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Novel amidino-substituted benzimidazoles: Synthesis of compounds and inhibition of dipeptidyl peptidase III

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Abstract

Dipeptidyl peptidase III (DPP III), also known as enkephalinase B, is a zinc-hydrolase with an indicated role in the mammalian pain modulatory system. In order to find a potent antagonist of this enzyme, we synthesized and screened the effect of a small set of benzimidazole derivatives on its activity. To improve the inhibitory potential, a cyclobutane ring was introduced as rigid conformation support to the diamidino substituted dibenzimidazoles. Two such compounds (**1′** and **4′**) from the group of cyclobutane derivatives containing amidino-substituted benzimidazole moieties, obtained by photochemical cyclization in water and by respecting rules of the "green chemistry" approach, were found to be strong DPP III inhibitors, with IC_{50} value below 5 μM. Compound **1′** displayed time-dependent inhibition towards human DPP III, characterized by the second-order rate constant of $6924 \pm 549\ M^{-1}\ min^{-1}$ ($K_i = 0.20$ μM). The peptide substrate valorphin protected the enzyme from inactivation by **1′**.

Keywords: Amidino-substituted benzimidazoles; Dipeptidyl peptidase III inhibitors; Green chemistry

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1. Introduction

Four types of dipeptidyl peptidases (DPPs) have been characterized originally in mammalian tissues, being designated as DPP I–IV and recognized through the hydrolysis of distinct synthetic substrates (dipeptidyl naphthylamides) [1]. Subsequently, several new mammalian dipeptidyl peptidase proteins were discovered (DPP6, DPP8, DPP9, and DPP10) [2–5] as the members of the group of "dipeptidyl peptidase IV activity and/or structure homologues", also known as the DASH family [6].

The dipeptidyl peptidase III (DPP III; EC 3.4.14.4; formerly: dipeptidyl arylamidase III, dipeptidyl aminopeptidase III) is a type of proteolytic enzymes discovered in extracts of bovine pituitary gland as the third in a series of dipeptidyl arylamidases through the hydrolysis of synthetic substrate Arg-Arg-2-naphthylamide (Arg_2-2NA) [7]. It was purified from several human and animal tissues and biochemically characterized to be a zinc-exopeptidase which cleaves dipeptides sequentially from the N-termini of its peptide substrates, consisting of three or more amino acids [8]. *In vitro* studies have shown that this enzyme displays a relatively broad specificity toward its substrates, optimally sized from tetra to octapeptides, including biologically active (neuro)peptides such as enkephalins and angiotensins [8–10]. However, the physiological substrates of this, in mammalian tissues broadly distributed, peptidase are mostly unknown, although a regulatory role for DPP III was suggested [9,10]. The importance of DPP III for cataractogenesis was pointed out recently [11]. A role in the metabolism of myotropic neuropeptide proctolin is well substantiated for the insect DPP III [12]. The high affinity shown by DPP III *in vitro* for enkephalins, and the evidence of a metabolic pathway involving a dipeptidyl aminopeptidase activity, indicated the role of DPP III (also reported as "enkephalinase B") in the metabolism of these opioid pentapeptides [10,13,14].

Human DPP III was recognized as a potential biomarker of malignancy, due to the finding of its enhanced expression in endometrial carcinomas and in malignant neoplasms of the ovary [15,16].

The 3-D structure of DPP III is not known. The amino acid sequence of rat and human DPP III has been determined and the amino acids involved in catalytic activity were established to be His^{451}, His^{456}, and Glu^{508}, which coordinate the active site Zn^{2+} ion, and Glu^{452} which is responsible for the general base catalysis of this enzyme [17–19]. Our previous results suggested the existence of hydrophobic subsite S_1' in the catalytic site of human DPP III [9].

Inhibition by non-specific chelating and thiol reagents is a feature of DPP III originating from various species [8,20]. However, the studies of more specific inhibitors of this enzyme are very rare. An early initiative by Nishikiori et al. [21] aimed at the discovery of DPP III inhibitors was based on the screening of microbial cultures and resulted in the isolation of acetyl-L-leucyl-L-argininal, which inhibited rat DPP III slightly less potently than structurally related leupeptin, a well known non-specific inhibitor of serine and thiol proteases. The same approach later revealed non-peptidic DPP III inhibitors of microbial origin, fluostatins A and B (in the fermentation broth of a *Streptomyces* sp. TA-3391), the compounds containing tetracyclic nucleus with fluorenone moiety [22]. Both fluostatins were reported to inhibit DPP III from human placenta, but not dipeptidyl peptidases of other types. Fluostatin A was shown to be the more potent, reversible, mixed-type inhibitor with K_i value of 14 μM. Its selectivity for DPP III was suggested.

The heptapeptide spinorphin (Leu-Val-Val-Tyr-Pro-Trp-Thr) was isolated from the bovine spinal cord as a potent endogenous inhibitor of enkephalin degrading enzymes purified from monkey brain [23]. It inhibited brain dipeptidyl aminopeptidase with $K_i = 0.5$ μM. Interestingly, the authors claimed that the enkephalin degrading enzymes (including aminopeptidase N and DPP III) isolated from peripheral tissues (kidney, blood) were unaffected by spinorphin. It was shown that spinorphin is easily degraded in the body by (amino)peptidases [24]. The synthetic pentapeptide tynorphin (Val-Val-Tyr-Pro-Trp), a truncated form of spinorphin, inhibited DPP III purified from monkey brain with an K_i of 7.5×10^{-8} M, suggesting that this pentapeptide is more specific for brain DPP III than spinorphin [25]. However, like spinorphin, tynorphin was unstable towards human serum peptidase action [25].

In the search for new and more potent non-peptidic inhibitors of DPP III, based on the knowledge of zinc ligands in the active site of this proteolytic enzyme (two His and one Glu), we attempted to investigate heterocyclic compounds which could form complexes with essential metal ions. In addition, as DPP III belongs to aminopeptidases, which require the free amino group in their substrates, since the preferred synthetic substrate of this enzyme is Arg_2-2NA, and a hydrophobic pocket in its catalytic site was indicated, in order to achieve selectivity it seemed reasonable to explore the effect of compounds which, besides metal ion-complexing functionalities also contain basic (e.g., amidino) and hydrophobic groups in the molecule.

