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# Synthesis of Tetrapeptide *p*-Nitrophenylanilides Containing Dehydroalanine and Dehydrophenylalanine and Their Influence on Cathepsin C Activity

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Abstract: Three dehydrotetrapeptides of rationally varying structure were prepared and tested as affectors of cathepsin C. These compounds appeared to be substrates of the enzyme, being equipotent with their classical counterparts. Thus, replacement of amino acid in a short peptide by corresponding dehydroamino acid does not prevent cathepsin C in recognizing dehydropeptide as its substrate. Copyright © 2001 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: dehydroamino acids; dipeptidyl-peptidase I; proteolytic enzymes

## INTRODUCTION

Cathepsin C (dipeptidyl-peptidase I, EC 3.4.14.1) sequentially removes dipeptides from the unsubstituted *N*-termini of polypeptide substrates with broad specificity [1]. This is a lysosomal peptidase which has been found in a number of mammals, as well as lower animals [2,3]. Its main function is protein degradation in lysosymes, but it was also found to participate in the activation of neuraminidase and proenzymes of serine proteinases (leukocyte elastase, cathepsin G, granzyme A) [1,4,5]. It is also well established that cathepsin C is involved in several pathological disorders, such as Duchenne muscular dystrophy, rheumatoid arthritis and basal cell carcinomas [6–8].

In general, cathepsin C shows an absolute requirement for a free amino terminus in its substrates. Most common assay substrates for this enzyme are substituted naphtyl- and phenylamides of glycylphenylalanine or glycylarginine [1]. This specificity is reflected in the structure of the most potent inhibitor of the enzyme, namely glycylphenylalanyl-diazomethane (compound **1**; Scheme 1) [9]. In this paper, we describe synthesis of tetrapeptide *p*-nitrophenylamides containing dehydroalanine and (*Z*)-dehydrophenylalanine in various positions of the chain (compounds **2**–**4**).

As dehydroamino acids are quite reactive, and various thiol nucleophiles are known to add to their double bonds [10–12], we hoped that these compounds might act as alkylating inhibitors of cathepsin C. This appeared to be a false idea, as our dehydropeptides acted as substrates of the enzyme, with activity comparable with their classic counterparts. However, their substrate activity also gives some additional, useful information about cathepsin C structural requirements.

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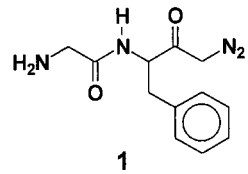

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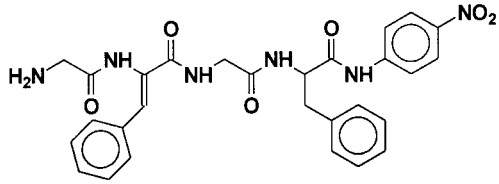

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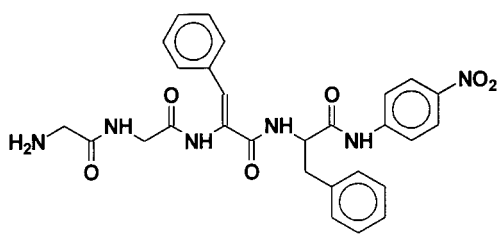
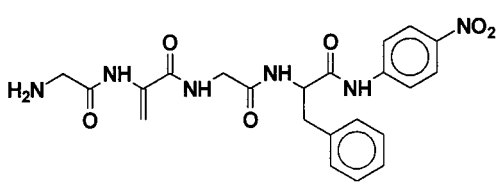


3, Gly-Gly-ΔPhe-Phe-pNA



4, Gly-ΔAla-Gly-Phe-pNA

Scheme 1 Potent cathepsin C inhibitor (**1**) and structurally related dehydropeptides synthesized in this work.

## MATERIALS AND METHODS

### General

All the starting materials were purchased from commercial suppliers, and were used without purification. All the solvents were additionally dried using standard procedures. Starting tri- and dipeptides were available from previous studies [13,14], or were obtained by standard methods [15].

NMR spectra were recorded on a Bruker 300 MHz instrument, operating at 300.13 MHz ($^1$H) and 75.46 MHz ($^{13}$C) in deuterated DMSO. Chemical shifts are given in relation to $SiMe_4$, and the central peak of deuterated chloroform triplet, respectively.

