, we calculated the energy of interaction E_{int}, which was defined as $E_{int} = E_{comp} - (E_{en} + E_{lig})$, where E_{comp} was the energy of the enzyme–ligand complex excluding the water layer; E_{en} was the energy of the enzyme; E_{lig} was the energy of the ligand.

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