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Pentapeptides containing two dehydrophenylalanine residues - synthesis, structural studies and evaluation of their activity towards cathepsin C

R. LATAJKA,[a]* M. JEWGINSKI,[b] M. MAKOWSKI,[b] M. PAWEŁCZAK,[b] T. HUBER,[c] N. SEWALD[c] and P. KAFARSKI[a,b]

[a] Department of Bioorganic Chemistry, Faculty of Chemistry, Wrocław University of Technology, Wybrzeze Wyspianskiego 27, 50-370 Wroclaw, Poland
[b] Institute of Chemistry, University of Opole, Oleska 48, 45-052 Opole, Poland
[c] University of Bielefeld, Department of Chemistry,33501 Bielefeld, Germany

Abstract: Synthesis, structural and biological studies of pentapeptides containing two ΔPhe residues (*Z* and *E* isomers) in position 2 and 4 in peptide chain were performed. All the investigated peptides adopted bent conformation and majority of them could exist as two different conformers in solution. Only pentapeptides, containing free *N*-termini appeared to act as weak inhibitors of cathepsin C with the slow-binding, competitive mechanism of inhibition, free acids being bound slightly better than their methyl esters. Results of molecular modeling suggested significant difference between peptides, depending of the type of amino acid residue in position 5 in peptide chain. Dehydropeptides containing Gly residue in this position may act as competitive slow-reacting substrates and therefore exhibit inhibitory-like properties. Copyright © 2008 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: dehydropeptide; conformation; inhibitors of cathepsin C

INTRODUCTION

Dehydroamino acids contribute in a catalytic role in the active sites of some yeast and bacterial enzymes [1], as well as occur 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 were found to influence the main chain and side chain dramatically, due to the presence of $C^{\alpha} = C^{\beta}$ double bond [3]. For example, (*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]. It suggests, that dehydroamino acid residues exert a powerful conformational influence, independent of other constraints. Thus, introduction of dehydroamino acid residues into bioactive peptide sequences has become a useful tool to study structure–function relationship and to provide new analogues of enhanced activity.

Cathepsins form quite a large family of lysosomal proteases involved in many physiological functions in 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 like arthritis and the accelerated breakdown of bone structure seen with osteoporosis. Cathepsin C (dipeptidyl dipeptidase I; EC 3.4.14.1) belongs to 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 hydrolyze out nearly every possible dipeptide unit, with the exception of those containing basic amino acids (Arg or Lys) at *N*-terminal position or Pro on either side of the scissile bond. It is also quite unusual in that for the requirement of the presence of halide ions for its activity. The main function of cathepsin C is protein degradation in lysosomes, but it is also found to participate in the activation of 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 showed that in majority of these peptides we observed conformations similar to these found for model peptides [14]. In the case of tetrapeptide

*Correspondence to: R. Latajka, Department of Bioorganic Chemistry, Faculty of Chemistry, Wrocław University of Technology, Wybrzeze Wyspianskiego 27, 50-370 Wroclaw, Poland;
e-mail: rafal.latajka@pwr.wroc.pl

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p-nitroanilides containing (*E*)-dehydrophenylalanine, *N*-terminal unprotected peptides appeared to be quite good substrates of the enzyme, whereas fully protected peptides acted as very weak inhibitors [15].

The next step of our investigations considered synthesis, structural and biological studies of pentapeptides containing two dehydrophenylalanine residues in position 2 and 4 in the peptide chain. In order to understand better the correlation between structure and activity, we synthesized a set of peptides (Figure 1) containing Δ^{E}Phe and Δ^{Z}Phe and determined their conformations in solution, evaluated their influence on cathepsin C and modeled their interactions with the enzyme by means of quantum chemistry methods. Assuming that in the case of dehydropeptides, containing two dehydrophenyalanine residues separated by more than one saturated residue, besides 3_{10}-helix, also α-helix could be found [16], this part of studies should be more interesting in the structural sense.

RESULTS AND DISCUSSION

Synthesis

Pentapeptides were synthesized in solution by classic (2 + 3) approach using mixed-anhydride procedure (Scheme 1) as described earlier [15]. Upon condensation of the part containing Boc-Gly-Δ^{E}Phe, substantial isomerization of the dehydroamino acid was observed and the desired pentapeptide No. 5 was accompanied by the formation of isomeric peptide containing Δ^{Z}Phe. Quite fortunately, we were able to resolve both isomers by column chromatography.

Structural and Conformational Studies

NMR studies were undertaken in order to determine structures and conformational preferences of dehydropeptides shown in Figure 1.

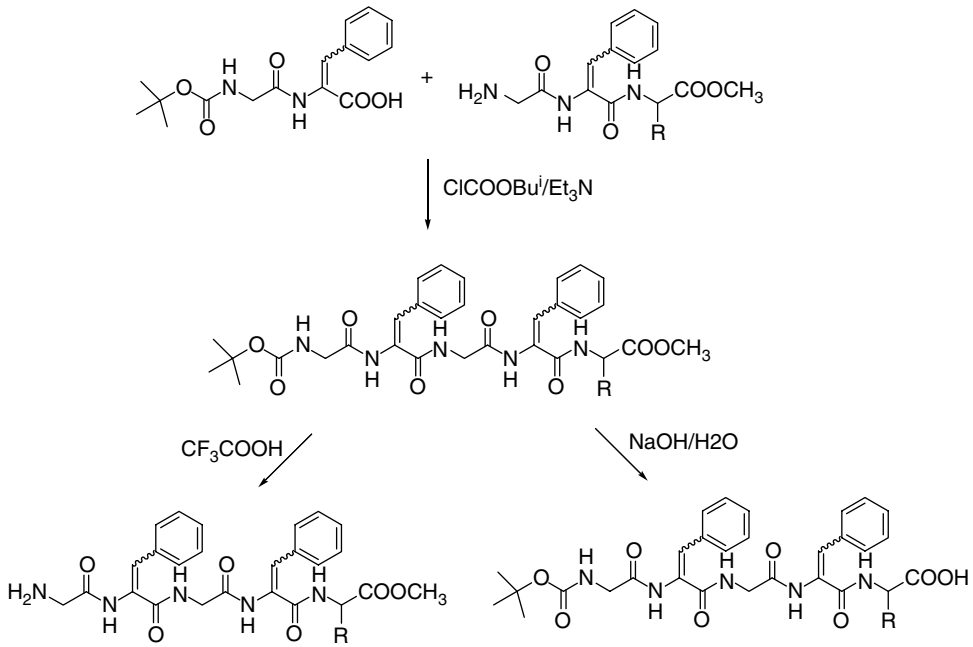
NMR Spectroscopy

In order to find some evidences of the presence of intramolecular hydrogen bonds in the studied peptides, we have performed measurements of the influence of temperature on chemical shifts of their amide protons. The experiments were performed in DMSO by increasing the temperature; the obtained results are presented in Table 1.