### Synthesis of Boc-Gly-ΔAla-Gly-Phe-pNA

Triethylamine (0.556 mL, 4. mmole) was added to a solution of Boc-Gly-ΔAla (0.488 g, 2 mmole) in 6 mL of DMF, and the solution was cooled to $-10$°C. Then, isobutyl chloroformate (0.26 mL, 2 mmole) was added and left for 5 min at this temperature. Finally, Gly-Phe-pNA trifluoroacetate salt (0.7989 g, 2 mmole) was added, and the reaction was carried out for 36 h at room temperature. The formed precipitate was then filtered off and the solvent was removed under reduced pressure. The resulting oil was dissolved in ethyl acetate (80 mL) and washed successively with 2 M hydrochloric acid ($2 \times 5$ mL), saturated potassium bicarbonate ($2 \times 5$ mL) and brine (saturated solution (or xyz) of NaCl) (5 mL). Organic layer was dried over magnesium sulphate, drying agent was removed by filtration, and the solvents were evaporated. Purification by means of column chromatography using silica gel $H_{60}$ and methanol-chloroform solution as an eluent afforded the desired peptides as white crystals melting at 183–186°C, in 38% yield. Elemental analysis calculated for $C_{27}H_{32}N_6O_8$ (568.6): 57.04% C and 5.67% H: found 57.34% C and 5.88% H.

$^1$H-NMR (DMSO): 10.54 (1H, s, NHpNA), 8.34 (1H, d, NHΔAla), 8.22–7.84 (6H, m, aromatic protons of pNA + NHPhe + NHGly(2)), 7.26–7.18 (5H, m, aromatic protons of Phe), 7.00 (1H, s , NHGly(1)), 4.65 (1H, m, $C^{\alpha}H$ Phe), 3.71 (2H, d, $C^{\beta}H_2$ ΔAla), 3.55 (1H, d, $CH_2$Gly(2)), 3.31 (1H, s, $CH_2$Gly(1)), 3.02 (2H, d, $C^{\beta}H_2$ Phe), 1.35 (9H, s, $3 \cdot CH_3$ of BOC) $^{13}$C-NMR (DMSO): 28.13 ($C^{I}$ of BOC), 55.74 ($C^{IV}$ of BOC), 78.00 ($C^{\beta}$ ΔAla), 125.36 ($C^{IV}$ ΔAla), 126.49; 128.14; 128.22 (aromatic ring of Phe), 119.53; 129.12; 136.93 (aromatic ring of pNA), 160.80; 168.30; 169.35; 170.44; 171.02 (C=O).

### Synthesis of Boc-Gly-ΔPhe-Gly-Phe-pNA

Triethylamine (0.584 mL, 2 mmole) was added to the solution of Boc-Gly-(Z)ΔPhe (0.640 g, 2 mmole) and trifluoroacetae of Gly-Phe-pNA (0.912 g, 2 mmole) in 8 mL of acetonitrile. After 15 min at room temperature, TBTU (0.774 g, 4.1 mmole) was added, and the resulting mixture kept at room temperature for 3 h. Then, acetonitrile was evaporated and the obtained dense oil was dissolved in 50 mL of ethyl acetate. The solution was worked-up, as above. White product was obtained by crystallization from 40:1 (v/v) mixture of chloroform and ethyl acetate. Yield 68%. M.p. 206–209°C. Elemental analysis calculated for $C_{33}H_{36}N_6O_8$ (664.6): 61.48% C and 5.63% H found 61.64% C and 5.38% H. $^1$H-NMR

(DMSO): 10.42 (1H, s, NHpNA), 8.41 (1H, s, NHΔPhe), 8.21–7.83 (6H, m, aromatic protons of pNa + NHGly(2) + NHPhe), 7.58 (1H, d, $C^{\beta}H$ ΔPhe), 7.39–7.14 (10H, m, aromatic protons of Phe and ΔPhe), 7.05 (1H, d, NHGly(1)), 4.62 (1H, d, $C^{\alpha}H$ Phe), 3.75 (4H, m, $2 \cdot CH_2$ of Gly(1) and Gly(2)), 3.01 (2H, m, $CH_2$ Phe), 1.32 (9H, s, $3 \cdot CH_3$ of BOC). $^{13}$C-NMR (DMSO): 28.13 ($C^{I}$ of BOC), 55.33 ($C^{IV}$ of BOC), 78.21(C ΔPhe), 124.89 ($C^{\alpha}$ ΔPhe), 129.73; 129.16; 128.81; 128.54; 128.17; 128.04 (aromatic rings of Phe and ΔPhe), 119.07; 124.89; 133.67 (aromatic ring of pNA), 156.03; 165.42; 169.05; 170.36; 170.96 (C=O).