As a lead structure, we chose benzimidazole moiety, which has been used already as a part of a central scaffold in some metallo- and serine peptidases inhibitors, due to its potential in H-bonding and π–π stacking interactions with the imidazole ring of His residues essential for the activity of these enzymes [26,27].

At first, we synthesized and examined a small set of benzimidazole derivatives and we report on the inhibitory potency against human DPP III of the most active ones, which contained one amidine group as substituent. Furthermore, assuming that the inhibitory effect towards this zinc-peptidase could be even higher with two amidino-substituted benzimidazole groups present in the inhibitor molecule, as was previously shown for several serine peptidases and potent zinc-mediated serine protease inhibitors [28], we introduced a cyclobutane ring into the inhibitor structure to provide rigid conformation support to the diamidino substituted dibenzimidazoles. Indeed, two compounds from this group, photochemically synthesized in water, were the most potent inhibitors of human DPP III which we prepared and we wish to report the results of this study as hitherto inhibitory activity of neither benzimidazole derivatives nor cyclobutane derivatives against human DPP III has been investigated.

2. Experimental section

2.1. General methods and chemicals

Melting points were obtained on an Original Kofler Mikroheitztisch apparatus (Reichert, Wien) and are uncorrected. The NMR spectra were recorded on Varian Gemini 300 or a Bruker Avance DPX 300 and 500 spectrometers at 300, 500, and 75 MHz, respectively. All NMR spectra were measured in DMSO-d_6 solutions using

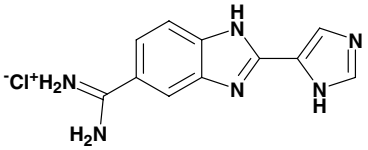
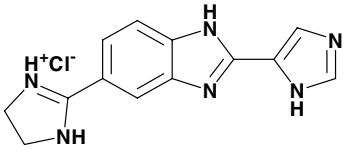
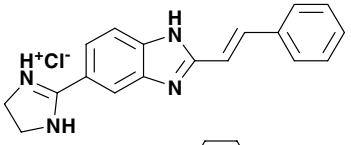
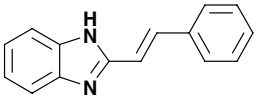
TMS as an internal standard. Mass spectra were recorded by using electrospray ionization technique (ESI) on the Micromass Platform LCZ single quadropole mass spectrometer. Elemental analysis for carbon, hydrogen, and nitrogen were performed on the Perkin-Elmer 2400 elemental analyzer. Where analyses are indicated only as symbols of elements, the obtained analytical results are within 0.4% of the theoretical value. Some compounds were routinely checked by TLC with Merck silica gel 60F-254 glass plates.

Arg-Arg-2-naphthylamide (Arg-Arg-2NA)·3HCl and Gly-Pro-2-naphthylamide (Gly-Pro-2NA) were supplied by Bachem AG (Bubendorf, Switzerland). Microcon YM-10 centrifugal filter devices (MWCO 10,000) were obtained from Millipore (Bedford, MA, USA). D-Tube Dialyzers were produced by Merck Biosciences (Darmstadt, Germany). ConA–Sepharose was purchased from Pharmacia Fine Chemicals AB (Uppsala, Sweden). All other materials used for this study were purchased from Sigma–Aldrich (St. Louis, MO, USA) unless stated otherwise.

2.2. Synthesis of compounds

Compounds **1** and **2** (Table 1) were prepared by condensation of 4-*N*-amidino substituted *o*-phenylenediamines and 4(5)-imidazolecarboxaldehyde in equimolar amounts according to the method of Fairley et al. [29].

Table 1
Effect of various benzimidazole derivatives on the activity of human DPP III[a]

Compound No.	Structure	Inhibition at 100 μM concentration	IC_{50} (μM)
1		Complete	53
2		Complete	18
3		Complete	~10
4		Partial	>100

[a] Enzyme (2.9×10^{-10} M) was preincubated with potential inhibitor at pH 7.4 for 10 min at 25 °C, followed by 5 min at 37 °C, before the addition of substrate. Activity assay was performed as described in the Section 2. The results are average values of 2–3 determinations.

*2.2.1. 2-(3H-Imidazol-4-yl)-1H-benzoimidazole-5-carboxamidine hydrochloride (**1**)*

Yield 0.108 g, 41.1%, mp 275–277 °C. MS *m/z*: 227.2 (MH^{+1}, −HCl); IR (cm^{-1}): 3153, 3005, 2771, 1691, 1432, 1605 1560; ^1H NMR (DMSO-d_6) (δ/ppm): 13.02 (br s, 1H, $NH_{imid.}$), 12.6 (br s, 1H, $NH_{benzimidazol}$), 9.20 (br s, 4H, NH_{amidin}) 8.90–8.85 (m, 2H, H_{arom}) 7.85 (d, 2H, $J = 8.2$ Hz, H_{arom}) 7.53 (s, 1H, H_{arom}); Anal. Calcd for ($C_{11}H_{11}ClN_6$): C, 54.08; H, 4.54; N, 29.11. Found: C, 54.35; H, 4.23; N, 29.49.

*2.2.2. 2-(3H-Imidazol-4-yl)-1H-benzoimidazole-5-imidazolinylamidine hydrochloride (**2**)*

Yield 0.09 g, 31.2%, mp > 290 °C. MS *m/z*: 253.2 (MH^{+1}, −HCl); IR (cm^{-1}): 3381, 3103, 2968, 2854, 1629, 1608, 1509; ^1H NMR (DMSO-d_6) (δ/ppm): 13.10 (br s, 1H, $NH_{imid.}$), 12.80 (br s, 1H, $NH_{benzimidazol}$), 10.47 (br s, 1H, NH_{amidin}), 8.16 (s, 1H, H_{arom}), 7.88 (s, 1H, H_{arom}), 7.86 (s, 1H, H_{arom}), 7.72 (d, 1H, $J = 8.4$ Hz, H_{arom}), 7.62 (s, 1H, H_{arom}), 3.96 (s, 4H, CH_2); Anal. Calcd for ($C_{13}H_{13}ClN_6$): C, 50.29; H, 4.22; N, 31.99. Found: C, 50.35; H, 4.33; N, 31.59.