The main parameter indicating the presence of hydrogen bond is the value of dδ/dT [ppm/K]. It is well established that the existence of hydrogen bond is reflected in the value of this coefficient lower than 0.004 [ppm K^{-1}]. Results obtained from

Table 1 Temperature dependence of chemical shifts of amide protons of the investigated peptides – temperature coefficient dδ/dT (ppmK^{-1})

Peptide	Gly[1]	ΔPhe[2]	Gly[3]	ΔPhe[4]	Gly[5]/Phe[5]
1	0.0060	0.0060	**0.0040**	**0.0045**	0.0073
2	0.0057	0.0060	**0.0040**	**0.0040**	0.0080
3	0.0007	0.0067	0.0048	0.0052	0.0111
4	0.0072	0.0069	0.0048	0.0047	0.0119
5	0.015	0.014	0.015	0.008	0.016
6	0.016	0.012	0.011	0.012	0.008



Scheme 1 Synthesis of investigated peptides.

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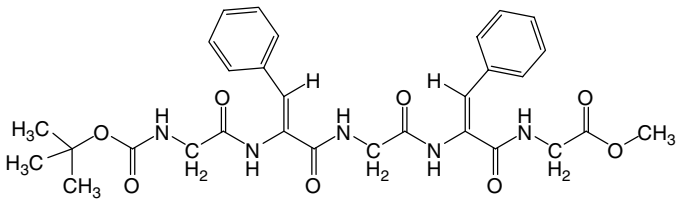
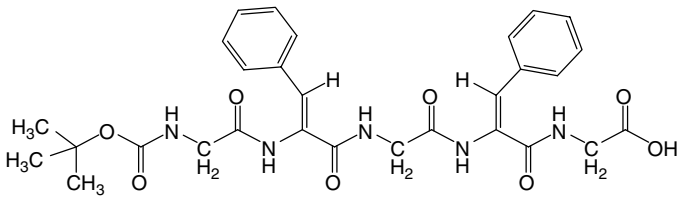
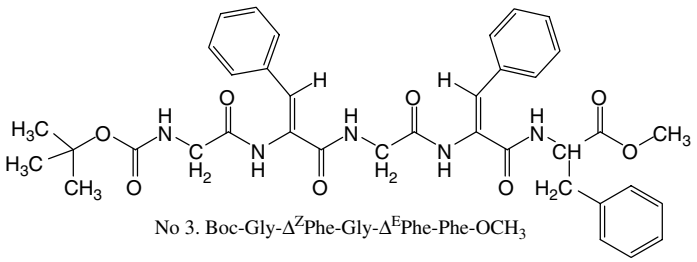


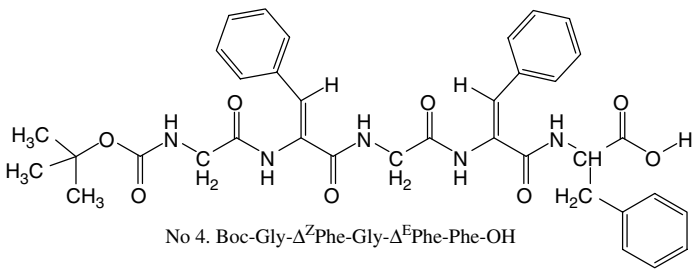
No 1. Boc-Gly-Δ^{Z}Phe-Gly-Δ^{E}Phe-Gly-OCH_3



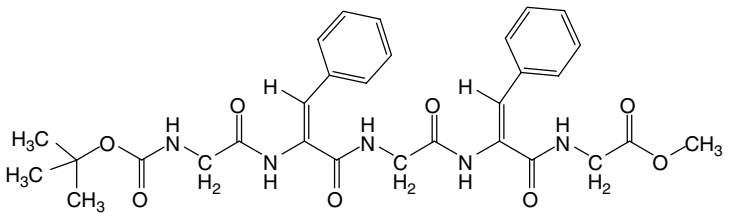
No 2. Boc-Gly-Δ^{Z}Phe-Gly-Δ^{E}Phe-Gly-OH



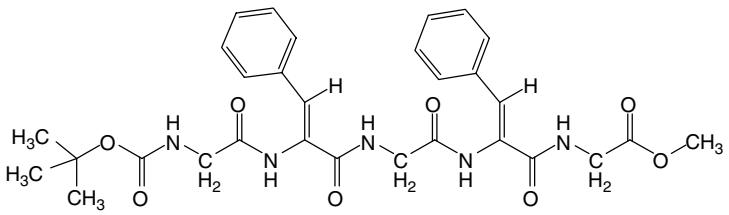
No 3. Boc-Gly-Δ^{Z}Phe-Gly-Δ^{E}Phe-Phe-OCH_3



No 4. Boc-Gly-Δ^{Z}Phe-Gly-Δ^{E}Phe-Phe-OH



No 5. Boc-Gly-Δ^{E}Phe-Gly-Δ^{E}Phe-Gly-OCH_3



No 6. Boc-Gly-Δ^{Z}Phe-Gly-Δ^{Z}PheGly-OCH_3

Figure 1 Structures of investigated peptides.

the experiments showed, that in the case of studied group of peptides, conformers were not stabilized by intramolecular hydrogen bonding. Only for peptides No. 2 and No. 3, amide protons of Gly(3) and ΔPhe(4) might be involved in such interactions.

The most important information about conformational preferences of investigated peptides were obtained from NOESY and ROESY experiments. On the basis of distance constraints and contacts, found in these spectra (see Table 2), optimization of fifty most

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stable conformers were calculated by the use of X-PLOR programme. The average values obtained for dihedral angles are presented in Table 3.

The obtained results suggested, that in every peptide bent conformation (Figures 2–7) was observed and values of dihedral angles were typical for the systems where two dehydrophenylalanine residues are separated by one amino acid residue [16,17]. What is interesting is that only one peptide (No. 1) existed as single conformer, whereas the rest of them most probably might exist in two different conformers. For peptides No. 2 and No. 5 (Figures 3 and 6), these