### Synthesis of Boc-Gly-ΔPhe-Phe-pNA

Triethylamine (0.584 mL, 2 mmole) was added to the solution of Boc-Gly-(Z)ΔPhe (0.640 g, 2 mmole) and trifluoroacetae of Phe-pNA (0.798 g, 2 mmole) in 8 mL of acetonitrile. After 15 min at room temperature, TBTU (0.774 g, 4.1 mmole) was added and kept at room temperature for 2.5 h. The standard work-up described above yields an oily product which was crystallized from 40:1 (v/v) mixture of chloroform and ethyl acetate. Yield 62%, m.p. 203–204.5°C. Elemental analysis calculated for $C_{31}H_{33}N_5O_7$ (664.6): 63.36% C and 5.66% H: found 63.46% C and 5.58% H. $^1$H-NMR (DMSO): 10.00 (1H, d, NHpNA), 8.32 (1H, d, NHΔPhe), 8.22–7.98 (5H, m, aromatic protons of pNa + NHPhe), 7.53 (1H, d, $C^{\beta}H$ ΔPhe), 7.39–7.19 (10H, m, aromatic protons of Phe and ΔPhe), 6.80 (1H, s, NHGly), 4.66 (1H, s, $C^{\alpha}H$ Phe), 3.28 (4H, m, $CH_2$ of Gly and $C^{\alpha}H_2$ of Phe), 1.34 (9H, m, $3 \cdot CH_3$ of BOC). $^{13}$C-NMR (DMSO): 28.28 ($CH_3$ of BOC), 55.95 ($C^{IV}$ of BOC), 78.55 ($C^{\beta}$ ΔPhe), 125.00 ($C^{\alpha}$ ΔPhe), 129.83; 129.21; 129.03; 128.92; 128.79; 128.46 (aromatic rings of Phe and ΔPhe), 119.41; 133.64; 137.99 (aromatic ring of pNA), 156.70; 165.35; 170.98; 171.07 (C=O).

### Synthesis of Boc-Gly-Gly-ΔPhe-Phe-pNA

Boc-Gly-ΔPhe-Phe-pNA (2 mmole) was dissolved in anhydrous trifluoroacetic acid (6 mL) and the mixture was stirred for 15 min at room temperature. Then, dichloromethane was added (20 mL) and volatile components of the reaction mixture evaporated *in vacuo*. The resulting oil was purified by dissolving in ethyl ether (20 mL) and evaporation of solvents (three times). The obtained oily product was then directly used in the next step of reaction. Thus, it was dissolved in 8 mL of acetonitrile, and triethylamine was added (0.584 mL, 4.1 mmole). After 15 min at room temperature, TBTU (0.674 g, 2.1 mmole) was added and reaction carried out for 2 h at room temperature. The crude oily product, which precipitated directly from the reaction mixture, was used in the next step of synthesis without purification. $^1$H-NMR (DMSO): 10.07 (1H, s, NHpNA), 8.36 (1H, s, NHΔPhe), 8.32–7.92 (6H, m, aromatic protons of pNA + NHPhe + NHGly(2)), 7.55 (1H, d, $C^{\alpha}H$ ΔPhe), 7.39–7.18 (10H, m, aromatic protons of Phe and ΔPhe), 6.98 (1H, s, NHGly(1)), 4.66 (1H, s, $C^{\beta}H$ Phe), 3.20 (4H, m, $2 \cdot CH_2$ of Gly(1) and Gly(2)), 3.09 (2H, m, $C^{\beta}H_2$ Phe), 1.30 (9H, s, $3 \cdot CH_3$ of BOC). $^{13}$C-NMR (DMSO): 28.10 ($C^{I}$ of BOC), 56.04 ($C^{IV}$ of BOC), 78.15 ($C^{\beta}$ ΔPhe), 124.93 ($C^{\alpha}$ ΔPhe), 129.71; 129.11; 128.93; 128.61; 128.48; 128.22 (aromatic rings of Phe and ΔPhe), 124.93; 133.31; 133.46 (aromatic ring of pNA), 155.81; 164.90; 170.30; 170.73; 170.95 (C=O).