Compound **3** was synthesized by condensation of commercially available 3-phenyl-propenal with 4-(2-imidazolinyl)-*o*-phenylenediamine [30] while compound **4** was prepared using the method by Ramaiah et al. [31].

Compounds **1′**–**8′** were prepared by photochemical synthesis.

*2.3. General method for preparing compounds **1′**–**6′***

A solution ($c = 1.3 \times 10^{-2}$ M) of corresponding *E*-2-styryl-5-*N*-amidino-substituted-benzimidazole hydrochloride (M. Hranjec, M. Kralj, K. Pavelić, G. Karminski-Zamola, *Patentni glasnik*, Patent Appl. P20050806A) in water was irradiated at room temperature with 400 W high-pressure mercury lamp using a Pyrex filter for 2 h. The air was bubbled through the solution. The solution was concentrated, precipitated with acetone and the resulting solid was filtered off. The product was dissolved in ethanol and precipitated with diethyl-ether, filtered off and dried. The procedure was repeated two times until the product was analytically pure.

*2.3.1. 1,3-Di-[5-(2-imidazolinyl)-2-benzimidazolyl]-2,4-di-phenyl-cyclobutane dihydrochloride (**1′**)*

Compound **1′** was prepared from *E*-5-(1*H*-imidazol-2-yl)-2-styryl-1*H*-benzoimidazole hydrochloride (0.150 g, 0.46 mmol) in 36 ml water after irradiation for 1.5 h: 0.120 g (80%) light violet powder; mp > 300 °C; MS *m/z*: 577 (M^{+1} (−2HCl) 10%), 289 ($M^{+2}/2$, 100%); ^1H NMR (DMSO-d_6) (δ/ppm) 13.25–13.00 (br s, 2H, $NH_{benzoimid.}$), 10.59 (br s, 4H, $NH_{amidine}$), 8.80–6.98 (m, 16H, H_{arom}), 5.06 (br s, 2H, $H_{cyclobut.}$), 4.91 (br s, 2H, $H_{cyclobut.}$), 4.06–4.00 (m, 8H, $H_{imid.}$); Anal. Calcd for ($C_{36}H_{34}Cl_2N_8$): C, 66.56; H, 5.28; N, 17.25. Found: C, 66.35; H, 5.03; N, 17.49.

*2.3.2. 1,3-Di-[5-(N-amidino)-2-benzimidazolyl]-2,4-di-phenyl-cyclobutane dihydrochloride (**2′**)*

Compound **2′** was prepared from *E*-2-styryl-3*H*-benzoimidazole-5-carboxamidine hydrochloride (0.140 g, 0.47 mmol) in 35 ml water after irradiation for 1.5 h: 0.130 g (93%) light violet powder; mp 254–257 °C; MS *m/z*: 525 (M^{+1} (−2HCl) 8%), 263 ($M^{+2}/2$, 100%); ^1H NMR (DMSO-d_6) (δ/ppm) 13.21 (br s, 2H, $NH_{benzoimid.}$), 9.30–9.09 (m, 8H, $NH_{amidine}$), 8.20–6.98 (m, 16H, H_{arom}), 5.01 (br s, 2H, $H_{cyclobut.}$), 4.92 (m, 2H, $H_{cyclobut.}$);

Anal. Calcd for ($C_{32}H_{30}Cl_2N_8$): C, 64.32; H, 5.06; N, 18.75; Found: C, 64.10; H, 4.90; N, 18.49.

*2.3.3. 1,3-Di-[5-(N-isopropylamidino)-2-benzimidazolyl]-2,4-di-phenyl-cyclobutane dihydrochloride (**3′**)*

Compound **3′** was prepared from *E*-*N*-Isopropyl-2-styryl-3*H*-benzoimidazole-5-carboxamidine hydrochloride (0.150 g, 0.44 mmol) in 35 ml water after irradiation for 1.5 h: 0.125 g (84%) yellow powder; mp 270–273 °C; MS *m*/*z*: 609 (M^{+1}(−2HCl) 20%), 305 (M^{+2}/2, 100%); ^{1}H NMR (DMSO-d_6) (δ/ppm) 13.20 (br s, 2H, $NH_{benzoimid.}$), 10.47–10,20 (br s, 6H, $NH_{amidine}$), 8.10–6.99 (m, 16H, H_{arom}), 5.25 (br s, 2H, $H_{cyclobut.}$), 4.92–4.89 (m, 2H, $H_{cyclobut.}$), 4.24–4.00 (m, 2H, $H_{i\text{-}Pr.}$), 1.43 (d, 12H, $H_{i\text{-}Pr.}$, $J = 6.3$ Hz); Anal. Calcd for ($C_{38}H_{42}Cl_2N_8$): C, 66.95; H, 6.21; N, 16.44. Found: C, 66.78; H, 6.03; N, 16.69.