Table 2 Interatomic distances found on ROESY spectra

Peptide	Atoms	Atoms	Distance (Å)
Boc-Gly-Δ^{Z}Phe-Gly-Δ^{E}Phe-Gly-Ome	HA# [Gly1]	HN [Gly1]	1.87
(No. 1)	HA# [Gly1]	HD# [Δ(Z)Phe2]	2.40
	HA# [Gly1]	HN [Δ (Z)Phe2]	1.87
	HN [Gly1]	HN [Δ (Z)Phe2]	2.75
	HN [Gly1]	HN [Gly3]	2.32
	HD# [d(Z)Phe2]	HN [Δ (Z)Phe2]	2.20
	HA# [Gly3]	HN [Gly3]	1.84
	HA [Gly3]	HN [Δ (E)Phe4]	1.81
	HN [Gly3]	HN [Δ (E)Phe4]	2.41
	HB [d(E)Phe4]	HN [Δ (E)Phe4]	2.06
	H# [OMe]	HD# [Δ (E)Phe4]	2.55
Boc-Gly-Δ^{Z}Phe-Gly-Δ^{E}Phe-Gly-OH	H## [Boc]	HD# [Δ (Z)Phe2]	2.40
No. 2	H## [Boc]	HD# [Δ (E)Phe4]	2.14
	HA# [Gly1]	HN [Gly1]	1.85
	HA# [Gly1]	HN [Δ (Z)Phe2]	1.85
	HA# [Gly1]	HN [Gly3]	2.09
	HN [Gly1]	HN [Δ (Z)Phe2]	2.49
	HB [d(Z)Phe2]	HN [Gly3]	2.00
	HD [d(Z)Phe2]	HN [Δ (Z)Phe2]	1.91
	HA# [Gly3]	HN [Gly3]	1.85
	HA# [Gly3]	HN [Δ (E)Phe4]	1.80
	HA# [Gly3]	HN [Gly5]	1.79
	HN [Gly3]	HN [Δ (Z)Phe2]	2.05
	HN [Gly3]	HN [Δ (E)Phe4]	2.11
	HB [d(E)Phe4]	HN [Δ (E)Phe4]	1.80
	HA# [Gly5]	HN [Δ (E)Phe4]	2.65
	HA# [Gly5]	HN [Gly5]	1.79
	HN [Gly5]	HN [Δ (E)Phe4]	2.24
Boc-Gly-Δ^{Z}Phe-Gly-Δ^{E}Phe-Phe-Ome	H## [Boc]	HN [Gly1]	2.09
No. 3	H## [Boc]	HD# [Δ (E)Phe]	1.97
	HA# [Gly1]	HN [Gly1]	1.80
	HA# [Gly1]	HD# [Δ (Z)Phe2]	2.15
	HA# [Gly1]	HN [Δ (Z)Phe2]	1.82
	HN [Gly1]	HN [Gly3]	1.87
	HD# [d(Z)Phe2]	HN [Δ (Z)Phe2]	1.79
	HA# [Gly3]	HN [Gly3]	1.79
	HA# [Gly3]	HN [Δ (E)Phe4]	1.80
	HN [Gly3]	HN [Δ (Z)Phe2]	1.79
	HN [Gly3]	HN [Phe5]	2.15
	HB [d(E)Phe4]	HD# [Δ (E)Phe4]	1.81
	HB [d(E)Phe4]	HN [Δ (E)Phe4]	1.78
	HA [Phe5]	HN [Gly1]	1.79
	HA [Phe5]	HD# [Δ (E)Phe4]	2.03
	HA [Phe5]	HN [Phe5]	1.81
	HB# [Phe5]	HA [Phe5]	1.79
	HB# [Phe5]	HN [Phe5]	1.78
	HN [Phe5]	HN [Δ (E)Phe4]	1.78
	H# [OMe]	HD# [Δ (E)Phe4]	2.06

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Table 2 (*Continued*)

Boc-Gly-Δ^{Z}Phe-Gly-Δ^{E}Phe-Phe-OH	H## [Boc]	HD# [Phe5]	2.26
No. 4	HA# [Gly1]	HN [Gly1]	1.74
	HA# [Gly1]	HD# [Δ (Z)Phe2]	2.22
	HA# [Gly1]	HN [Δ (Z)Phe2]	1.81
	HN [Gly1]	HN [Δ (Z)Phe2]	2.46
	HN [Gly1]	HN [Gly3]	2.01
	HD# [d(Z)Phe2]	HN [Δ (Z)Phe2]	1.96
	HA# [Gly3]	HN [Gly3]	1.74
	HA# [Gly3]	HN [Δ (E)Phe4]	1.78
	HN [Gly3]	HN [Δ (Z)Phe2]	2.23
	HN [Gly3]	HN [Δ (E)Phe4]	2.01
	HB [d(E)Phe4]	HN [Δ (E)Phe4]	1.79
Boc-Gly-Δ^{E}Phe-Gly-Δ^{E}Phe-Gly-Ome	H## [Boc]	HN [Gly1]	1.81
No. 5	H## [Boc]	HD# [Δ (E)Phe2]	2.01
	H## [Boc]	HB [Δ (E)Phe4]	1.79
	H## [Boc]	HD# [Δ (E)Phe4]	1.79
	HA# [Gly1]	HN [Gly1]	1.78
	HA# [Gly1]	HB [Δ (E)Phe2]	2.36
	HA# [Gly1]	HN [Δ (E)Phe2]	1.89
	HN [Gly1]	HN [Δ (E)Phe2]	2.97
	HB [d(E)Phe2]	HN [Δ (E)Phe2]	2.00
	HB [d(E)Phe2]	HN [Gly3]	2.76
	HD# [d(E)Phe2]	HN [Gly3]	2.26
	HA# [Gly3]	HN [Gly3]	1.78
	HA# [Gly3]	HN [Δ (E)Phe4]	1.97
	HA# [Gly3]	HN [Gly5]	1.79
	HN [Gly3]	HN [Δ (E)Phe2]	3.02
	HN [Gly3]	HN [Δ (E)Phe4]	2.45
	HB [d(E)Phe4]	HD# [Δ (E)Phe4]	1.78
	HB [d(E)Phe4]	HN [Δ (E)Phe4]	1.94
	HB [d(E)Phe4]	H N [Gly5]	2.83
	HD# [d(E)Phe4]	HN [Gly5]	2.47
	HA# [Gly5]	HD# [Δ (E)Phe4]	2.38
	HN [Gly5]	HN [Δ (E)Phe4]	2.68
	H# [OMe]	HN [Δ (E)Phe2]	2.64
	H# [OMe]	HA [Gly5]	2.54
Boc-Gly-Δ^{Z}Phe-Gly-Δ^{Z}Phe-Gly-Ome	H## [Boc]	HA# [Gly1]	3.14
No. 6	H## [Boc]	HN [Gly1]	1.79
	H## [Boc]	HB [Δ(Z)Phe4]	1.78
	H## [Boc]	HA# [Gly5]	1.79
	H## [Boc]	H# [OMe]	2.04
	HA# [Gly1]	HN [Gly1]	1.78
	HA# [Gly1]	HN [Δ (Z)Phe2]	1.87
	HA# [Gly1]	HN [Gly3]	2.53
	HB [d(Z)Phe2]	HN [Gly3]	2.39
	HA# [Gly3]	HN [Gly3]	1.79
	HA# [Gly3]	HN [Δ (Z)Phe4]	1.88
	HN [Gly3]	HN [Δ (Z)Phe2]	2.52
	HB [d(Z)Phe4]	HN [Gly5]	2.31
	HN [d(Z)Phe4]	HN [Gly3]	2.81
	HN [d(Z)Phe4]	HN [Gly5]	2.51
	HA# [Gly5]	HN [Gly1]	2.73
	HA# [Gly5]	HB [Δ (Z)Phe2]	2.53
	HA# [Gly5]	HB [Δ (Z)Phe4]	2.49
	HA# [Gly5]	HN [Gly5]	1.78
	H# [OMe]	HN [Gly3]	2.63
	H# [OMe]	HN [Δ (Z)Phe4]	2.13
	H# [OMe]	HN [Gly5]	2.31

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Table 3 Average values of dihedral angle [°] obtained on base of X-PLOR calculation