### Synthesis of Dehydrotetrapeptide Trifluoroacetates

Protected dehydrotetrapeptide trifluoroacetate (1 mmole) was dissolved in 3 mL of anhydrous trifluoroacetic acid, and stirred at room temperature for 15 min. Then dichloromethane (10 mL) was added, and volatile components of reaction mixture removed under reduced pressure. Glassy product was purified by dissolving in ether (10 mL) and evaporation of solvents. This procedure was repeated three times. The resulting trifluoroacetates were, additionally, dried in vacuum dessicator over phosphorus pentaoxide yielding glassy substances of very broad melting points.

***Gly-ΔAla-Gly-Phe-pNA Trifluoroacetate***. Yield 93%. Elemental analysis calculated for $C_{24}H_{25}N_6O_8F_3$ (582.49): 49.49% C and 4.33% H found 49.35% C and 4.55% H. $^1$H-NMR (DMSO): 10.75 (1H, s, NHpNA), 8.54 (2H, d, NHΔAla), 8.21–8.18 (5H, m, aromatic protons of pNA and NHPhe), 7.81 (2H, d, NHGly(1)), 7.27–7.20 (5H, m, aromatic protons of Phe), 7.18 (1H, d, NHGly(2)), 4.68 (1H, m, $C^{\alpha}H$ Phe), 3.70 (2H, m, $C^{\beta}H_2$ Phe), 3.55 (2H, s, $C^{\beta}H_2$ ΔAla), 3.18–2.84 (4H, m, $2 \cdot CH_2$ Gly(1) and Gly(2)). $^{13}$C-NMR (DMSO): 55.69 ($C^{\beta}$ ΔAla), 119.74 ($C^{\alpha}$ ΔAla), 129.60; 129.64; 127.00 (aromatic rings of Phe), 115.76; 125.41; 137.62 (aromatic ring of pNA), 145.30; 164.64; 168.80; 171.47 (C=O).

***Gly-ΔPhe-Gly-Phe-pNA Trifluoroacetate***. Yield 97%. Elemental analysis calculated for $C_{30}H_{29}N_6O_8F_3$ (658.59): 54.71% C and 4.44% H found 54.85% C and 4.67% H. $^1$H-NMR (DMSO): 10.67 (1H, s, NHpNA), 8.40 (1H, s, NH ΔPhe), 8.29–8.12 (5H, m, aromatic protons of pNA and NHPhe),

7.82 (2H, d, NHGly(1)), 7.55 (1H, d, $C^{\beta}H$ ΔPhe), 7.38–7.18 (10H, m, aromatic protons of Phe and ΔPhe), 7.13 (1H, s, NHGly(2)), 4.66 (1H, s, $C^{\alpha}H$ Phe), 3.51 (4H, m, $2 \cdot CH_2$ of Gly(1) and Gly(2)), 3.06 (2H, d, $C^{\beta}H_2$ Phe). $^{13}$C-NMR (DMSO): 55.65 ($C^{\beta}$ ΔPhe), 123.39 ($C^{\alpha}$ ΔPhe), 129.75; 129.62; 129.47; 129.12; 128.61; 128.40 (aromatic rings of Phe and ΔPhe), 119.53; 133.89; 137.64 (aromatic ring of pNA), 165.08; 166.92; 169.39; 171.56 (C=O).

***Gly-Gly-ΔPhe-Phe-pNA Trifluoroacetate***. Yield 94%. Elemental analysis calculated for $C_{30}H_{29}N_6O_8F_3$ (658.59): 54.71% C and 4.44% H: found 54.35% C and 4.31%H. $^1$H-NMR (DMSO): 10.40 (1H, s, NHpNA), 8.82 (1H, s, NHΔPhe), 8.47 (1H, s, NHGly(1)), 8.24–7.92 (5H, m, aromatic protons of pNA + NHPhe), 7.54 (1H, s, $C^{\beta}H$ ΔPhe), 7.39–7.20 (10H, m, aromatic protons of Phe and ΔPhe), 6.89 (1H, s, NHGly(2)), 4.69 (1H, s, $C^{\alpha}H$ Phe), 3.63 (2H, s, $C^{\beta}H_2$ Phe), 3.35 (4H, m, $2 \cdot CH_2$ of Gly(1) and Gly(2)). $^{13}$C-NMR (DMSO): 56.32 ($C^{\beta}$ ΔPhe), 125.04 ($C^{\alpha}$ ΔPhe), 129.61; 129.18; 128.85; 128.62; 128.22; 127.98 (aromatic rings of Phe and ΔPhe), 119.13; 133.60; 137.79 (aromatic ring of pNA), 165.10; 166.65; 169.05; 171.03 (C=O).