*2.3.4. 1,3-Di-[5-(2-imidazolinyl)-2-benzimidazolyl]-2,4-di-o-chlorophenyl-cyclobutane dihydrochloride (**4′**)*

Compound **4′** was prepared from *E*-2-[2-(2-chloro-phenyl)-vinyl]-5-(1*H*-imidazol-2-yl)-1*H*-benzimidazole hydrochloride (0.160 g, 0.45 mmol) in 35 ml water after irradiation for 1.5 h: 0.125 g (78%) light brown powder; mp > 300 °C; MS *m*/*z*: 645 (M^{+1} (−2HCl) 5%), 323 (M^{+2}/2, 100%); ^{1}H NMR (DMSO-d_6) (δ/ppm) 13.18–12.97 (m, 2H, $NH_{benzoimid.}$), 10.60 (br s, 4H, $NH_{amidine}$), 8.40–6.93 (m, 14H, H_{arom}), 5.34 (br s, 2H, $H_{cyclobut.}$), 5.01 (br s, 2H, $H_{cyclobut.}$), 3.94–3.90 (m, 8H, $H_{imid.}$); Anal. Calcd for ($C_{36}H_{32}Cl_4N_8$): C, 60.18; H, 4.69; N, 15.60. Found: C, 60.32; H, 4.93; N, 15.29.

*2.3.5. 1,3-Di-[5-(N-amidino)-2-benzimidazolyl]-2,4-di-o-chlorophenyl-cyclobutane dihydrochloride (**5′**)*

Compound **5′** was prepared from *E*-2-[2-(2-chloro-phenyl)-vinyl]-3*H*-benzoimidazole-5-carboxamidine hydrochloride (0.150 g, 0.45 mmol) in 35 ml water after irradiation for 1.5 h: 0.140 g (93%) light brown powder; mp 263–265 °C; MS *m*/*z*: 593 (M^{+}(−2HCl) 20%), 297 (M^{+2}/2, 100%); ^{1}H NMR (DMSO-d_6) (δ/ppm) 13.02 (br s, 2H, $NH_{benzoimid.}$), 9.38–9.23 (m, 4H, $NH_{amidine}$), 9.09–9.03 (m, 4H, $NH_{amidine}$), 8.14–7.08 (m, 14H, H_{arom}), 5.46 (d, 2H, $H_{cyclobut.}$), 5.19 (br s, 2H, $H_{cyclobut.}$); Anal. Calcd for ($C_{32}H_{28}Cl_4N_8$): C, 57.67; H, 4.23; N, 16.81. Found: C, 57.40; H, 4.51; N, 16.59.

*2.3.6. 1,3-Di-[5-(N-isopropylamidino)-2-benzimidazolyl]-2,4-di-o-chlorophenyl-cyclobutane dihydrochloride (**6′**)*

Compound **6′** was prepared from *E*-2-[2-(2-chloro-phenyl)-vinyl]-*N*-isopropyl-3*H*-benzoimidazole-5-carboxamidine hydrochloride (0.100 g, 0.27 mmol) in 20 ml water after irradiation for 2 h: 0.70 g (70%) light yellow powder; mp 269–271 °C; MS *m*/*z*: 677 (M^{+1} (−2HCl) 30%), 339 (M^{+2}/2, 100%); ^{1}H NMR (DMSO-d_6) (δ/ppm) 13.29–13.04 (m, 2H, $NH_{benzoimid.}$), 9.49–9.37 (m, 2H, $NH_{amidine}$), 9.09 (br s, 4H, $NH_{amidine}$), 7.94–7.05 (m, 14H, H_{arom}), 5.16 (br s, 2H, $H_{cyclobut.}$), 5.02 (br s, 2H, $H_{cyclobut.}$), 4.20–4.11 (m, 2H, $H_{i\text{-}Pr.}$), 1.30 (d, 12H, $H_{i\text{-}Pr}$, $J = 6.2$ Hz); Anal. Calcd for ($C_{38}H_{40}Cl_4N_8$): C, 60.81; H, 5.37; N, 14.93. Found: C, 60.70; H, 5.08; N, 14.68.

2.4. General method for preparing compounds 7′ and 8′

A solution ($c = 1 \times 10^{-2}$ M) of 5-(4,5-dihydro-1*H*-imidazol-2-yl)-2-(2-furan-2-yl-vinyl)-1*H*-benzoimidazole hydrochloride and 5-(4,5-dihydro-1*H*-imidazol-2-yl)-2-(2-thiophen-2-yl-vinyl)-3*H*-benzoimidazole hydrochloride (M. Hranjec, M. Kralj, K. Pavelić, G. Karminski-Zamola, *Patentni glasnik*, Patent Appl. P20050806A) in water, was irradiated at room temperature with 400 W high-pressure mercury lamp using a Pyrex filter for 3 h. The air was bubbled through the solution. The solution was concentrated, precipitated with acetone and the resulting solid was filtered off. The product was dissolved in water and precipitated with acetone, filtered off and dried. The procedure was repeated two times until the product was analytically pure.

2.4.1. 1,3-Di-[5-(2-imidazolinyl)-2-benzoimidazolyl]-2,4-di-(2-furyl)-cyclobutane dihydrochloride (7′)

Compound **7′** was prepared from 5-(4,5-dihydro-1*H*-imidazol-2-yl)-2-(2-furan-2-yl-vinyl)-1*H*-benzoimidazole hydrochloride (0.090 g, 0.29 mmol) in 30 ml water after irradiation for 2 h: 0.070 g (78%) light brown powder; mp > 300 °C; MS *m*/*z*: 558 (M^{+2} (−2HCl) 50%), 280 ($M^{+4}/2$, 100%); ^{1}H NMR (DMSO-d_6) (δ/ppm) 13.20 (br s, 2H, $H_{benzoimid.}$), 10.80 (br s, 4H, $NH_{amidine}$), 8.42–7.33 (m, 10H_{arom}), 6.20–6.14 (m, 2H, $H_{fur.}$, $J = 2.80$ Hz), 5.02 (t, 2H, $H_{cyclobut.}$, $J = 8.10$ Hz), 4.70 (br s, 2H, $H_{cyclobut.}$), 3.90 (br s, 8H, $H_{imid.}$); Anal. Calcd for ($C_{32}H_{30}Cl_2N_8O_2$): C, 61.05; H, 4.80; N, 17.80. Found: C, 61.32; H, 5.03; N, 17.58.