Peptide[a]	Φ^1	ψ^1	φ^2	ψ^2	φ^3	ψ^3	φ^4	ψ^4	φ^5	ψ^T
1	−127.1	−93.6	−94.7	−2.1	−95.9	−77.4	7.5	−88.3	104.5	42.6
2a	58.1	22.8	47.6	33.4	−76.3	−70.2	101.8	−4.1	57.9	58.1
2b	−47.8	−47.4	−25,7	−41.2	83.4	72.6	−101.8	2.7	−59.1	−47.8
3a	117.6	116.1	84.2	−1.1	−136.9	−103.0	−56.4	−40.5	−12.4	107.2
3b	114.9	−69.6	−90.7	−2.4	−129.3	−95.4	−54.2	−26.3	−10.5	107.1
4a	121.1	−47.5	−80.4	−17.2	178.1	−50.9	−67.8	−49.7	69.3	121.1
4b	−47.5	43.7	83.1	−3.3	159.8	−43.9	−71.3	−50.2	77.2	−123.1
5a	43.7	−66.0	133.8	98.0	−176.6	49.8	−10.9	61.2	−172.2	13.4
5b	−111.4	65.0	−132.8	−100.9	172.9	−48.8	10.6	−62.1	174.7	3.1
6a	−147.7	166.5	107.2	−48.1	107.3	65.0	51.7	93.1	27.2	58.9
6b	147.4	−163.2	−109.8	48.3	−112.3	−50.0	−36.3	−91.8	−29.3	−58.8

[a] a, conformation with lower energy; b, conformation with higher energy.

two possible forms (of lower and higher energy) were symmetrical, which resulted from the analysis of the values of Φ and ψ dihedral angles. It is well known that for the peptides containing two dehydroamino acid residues, the size of substituent on C^{β} in saturated, separating residue has great importance on adopted conformation [16,17]. In the case of pentapeptides studied in this work, the peptides differ from each other by the structure of *C*-terminal amino acid (glycine vs phenylalanine) and its form (free acid vs methyl ester) and the type of dehydrophenylalanine isomer. Although obtained conformational differences observed suggested that structural factors had a vital importance, there was no clear structure–conformation relationship.

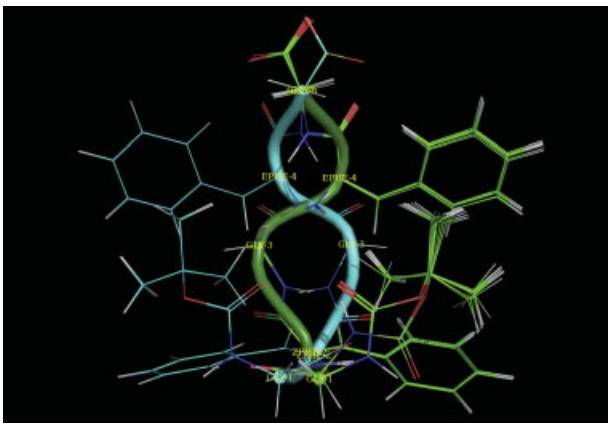

Figure 3 Twenty of the most stable conformations of peptide No. 2 proposed on the basis of X-PLOR calculation - superposition of both (higher and lower energy) conformations.

Inhibitory Activity

Molecular modeling predicted that *N*-protected peptides studied in this work might act as inhibitors susceptible to nucleophilic attack of thiol cysteine. Therefore, we studied the activity of two peptides (No. 1 and No. 3) for which different bonding mode was predicted by the calculations (see next paragraph). Unfortunately, none of them affected enzyme activity when applied at a concentration of 0.01 mM (highest possible concentration obtainable). This was somewhat surprising since our previous experience [15] showed that predictions were in a good agreement with calculations.

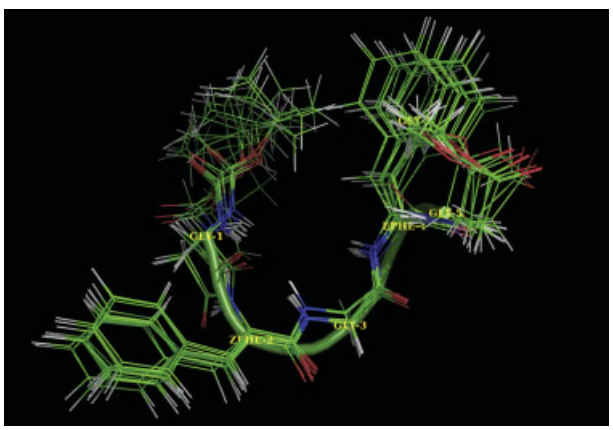

Figure 2 The most stable conformations of peptide No. 1 proposed on the basis of X-PLOR calculations.

In the preceding studies, we found that dehydrotetrapeptides containing free, underivatized amino group acted as good substrates of cathepsin C [15]. Therefore, we decided to study the activity of analogous pentapeptides. They were found to act as weak inhibitors of cathepsin C (Table 4 – for comparison, results obtained for phosphonodehydropeptides are included). Despite of weak activity, the slow-binding competitive mechanism of inhibition was found with free acids being bound slightly better than their corresponding methyl esters. As it is usual in the case of cathepsin C, [18] even weak inhibitors are acting as slow-binding ones and in this case they followed type A of inhibition (inhibitor binding

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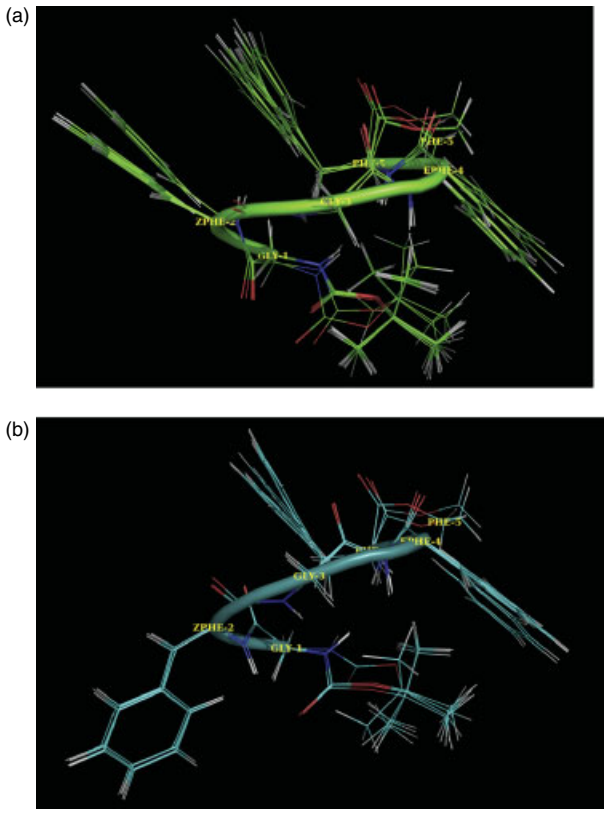

Figure 4 Twenty of the most stable conformations of peptide No. 3 proposed on the basis of X-PLOR calculation; (a) lower energy conformation, (b) higher energy conformation.