### Assay of Inhibitory Activity

Cathepsin C was isolated according to the described procedure [16]. Its activity was equal 2.26 mU. Enzymatic reaction was assayed at 25°C in acetate buffer (pH 5) containing NaCl (10 mM final concentration) and 2-mercaptoethanol (5 mM final concentration). The assay mixture contained tetrapeptide *p*-nitroanilide (1–7 mM final concentration) and the course of reaction was monitored by following the change in absorbance at 405 nm. Michaelis constants ($K_M$) and maximal velocities of the reaction ($V_{max}$) were obtained using the computer programme kindly provided by Dr J. Hurek (University of Opole).

## RESULTS AND DISCUSSION

### Dehydropeptide Synthesis

Cathepsin C is known to degrade peptide by sequential removal of dipeptides from their *N*-terminus. As the best substrates are those containing repeatable Gly-Phe motifs, we have synthesized compound **2**, which is a formal analogue of Gly-Phe-Gly-Phe-pNA. Structurally similar peptide **3** represents an analogue in which (Z)-dehydrophenylalanine was shifted towards *C*-termini, whereas in compound **4**, dehydroalanine was introduced in place of *N*-terminal phenylalanine of the model peptide. For comparison, their structural counterparts, namely Gly-Phe-Gly-Phe-pNA, Gly-Gly-Phe-Phe-pNA and Gly-Ala-Gly-Phe-pNA were also prepared and assayed. Dehydropeptides were obtained by standard solution method by coupling N-butoxycarbonyl peptides containing *C*-terminal dehydroamino acids, with corresponding peptide *p*-nitrophenylamides, using mixed carboxylic-carbonic anhydride method, or using TBTU as a condensing agent. Removal of butoxycarbonyl protection was easily achieved by action of trifluoroacetic acid with no effect on *p*-nitropenyl amide moiety. Removal of the latter group could be also easily achieved by mild alkaline hydrolysis.

### Enzymatic Studies

All the studied peptides (tested as trifluoroacetate salts) exerted quite good substrate activity (Table 1) towards cathepsin C from bovine spleen. As seen from the Table 1, the affinities of dehydropeptides are identical or even higher than these observed for model peptides and comparable with that given by Gly-Phe-pNA, the standard synthetic substrate of the enzyme. The most striking effect was observed when comparing Gly-Phe-Gly-Phe-pNA with Gly-ΔPhe-Gly-Phe-pNA, because dehydropeptide is bound by cathepsin C three times stronger than its counterpart, whereas its higher $V_{max}$ value indicates that it is a better substrate for the enzyme. In order to hydrolyse this peptide, the enzyme had to split the bond between (Z)-dehydrophenylalanine and glycine in the first step of the reaction, which is then followed by hydrolysis of the dipeptide anilide containing *N*-terminal (Z)-dehydrophenylalanine.

Table 1 Substrate Activity Dehydrotetrapeptides

| Peptide | Michaelis constant $K_M$ [mM] | $V_{max}$ |
|---|---|---|
| Gly-Phe-pNA | 3.1 | 1.039 |
| Gly-ΔPhe-Gly-Phe-pNA | 7.8 | 0.224 |
| Gly-Phe-Gly-Phe-pNA | 22.5 | 0.075 |
| Gly-ΔAla-Gly-Phe-pNA | 5.0 | 0.231 |
| Gly-Ala-Gly-Phe-pNA | 5.6 | 0.257 |
| Gly-Gly-ΔPhe-Phe-pNA | 7.8 | 0.003 |
| Gly-Gly-Phe-Phe-pNA | Insoluble in water | |

Thus, two different dehydropeptides act as cathepsin C substrates before *p*-nitrophenylalanine is released. Other data given in Table 1 also suggest that replacement of amino acid in a short peptide by corresponding dehydroamino acid does not result in a drastic change in its structure. This is quite surprising if considering well established antimetabolite activity of dehydroamino acids and their peptides.

## CONCLUSIONS

Tetrapeptides containing (Z)-dehydrophenylalanine and dehydroalanine appear to be good synthetic substrates of dipeptidyl-peptidase I (cathepsin C), being equipotent or better than their classical counterparts.

## Acknowledgements

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