2.4.2. 1,3-Di-[5-(2-imidazolinyl)-2-benzoimidazolyl]-2,4-di-(2-thienyl)-cyclobutane dihydrochloride (8′)

Compound **8′** was prepared from 5-(4,5-dihydro-1*H*-imidazol-2-yl)-2-(2-thiophen-2-yl-vinyl)-3*H*-benzoimidazole hydrochloride (0.100 g, 0.30 mmol) in 30 ml water after irradiation for 3 h: 0.065 g (65%) dark gray powder; mp 276–280 °C; MS *m*/*z*: 589 (M^{+1} (−2HCl) 100%), 295(M^{+2}/2, 70%); ^{1}H NMR (DMSO-d_6) (δ/ppm) 13.20 (br s, $H_{benzoimid.}$), 13.04 (br s, 2H, $H_{benzoimid.}$), 10.70 (br s, 4H, $NH_{amidine}$), 9.02 (br s, 4H, $NH_{amidine}$), 8.30–6.88 (m, 12H, H_{arom}), 5.24 (t, 2H, $H_{cyclobut.}$, $J = 7.60$ Hz), 4.72 (t, 2H, $H_{cyclobut.}$, $J = 7.50$ Hz), 4.08 (br s, 8H, $H_{imid.}$); Anal. Calcd for ($C_{32}H_{30}Cl_2N_8S_2$): C, 58.09; H, 4.57; N, 16.93. Found: C, 58.31; H, 4.28; N, 16.66.

2.5. Biochemical methods

2.5.1. Purification of enzymes

Human DPP III was purified from healthy blood donor erythrocytes as described before [20]. Dipeptidyl peptidase IV (DPP IV, EC 3.4.14.5) was isolated from the rat kidney membranes. Rat kidney homogenate and membrane pellet were prepared as described earlier [32]. Membranes were solubilized with detergent (1% Triton X-100) according to Barelli et al. [33]. Detergent-form of DPP IV was purified by four-step chromatographic procedure. The first step, chromatography on the column of DEAE-cellulose equilibrated in 50 mM Tris/HCl (with 1% Triton X-100) pH 7.7, was performed according to Gorenstein and Snyder [34]. The DPP IV was eluted with a linear gradient of sodium chloride 0–0.4 M, and applied to the concanavalin A-Sepharose column equilibrated in 20 mM Tris/HCl buffer pH 7.4, containing 0.5 M NaCl and 0.5% Triton X-100. DPP

IV activity was eluted with a gradient 0–0.5 M methyl-α-D-glucopyranoside. Last two purification steps were gel filtration (column of Sephacryl S-200 superfine in 50 mM Tris/HCl buffer pH 7.5, containing 0.2 M NaCl) and chromatography on hydroxyapatite performed on Econo-Pac CHT-II Cartridge according to the manufacturer's (Bio-Rad Laboratories, Hercules, CA, USA) instruction manual.

2.5.2. Enzyme activity measurements and IC_{50} determination

The IC_{50} value is defined as the concentration of an inhibitor which caused 50% reduction of the enzyme activity under specific assay conditions. The assay conditions for DPP III were: 50 mM Tris/HCl buffer, pH 7.4. DPP IV was assayed in 50 mM sodium phosphate buffer of the same pH 7.4. For IC_{50} determination, the enzyme (0.1–0.3 nM) was incubated with increasing concentrations of inhibitor for 10 min at 25 °C, followed by 5 min at 37 °C in the total volume of 950 μl of buffer pH 7.4. The enzymatic reaction was started by addition of 50 μl of appropriate naphthylamide (NA) substrate solution (final concentrations were: 40 μM Arg-Arg-2NA, DPP III substrate; 0.5 mM Gly-Pro-2NA, DPP IV substrate) and after 15 min incubation at 37 °C, the reaction was stopped and quantified using the spectrophotometric method described previously [9]. The principle of these assays with synthetic 2-naphthylamide substrates is based on the absorbance at 530 nm of a chromophoric complex formed by the reaction product 2-naphthylamine with Fast Blue B salt. The stock solutions (7 mM) of inhibitors were prepared in dimethyl sulfoxide and further diluted with buffer, before the addition to the assay mixture. Inhibitor IC_{50} values were determined by semi-log plots with five different concentrations.

*2.5.3. Inactivation of DPP III by compound **1′***

The enzyme (14.5 nM) was equilibrated in 20 mM sodium phosphate buffer, pH 7.4 at 25 °C. Then the inhibitor was added to the appropriate concentration (3–20 μM). Aliquots of 10 μl were taken periodically and the residual enzymatic activity determined in a 1 ml reaction mixture by incubation at 37 °C for 15 min at pH 8.6, with Arg-Arg-2NA (40 μM) as substrate. The activity was expressed in percentages of the enzyme control (the enzyme activity in the absence of the inhibitor). The enzyme control sample contained dimethyl sulfoxide in the same % as the inhibitor-treated enzyme sample. When substrate protection was investigated, peptide solution was added to the enzyme equilibrated in buffer at 25 °C, immediately before the addition of the inhibitor.

2.5.4. K_i value determination

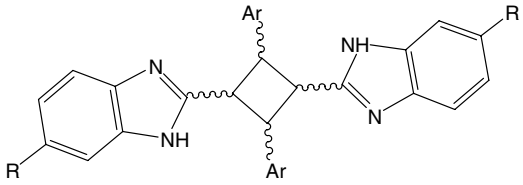
The dissociation constant for the initial reversible complex $E \cdot I$ was determined using high and competitive concentrations of the substrate (Arg_2-2NA) and inhibitor according to the method by Tornheim [35].