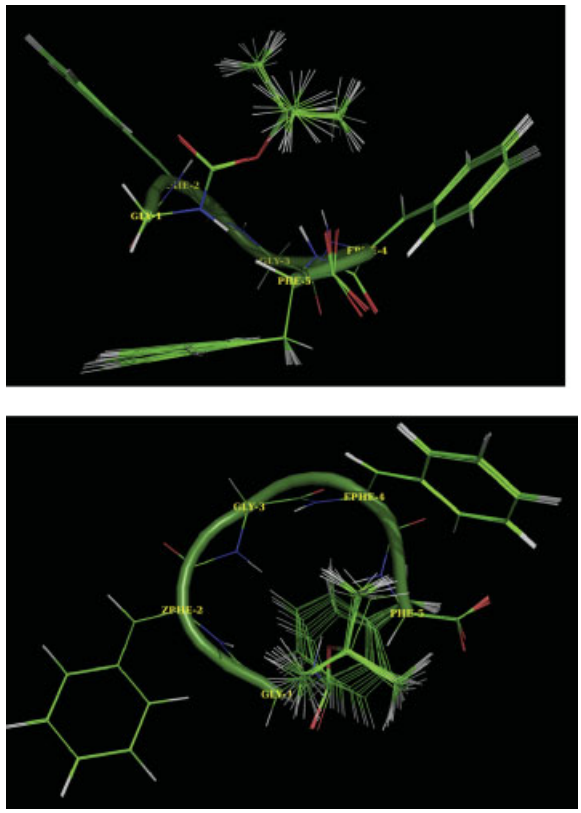

Figure 5 Ten of the most stable structures of No. 4, proposed on the basis of X-PLOR calculation, conformation with the lower energetic No. 4a.

is slower than the diffusion-limited) indicating that the inhibitor reaches optimal placement in the active site of the enzyme reasonably slowly.

Molecular Modeling and Enzymatic Activity

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

Optimal conformations of the inhibitory dehydrotetrapeptides were obtained by use of Gaussian 03 at the HF/6–31g (d,p) level [19] 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 [20] by using AutoDock programme [21,22]. These structures were chosen for docking process, because they were in good agreement with structures predicted by the NMR experiment. AutoDock for docking process requires good assignment of charge, and therefore they were obtained from the *ab initio* calculations with Merz-Singh-Kollman scheme [23]. 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. As a result of docking process, for each peptide we got clusters, where

Table 4 Enzymatic activity of investigated peptides towards cathepsin C

Peptide	KI (μM)	S-B
Gly-Δ^{Z}Phe-Gly-Δ^{E}Phe-Gly-OMe	173	$k_1 = 98.15\ M^{-1}\ s^{-1}\ k_2 = 0.008\ s^{-1}$
Gly-Δ^{Z}Phe-Gly-Δ^{E}Phe-Phe-OMe	324	$k_1 = 12.35\ M^{-1}\ s^{-1}\ k_2 = 0.005\ s^{-1}$
Gly-Δ^{Z}Phe-Gly-Δ^{E}Phe-Gly	50	$k_1 = 61.16\ M^{-1}\ s^{-1}\ k_2 = 0.006\ s^{-1}$
Gly-Δ^{Z}Phe-Gly-Δ^{E}Phe-Phe	122	$k_1 = 13.09\ M^{-1}\ s^{-1}\ k_2 = 0.006\ s^{-1}$
Boc-Gly-Δ(Z)Phe-AbuPO$(OMe)_2$	681	$k_1 = 9.51\ M^{-1}\ s^{-1}\ k_2 = 0.0055\ s^{-1}$
Gly-Δ(Z)Phe-AlaPO$(OEt)_2$ * TFA	1563	$k_1 = 1764\ M^{-1}\ min^{-1}\ k_2 = 0.44\ min^{-1}$

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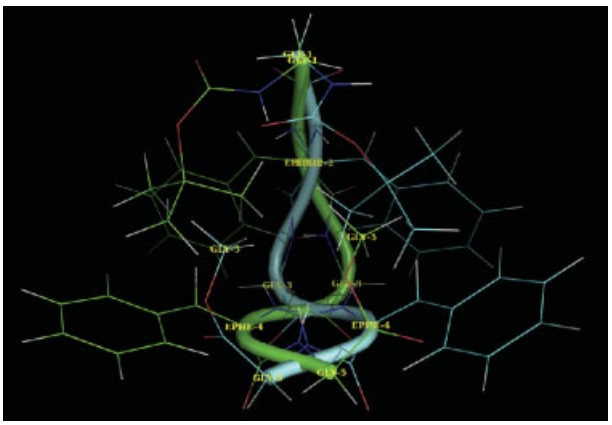

Figure 6 Superposition of lower (green) and higher (light blue) energy conformers of peptide No. 5 with main chain marked.

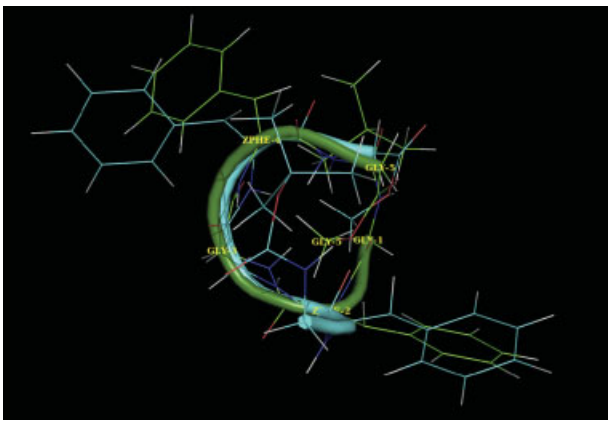

Figure 7 Superposition of lower (green) and higher (light blue) energy conformers of peptide No. 6 with main chain marked.

every one represented different binding site. In the next stage, the structures were selected and docked in the most probable places and after optimalization, those of the best ligand–enzyme interaction (the lowest interaction energy) were chosen. As it could be seen from Figures 8 and 9, docked peptides were stabilized by intermolecular hydrogen bonds and two types of possible location in the active site of the enzyme were observed.

In the case of peptides No. 1 and No. 2 (Figure 8), the aromatic ring of Δ^{Z}Phe was located in the active site, and the distance between Cys234 sulfur atom and C^{α} and C^{β} of Δ^{Z}Phe(2) were 3.66 A and 4.85 A in peptide No. 1 and 4.06 A and 5.20 A in peptide No.

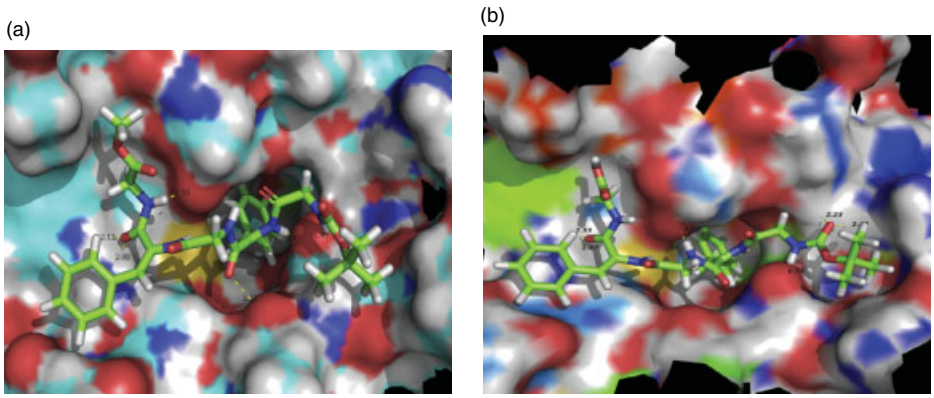

Figure 8 Location of peptides No. 1 (b) and No. 2 (a) in the active site of DPPI, obtained by use of molecular modeling.

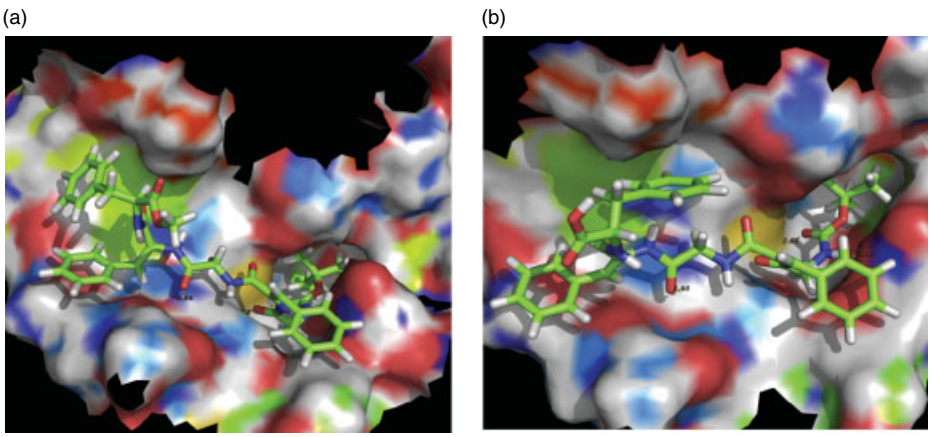

Figure 9 Location of peptides No. 3 (a) and No. 4 (b) in the active site of DPPI, obtained by use of molecular modeling.

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2. It suggested that addition of thiol moiety to double bond might be possible. On the other hand, nitrogen atoms from peptide bonds which potentially could be hydrolyzed (Δ^{Z}Phe[2]-Gly[3] and Gly[3]-Δ^{E}Phe[4]) were located at a larger distance, respectively 5.99 A, 5.22 A and 5.54 A, 5.17 A from sulfur atom of Cys234.

Totally different location in the active site of the enzyme was observed for peptides No. 3 and No. 4 (Figure 9). In these cases, aromatic rings were located 'outside' the active site, whereas methyl groups of Boc were located in small hydrophobic site. Obtained results suggested, that Gly[1], Δ^{Z}Phe[2] and Gly[3] residues were involved in binding.

In order to find correlation between molecular modeling and results of experimental studies, the docking process was performed also for the group of *N*-terminal unblocked analogues of investigated peptides. These compounds were found to act as moderate inhibitors of the enzyme. In the case of all studied compounds, numerous intermolecular hydrogen-bonds-binding peptide in the active site of enzyme were found (see Table 5).

The comparison of obtained results suggested that there was a significant difference between peptides depending of the type of amino acid residue in position 5 in peptide chain. Dehydropeptides containing Gly in this position have such a location in the active site of the enzyme that their hydrolysis could be more probable – the distances between peptide bonds and sulfur atom of Cys234 are respectively 3.67 Å for Gly-Δ^{Z}Phe-Gly-Δ^{E}Phe-Gly-OMe and 3.40 Å for its *C*-terminal unblocked analogue - see Figure 10. Thus they may act as competitively slow-reacting substrates and therefore exhibit inhibitory-like properties.

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).

Peptide No. 1. Obtained in 80% yield by crystallization from ethyl acetate–*iso*octane mixture. mp 191.5–193 °C. ^{1}H NMR (DMSO, TMS): 1.40 ppm, s, 9H (Boc); 3.66 ppm, s, 3H (–CH_3 Gly[5]); 3.81 ppm, d (5.68 Hz), 2H (HA Gly[1]); 3.86 ppm, d (5.67 Hz), 2H (HA Gly[5]); 3.89 ppm, d (5.78 Hz), 2H (HA Gly[3]); 6.84 ppm, s, 1H (HB Δ^{E}Phe[4]); 7.08 ppm, t (5.55 Hz); 1H (HN Gly[1]); 7.16 ppm, s, 1H (HB Δ^{Z}Phe[2]);

Table 5 Parameters of location of *N*-terminal unblocked peptides in the active site of DPPI

Peptide	Intermolecular hydrogen bonds
Gly-Δ^{Z}Phe-Gly-Δ^{E}Phe-Gly-OMe	HE1 [Trp405] → CO [d(Z)Phe4]; HN [d(Z)Phe7] → OE1 [Gln228]; HN [Cys234] → CO [d(E)Phe2]; HN [Gly277] → CO [Gly1]; H_3N [Gly1] → CO [Gly277]; H_3N [Gly1] → COO^- [Asp1]; H_3N [Gly1] → H_2O → OH[Thr279]
Gly-Δ^{Z}Phe-Gly-Δ^{E}Phe-Gly	HE1 [Trp405] → CO [Gly3]; HN [d(E)Phe] → CO [Asn380]; H_3N [Gly1] → COO^-[Asp1]
Gly-Δ^{Z}Phe-Gly-Δ^{E}Phe-Phe-OMe	H_3N [Gly1] → COO^-[Asp1]; H_3N [Gly1] → CO [Thr379]; HN [Val352] → CO [Phe5]; H_2N [Asn380] → CO [Phe5]
Gly-Δ^{Z}Phe-Gly-Δ^{E}Phe-Phe	HE1 [Trp405] → COO^- [Phe5]; HN [Cys231] → COO^- [Phe5]; HN [d(E)Phe2] → COO^- [Asp1]; H_3N [Gly1] → COO^- [Asp1]; H_3N [Gly1] → CO [Thr2]

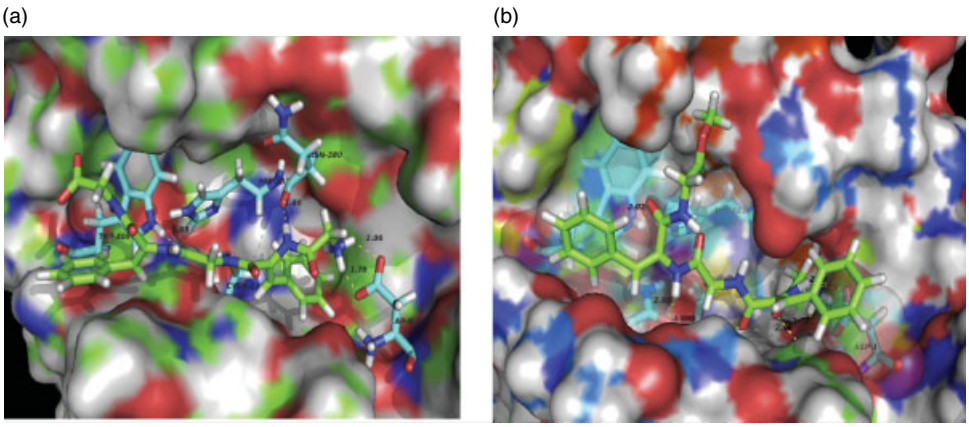

Figure 10 Location of peptides Gly-Δ^{Z}Phe-Gly-Δ^{E}Phe-Gly (a) and Gly-Δ^{Z}Phe-Gly-Δ^{E}Phe-Gly-OMe (b) in the active site of DPPI, obtained by use of molecular modeling.