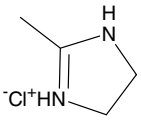
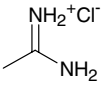
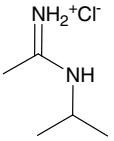
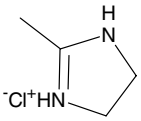
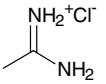
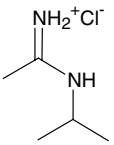
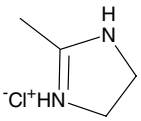
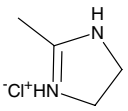
3. Results and discussion

3.1. Synthesis of inhibitors

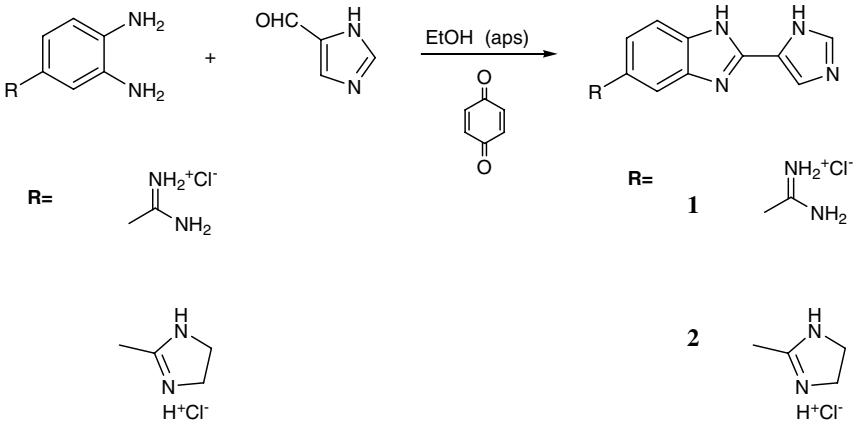
We examined altogether 12 benzimidazole compounds (Tables 1 and 2) for their potential inhibitory activity towards human DPP III. The synthesis of compounds is presented in Schemes 1 (compounds **1** and **2**), 2 (compound **3**) and 3 (compounds **1′**–**8′**).

Table 2
Effect of 2,4-disubstituted-1,3-di-(5-amidino-2-benzoimidazolyl)-cyclobutane hydrochlorides on the activity of human erythrocyte DPP III[a]

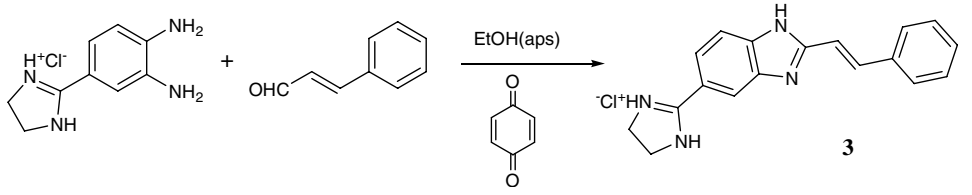


Compound No.	Ar	R	IC_{50} (μM)
1′	Phenyl		2.8
2′	Phenyl		5.6
3′	Phenyl		>10
4′	*o*-Cl-phenyl		1.7
5′	*o*-Cl-phenyl		~7
6′	*o*-Cl-phenyl		~6
7′	2-Furyl		~8
8′	2-Thienyl		~10

[a] Enzyme (0.3 nM) was preincubated with potential inhibitor at pH 7.4 for 10 min at 25 °C, followed by 5 min at 37 °C, before the addition of substrate. Activity assay was performed as described in the Section 2. The results are average values of 2–3 determinations.



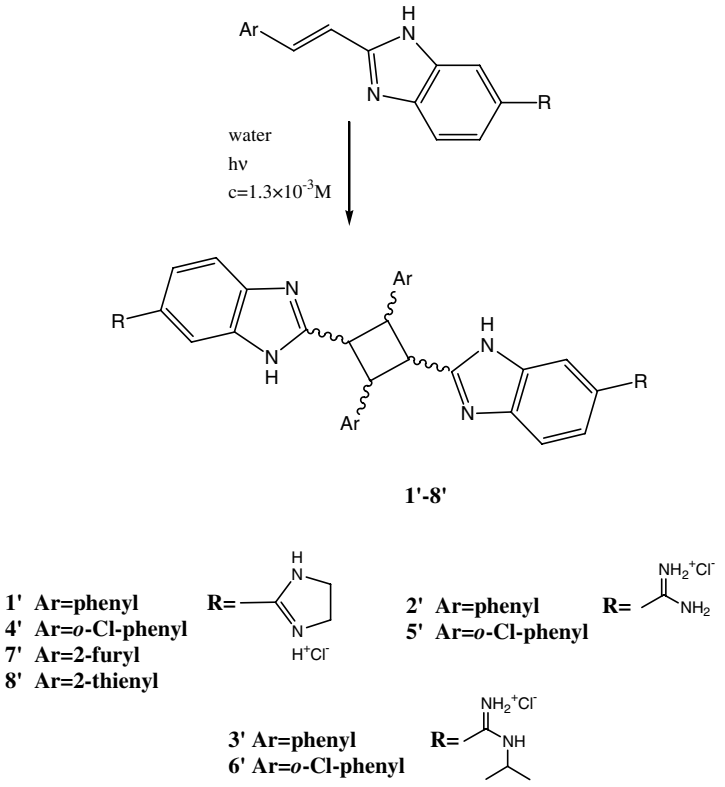
Scheme 1.



Scheme 2.

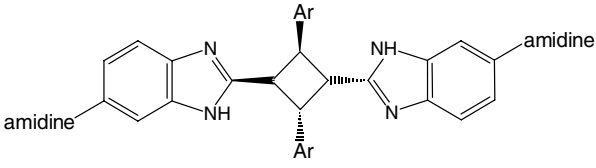
Water plays an essential role in life processes, yet its use as a solvent has been limited in organic synthesis despite the fact that it is the cheapest, safest and most non-toxic solvent. The use of water as a medium for organic reactions is therefore one of the latest challenges for modern organic chemists. We applied green chemistry approach for the synthesis of compounds **1′**–**8′** (Table 2), which have been prepared in a very high yield (70–93%) from the corresponding monomers by the reaction of photochemical [2 + 2] cycloaddition in water according to Scheme 3 [36]. The same reaction took place in concentrated and also in diluted solution. This is the first example of preparing cyclobutane derivatives with amidino-substituted benzimidazole nuclei.

Prepared compounds **1′**–**8′** appeared as a constant photochemical mixture of two stereoisomers of the head-to-tail type, detected by the presence of the same *m*/*z* fragment ion in the mass spectrometry. This constant mixture contained a very high amount of one isomer (>98%). Isomers could not be separated either by column chromatography or the HPLC/MS method. All cyclobutane derivatives showed small molecular ion and high base peaks *m*/*z* $M^+/2$ so we assigned them as head-to-tail adducts. The NMR data pointed to the symmetrical cyclobutane structure. The direct elucidation of cyclobutane stereochemistry was very difficult due to the highly overlapped ^{1}H NMR, especially in the region of aryl and benzimidazolyl protons. Signals for *trans*-ethylenic double bond with the coupling constant of about 16 Hz disappeared and two signals appeared for four cyclobutane protons in 1:1 ratio at about 4–5 ppm, thus indicating the symmetrical structure.



Scheme 3.

According to the NMR and literature data on the previously obtained similar compounds [37–42] and using some 2D NMR tecniques, like COSY, HETCOR and especially NOESY, we assume that stereochemistry of cyclobutane derivatives should correspond to the structure presented in Fig. 1, which is also energetically the most stable and most likely isomer. NOESY experiments showed some NOE interactions between cyclobutane protons and NH of benzimidazole nuclei as well as between cyclobutane protons and aryl protons.



Ar= phenyl, o-Cl-phenyl
2-furyl, 2-thienyl

Fig. 1. Structure of 2,4-disubstituted-1,3-di-(5-amidino-2-benzimidazolyl) cyclobutanes prepared by photochemical [2 + 2] cycloaddition in water.

3.2. Screening for inhibitory activity towards human DPP III

The screening was performed by examining the effect of 100 μM compound on the activity of human DPP III as described under the Section 2 at pH 7.4, using Arg_2-2NA as a substrate. IC_{50} was determined for those compounds which were found inhibitory.

Initially, we investigated a small set of four benzimidazole derivatives for their inhibitory activity towards human DPP III (Table 1). Compounds **1** and **2** contained an imidazole ring coupled directly to the amidino-substituted benzimidazole (Table 1). Compounds **3** and **4** contained benzimidazole and phenyl group linked through etenyl spacer (Table 1).

The data in Table 1 show that both compounds with the imidazole ring coupled to amidino-substituted benzimidazole were inhibitory. By comparing the effect of compounds **1** and **2** on the activity of human DPP III, it is obvious that cyclization of amidino functionalities significantly increased the inhibitory potency.

Examination of the two benzimidazole compounds with the phenyl ring coupled through a short spacer (compounds **3** and **4**; Table 1) revealed that the presence of amidino group is essential for obtaining the potent DPP III inhibition by benzimidazole derivatives, thus confirming our starting assumption on the necessity of the basic group presence in the inhibitor molecule.

Strong inhibition obtained with compound **3**, and previous findings by Katz et al. who reported high-affinity binding of zinc-mediated inhibitor bis-(5-amidino-2-benzimidazolyl)methane (BABIM) to trypsin [28], prompted us to prepare and investigate the inhibitory activity of a molecule formed by compound **3** dimerization. We assumed that such compound, possibly due to the participation of two benzimidazole moieties in tetrahedral coordination of the essential Zn^{2+}, would bind to the active site of human DPP III more firmly.

Table 2 shows that thus prepared symmetrical cyclobutane derivative (substance **1′**) was a much stronger enzyme inhibitor than the parent monomer (compound **3**, Table 1). We further varied aromatic substituent in positions 2 and 4 of cyclobutane ring, and amidino substitutent on the benzimidazole moiety, to investigate the influence of these structural changes on inhibitory activity.

Altogether eight symmetrical cyclobutane derivatives bearing two identical amidino-substituted benzimidazole rings and two identical phenyl or furyl or thienyl groups as substituents were synthesized and their effect on the activity of human DPP III at pH 7.4 was examined (Table 2). Among all these compounds, the most potent inhibition was caused by compounds **1′** and **4′**, with IC_{50} below 5 μM, followed by **2′**, **6′**, **5′**, and **7′**, with IC_{50} value between 5 and 10 μM.

The data in Table 2 show that the most potent DPP III inhibitors were the cyclobutane derivatives with two identical amidino-substituted benzimidazole moieties, which also contained two identical phenyl substituents, rather than furyl or thienyl. The cyclization of amidino functionalities increased the inhibitory potency of compounds with phenyl substituents (**1′** and **4′** had lower IC_{50} values than their ''non-cyclized'' counterparts **2′** and **5′**). The binding of the amidino group to isopropyl decreased the inhibitory effect of prepared cyclobutane derivative. The classical chelating agent and non-selective inhibitor of metallopeptidases, 1,10-phenanthroline, inhibited human DPP III under the chosen conditions much less potently, than any of the compounds listed in Table 2, with IC_{50} value of 92 μM.

In addition, we evaluated the five most potent new inhibitors of DPP III, compounds **1′**, **2′**, **4′**, **5′**, and **6′** for their ability to affect the activity of mammalian dipeptidyl peptidase IV (DPP IV). However, this serine peptidase was not inhibited, even at 100 μM concentration of the same heterocyclic compounds (data not shown). This indicates the specificity of action towards DPP III type of the proteolytic enzyme.

3.3. Inhibition type

Compound **1′** was chosen for further investigation of the type and mechanism of human DPP III inhibition by amidino benzimidazole compounds. The reversibility of the inhibition by compound **1′** was tested first by dilution of nearly completely inhibited enzymes, followed by residual activity determination. The extent of DPP III inhibition remained the same after significant (200-fold) dilution of preincubation mixture of the enzyme with the inhibitor in μM concentration range. Then we checked the reversibility of DPP III inhibition by **1′**, by dialysis against 10 mM Tris/HCl buffer pH 7.4 at 4 °C with or without the addition of 10 μM Zn acetate. No significant reactivation of the enzyme was found even at the prolonged time of dialysis (up to 94 h). Furthermore, the enzymatic activity of inhibitor-treated DPP III was not restored even after 5 cycles of ultrafiltration performed on Microcon YM-10 centrifugal filter device (MWCO 10,000). The results obtained imply irreversible inhibition of human DPP III by this type of compounds.