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7.21–7.64 ppm, m, 10H (aromatic); 8.35 ppm, t (4.84 Hz), 1H (HN Gly[3]); 8.57 ppm, t (5.45 Hz), 1H (HN Gly[5]); 9.39 ppm, s, 1H (HN Δ^{E}Phe[4]); 9.74 ppm, s, 1H (HN Δ^{Z}Phe[2]). ^{13}C NMR (DMSO, TMS): 28.7 ppm (CH_3 Boc); 41.7 ppm (CA Gly[5]); 43.5 ppm (CA Gly[1]); 44.1 ppm (CA Gly[3]); 52.1 ppm (CH_3 Gly[5]); 78.8 ppm (C IV Boc); 118.1 ppm (CB Δ^{E}Phe[4]); 127.3–135.0 ppm (aromatic + CB Δ^{Z}Phe[2]); 131.8 ppm (CA Δ^{E}Phe[4]); 156.5 (C Boc); 165.4 ppm (C Δ^{E}Phe[4]); 165.5 ppm (C Δ^{Z}Phe[2]); 167.9 ppm (C Gly[3]); 170.3 ppm (C Gly[5]); 170.7 ppm (C Gly[1]). Elemental analysis for $C_{30}H_{35}N_5O_8$ calculated: 60.7% C, 5.94% H and 11.79% N, found: 60.95% C, 6.14% H and 11.5% N.

Peptide No. 2. ^{1}H NMR (DMSO, TMS): 1.40 ppm s, 9H (CH_3 Boc); 3.78 ppm, d (5.67 Hz), 2H (HA Gly[5]); 3.80 ppm, d (5.74 Hz), 2H (HA Gly[1]); 3.89 ppm, d (5.81 Hz), 2H (HA Gly[3]); 6.84 ppm, s, 1H (HB Δ^{E}Phe[4]); 7.11 ppm, t (5.74HZ), 1H (HN Gly[1]); 7.16 ppm, s, 1H (Hb Δ^{Z}Phe[2]); 7.16–7.64 ppm, m, 10H (aromatic); 8.35 ppm, t (5.26), 1H (HN Gly[3]); 8.45 ppm, t (5.48 Hz), 1H (HN Gly[5]); 9.41 ppm, s, 1H (HN Δ^{E}Phe[4]); 9.76 ppm, s, 1H (HN Δ^{Z}Phe[2]); 12.50 ppm, s (narrow), 1H (HOOC Gly[5]) ^{13}C NMR (DMSO, TMS): 28.7 ppm (CH_3 Boc); 41.6 ppm (CA Gly[5]); 43.5 ppm (CA Gly[3]); 44.1 ppm (CA Gly[1]); 78.8 ppm (C IV Boc); 118.1 ppm (CB Δ^{E}Phe[4]); 128.9 ppm (CB Δ^{Z}Phe[2]); 127.4–130.9 aromatic + CA Δ^{Z}Phe[2]); 132 ppm (CA Δ^{E}Phe[4]); 134.3, 135 ppm (CG Δ^{E}Phe[4] and CG Δ^{Z}Phe[2]); 156.5 ppm (C Boc); 165.2 ppm (C Δ^{E}Phe[4]); 165.6 ppm (C Δ^{Z}Phe[2]); 168.0 ppm (C Gly[3]); 170.7 ppm (C Gly[1]); 171.4 ppm (C Gly[5]). Elemental analysis for $C_{29}H_{33}N_5O_8$ calculated: 60.09% C, 5.74% H and 12.08% N, found: 58.28% C, 6.02% H and 11.16% N.

Peptide No. 3. Obtained in 75% yield by crystallization from ethyl acetate–methanol–hexane mixture. mp 198–200 °C. ^{1}H NMR (DMSO, TMS): 1.37 ppm, s, 9H (CH_3 Boc); 2.90 ppm, d, (7.20 Hz) 2H (HB Phe[5]); 3.51 ppm, s, 3H (CH_3 Phe[5]); 3.79 ppm, d (5.22 Hz), 2H (HA Gly[1]); 3.86 ppm, d, (5.29 Hz) 2H (HA Gly[3]); 4.53 ppm, m, 1H (HA Phe[5]); 6.77 ppm, s, 1H (HB Δ^{E}Phe[4]); 7.13–7.63 ppm, m (aromatic + HB Δ^{Z}Phe[2]); 7.14 ppm, t, 1H (HN Gly[1]); 8.34 ppm, t, (5.09 Hz), 1H (HN Gly[3]); 8.61 ppm, d (7.13 Hz) 1H (HN Phe[5]); 9.41 ppm, s, 1H (HN Δ^{E}Phe[4]); 9.81 ppm, s, 1H (HN Δ^{Z}Phe[2]). ^{13}C NMR (DMSO, TMS): 28.2 ppm (CH_3 Boc); 36.7 ppm (CB Phe[5]); 43.0 ppm (CA Gly[3]); 43.6 ppm (CA Gly[1]); 51.7 ppm (CH_3 Phe[5]); 53.9 ppm (CA Phe[5]); 78.2 ppm (C IV Boc); 117.3 ppm (CB Δ^{E}Phe[4]); 126.6–129 ppm (aromatic); 129.8 ppm (CA Δ^{E}Phe[4]); 131.7–136.9 ppm (aromatic + CB Δ^{Z}Phe[2]); 156.1 ppm (C Boc); 164.3 ppm (C Δ^{Z}Phe[2]); 165.0 ppm (C Δ^{E}Phe[4]); 167.5 ppm (C Gly[3]); 170.3 ppm (C Gly[1]); 171.0 ppm (C Phe[5]). Elemental analysis for $C_{37}H_{41}N_5O_8$ calculated: 64.99% C, 6.04% H and 10.02% N, found: 63.57% C, 6.29% H and 11.04% N.

Peptide No. 4. ^{1}H NMR (DMSO, TMS): 1.37 ppm, s, 9H (CH_3 Boc); 2.92 ppm, d, 2H (HB Phe[5]); 3.79 ppm, d (4.55 Hz), 2H (HA Gly[1]); 3.78 ppm, d (4.69 Hz), 2H (HA Gly[3]); 4.49 ppm, m, 1H (Ha Phe[5]); 6.78 ppm, s, 1H (HB Δ^{E}Phe[4]); 7.15–7.63 ppm, m, 16H (aromatic + HB Δ^{Z}Phe[2]); 7.17 ppm, 1H (HN Gly[1]); 8.30 ppm, d (6.68 Hz), 1H (HN Phe[5]); 8.39 ppm, t (5.40 Hz), 1H (HN Gly[3]); 9.41 ppm, s, 1H (HN Δ^{E}Phe[3]); 9.83 ppm, s, 1H (HN Δ^{Z}Phe[2]); 12.61 ppm, s (narrow), 1H (HOOC Phe[5]). ^{13}C NMR (DMSO, TMS): 28.1 ppm (CH_3 Boc); 36.7 ppm (CB Phe[5]); 43.0 ppm (CA Gly[3]); 43.69 ppm (CA Gly[1]); 54.3 ppm (CA Phe[5]); 78.3 ppm (C IV Boc); 117.7 ppm (CB Δ^{E}Phe[4]); 126.4–134.6 ppm (aromatic + CB Δ^{Z}Phe[2] + CA Δ^{Z}Phe[2]); 131.7 ppm (CA Δ^{E}Phe[4]); 137.3 ppm (CG Phe[5]); 156.1 ppm (C Boc); 164.2 ppm (C Δ^{E}Phe[4]); 165.1 ppm (C Δ^{Z}Phe[2]); 167.6 ppm (C Gly[3]); 170.4 (C Gly[1]); 172.1 ppm (C Phe[5]).

Peptide No. 5. Obtained in 51% yield as mixture of *ZE*/*EE* peptide. They were separated by means of column chromatography using H60 silica and gradient of benzene–ethyl acetate solution (from 5 to 60%) as eluent. Desired isomer was obtained in 21% yield. mp 167–170 °C. ^{1}H NMR (DMSO, TMS): 1.40 ppm, s, 9H (Boc); 3.66 ppm, s, 3H (–CH_3 Gly[5]); 3.81 ppm, d (5.68 Hz), 2H (HA Gly[1]); 3.86 ppm, d (5.67 Hz), 2H (HA Gly[5]); 3.89 ppm, d (5.78 Hz), 2H (HA Gly[3]); 6.84 ppm, s, 1H (HB Δ^{E}Phe[4]); 7.08 ppm, t (5.55 Hz); 1H (HN Gly[1]); 7.16 ppm, s, 1H (HB Δ^{Z}Phe[2]); 7.21–7.64 ppm, m, 10H (aromatic); 8.35 ppm, t (4.84 Hz), 1H (HN Gly[3]); 8.57 ppm, t (5.45 Hz), 1H (HN Gly[5]); 9.39 ppm, s, 1H (HN Δ^{E}Phe[4]); 9.74 ppm, s, 1H (HN Δ^{Z}Phe[2]). ^{13}C NMR (DMSO, TMS): 28.7 ppm (CH_3 Boc); 41.7 ppm (CA Gly[5]); 43.5 ppm (CA Gly[1]); 44.1 ppm (CA Gly[3]); 52.1 ppm (CH_3 Gly[5]); 78.8 ppm (C IV Boc); 118.1 ppm (CB Δ^{E}Phe[4]); 127.3–135.0 ppm (aromatic + CB Δ^{Z}Phe[2]); 131.8 ppm (CA Δ^{E}Phe[4]); 156.5 (C Boc); 165.4 ppm (C Δ^{E}Phe[4]); 165.5 ppm (C Δ^{Z}Phe[2]); 167.9 ppm (C Gly[3]); 170.3 ppm (C Gly[5]); 170.7 ppm (C Gly[1]). Elemental analysis for $C_{30}H_{35}N_5O_8$ calculated: 60.7% C, 5.94% H and 11.79% N, found: 60.19% C, 6.05% H and 11.52% N.

Peptide No. 6. Obtained in 70% yield. mp 181–183 °C ^{1}H NMR (DMSO, TMS): 1.39 ppm, s, 9H (Boc); 3.64 ppm, s, 3H (–CH_3 Gly[5]); 3.75 ppm, d (3.69 Hz), 2H (HA Gly[1]); 3.93 ppm, d (2.52 Hz), 2H (HA Gly[5]); 4.03 ppm, d (7.08 Hz), 2H (HA Gly[3]); 7.19 ppm, s, 1H (HB Δ^{E}Phe[4]); 7.04 ppm, t (5.76 Hz); 1H (HN Gly[1]); 7.23 ppm, s, 1H (HB Δ^{Z}Phe[2]); 7.33–7.63 ppm, m, 10H (aromatic); 8.37 ppm, t (5.82 Hz), 1H (HN Gly[3]); 8.42 ppm, t (5.58 Hz), 1H (HN Gly[5]); 9.45 ppm, s, 1H (HN Δ^{E}Phe[4]); 9.64 ppm, s, 1H (HN Δ^{Z}Phe[2]). ^{13}C NMR (DMSO, TMS): 28.6 ppm (CH_3 Boc); 41.8 ppm (CA Gly[5]); 43.8 ppm (CA Gly[1]); 44.2 ppm (CA Gly[3]); 52.2 ppm (CH_3 Gly[5]); 78.8 ppm (C IV Boc); 118.1 ppm (CB Δ^{E}Phe[4]); 128.7–130.4 ppm (aromatic + CB Δ^{Z}Phe[2]); 134.15 ppm (CA Δ^{E}Phe[4]); 156.5 (C Boc); 165.5 ppm (C Δ^{E}Phe[4]); 166 ppm (C Δ^{Z}Phe[2]); 169.5 ppm (C Gly[3]); 170.5 ppm (C Gly[5]); 170.7 ppm (C Gly[1]). Elemental analysis for $C_{30}H_{35}N_5O_8$ calculated: 60.7% C, 5.94% H and 11.79% N, found: 60.27% C, 6.23% H and 11.59% N.

NMR Spectroscopy

NMR spectra were recorded on Bruker Avance DRX300, Bruker AMX600 and Bruker AMX400 instruments in deuterated DMSO, chemical shifts are given in relation to $SiMe_4$. In all cases, 15 mM peptide solutions were prepared. NMR spectral signal assignments and integrations were carried out with SPARKY [24]. The separation between two geminal proton in –CH_2– group was used as a reference in distance calculations. Conformational calculations were carried out with X-PLOR [25]. In view of the investigated peptides containing unnatural aminoacid residues, it was neccessary to modify the topology file available in distribution. To build the required topology for Δ^{Z}Phe and Δ^{E}Phe, the available topology for Phe was used.

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In addition, a large force constant warranting planarity of the dehydroaminoacids residue was added.

Assay of the Enzymatic Activity

Cathepsin C was isolated according to the reported procedure [26]. 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 dehydropeptide (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 provided kindly by Dr J. Hurek (University of Opole).

Molecular Modeling

The structures of the studied dehydropeptides were optimized in program Gaussian 03 at the HF/6–31g (d,p) level [19] in gas phase by using Merz-Singh-Kollman scheme [23] for the determination of the atomic charges. The calculations of the docking process were performed using AutoDock program [21]. 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 Protein Data Bank [20]. During the docking process, main chain of the dehydropeptide was fixed, whereas side chains and the terminal groups ($-NH_2$, –pNA, –Boc) were left as variable. The coordinates of the SH proton from the Cys234 were taken as a grid center in the docking process. Several possible structures of ligand-enzyme complex for each dehydropeptide were obtained in that manner, which were grouped in the clusters. In the next step, the structure was selected and optimized by the use of Accelrys's DISCOVER program with the cff97 force field, at neutral pH and considering 10 ´Å water layer. In the first step, all heavy atoms were frozen and the steep descents algorithm with maximum derivative equal to 0.1 was used. In the next steps, we unfroze the side chain atoms from active side and all the atoms of the dehydropeptide were subjected to conjugate gradient algorithm and tether constrains on the unfrozen heavy atoms.

CONCLUSIONS

Spectroscopic and theoretical studies showed that all the investigated peptides adopted bent conformations and majority of them could exist as two different conformers in solution. The obtained values of dihedral angles were typical for the systems, where two dehydrophenylalanine residues were separated by one amino acid residue.

Only pentapeptides, containing free *N*-termini appeared to act as weak inhibitors of cathepsin C with the slow-binding, competitive mechanism of inhibition, free acids bound slightly better than their methyl esters. Results of molecular modeling suggested that there was a significant difference in binding to enzyme between peptides, depending on the type of amino acid residue in position 5 in peptide chain. Dehydropeptides, containing Gly residue in this position, had such a location in the active site of the enzyme that their hydrolysis could be more probable and might act as competitive slow-reacting substrates and therefore exhibit weak inhibitory-like properties.

Acknowledgements

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