3.4. Kinetics of the inactivation of DPP III by compound *1′*

The time dependency of DPP III inhibition by compound **1′** was measured under the pseudo-first-order conditions, as described in the Section 2.

Time course of inactivation of human DPP III caused by 5 μM compound **1′** is shown in Fig. 2A. In accordance to the high (300-fold) molar excess of the inhibitor over the enzyme, semilogarithmic plot of residual activity against time was found to be linear (not shown), indicating that enzyme inactivation under the chosen conditions exhibited pseudo-first-order kinetics. The data given in Fig. 2A were plotted according to a second-order rate reaction model using the equation:

$$\log[(a_0 - x)/(b_0 - x)] = (k/2.303) \cdot (a_0 - b_0) \cdot t + \log(a_0/b_0)$$

where a_0 is the initial concentration of inhibitor, b_0 is the initial concentration of enzyme, x corresponds to the concentration of the inhibited enzyme at time t, and k represents the overall second-order rate constant for the reaction (Fig. 2B).

The value of the determined second-order rate constant for human DPP III inactivation by compound **1′**, obtained from the slopes of linear plots of log $[(a_0 - x)/(b_0 - x)]$ versus time was $6924 \pm 549\ M^{-1}\ min^{-1}$ (the mean value from three determinations ± SD). The obtained second-order-rate constant for inactivation of human DPP III by **1′** compares well with the second-order-rate constant for inactivation of the same enzyme with sulfhydryl reagent *p*-hydroxy-mercuribenzoate (*p*HMB) ($3523\ M^{-1}\ min^{-1}$) [32]. However, unlike with *p*HMB, thiol compound dithiothreitol did not protect the enzyme from inactivation by **1′** (data not shown).

To gain further insight into the inactivation mechanism of human DPP III by heterocyclic compound **1′**, the effect of several additions to the preincubation mixture was investigated. Sodium chloride (0.15 M), added to the preincubation mixture with inhibitor

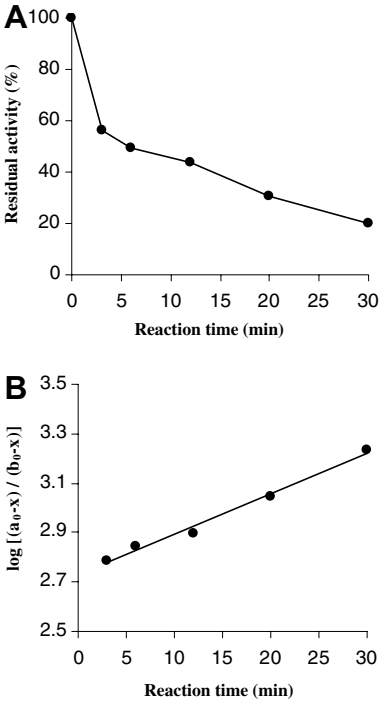


Fig. 2. Progressive inhibition of human dipeptidyl peptidase III by compound **1′**. (A) Time course of DPP III inactivation by compound **1′**. Incubation conditions: 14.5 nM DPP III, 5 μM inhibitor; pH 7.4; temperature, 25 °C. Aliquots were drawn at different time intervals and the residual activity was determined as described in the Section 2. (B) Determination of the second-order rate constant of DPP III inactivation by compound **1′**. Assay conditions: as for (A). The kinetics shown in (A) was plotted according to the second-order reaction model: $\log[(a_0 - x)/(b_0 - x)] = (k/2.303)\cdot(a_0 - b_0)\cdot t + \log(a_0/b_0)$ and the second-order rate constant was determined from the slope of the line drawn on the basis of linear regression analysis.

exerted partial protection of DPP III activity. Cobalt and zinc salts (up to 100 μM) were uneffective, showing that the mode of action of compound **1′** was not via non-specific chelation of the active site metal ion. On the contrary, 1,10-phenanthroline inhibited DPP III reversibly (the addition of Co^{2+} ions abolished the inhibitory effect; data not shown).

DPPs III peptide substrate, heptapeptide valorphin, when introduced into the preincubation mixture in 1 μM concentration, protected the enzyme from inactivation (after 30 min preincubation with the inihibitor, instead of 80%, inhibition extent was 40% in the presence of valorphin), indicating the competitive nature of inhibition.

The dissociation constant of 0.20 ± 0.007 μM for the initial reversible complex DPP III and compound **1′**, was obtained following the method by Tornheim [35], according to the equation:

$$K_i = [I]/((V_0/V - 1)(1 + [S]/K_m))$$

where V_0 and V are the rates in the absence and presence of the inhibitor, [S] is the initial concentration of substrate and the K_m is Michaelis constant. The applied concentration of the inhibitor, [I], inhibited the enzyme for about 50%, and the substrate concentration [S]

was 10-fold that of K_m. Michaelis constant for the substrate Arg_2-2NA was determined separately, from the initial reaction rates by varying substrate concentrations from 4 to 40 μM, using Hanes plot, to be 12.00 ± 1.58 μM.

The inactivation mechanism of DPP III by compound **1′** is not clear. We showed that inactivation did not occur via non-specific chelation of the active site Zn^{2+}. The observed enzyme protection by peptide substrate and by the increased salt concentration strongly suggests that the inactivator (compound **1′**) is active site directed and that the enzyme-inhibitor binding involves electrostatic interaction(s), respectively. Studies beyond the scope of this investigation would be required to determine the inactivation mechanism of **1′**.

In the absence of data on the 3-D structure of DPP III, we may, based on the presented experimental results, speculate that compound **1′** interacts with this metallo-peptidase through multiple sites. Electrostatic interaction between substituted amidino (imidazoline) group of the inhibitor and, yet unknown, counterpart in the enzyme active site, and the hydrophobic interaction of phenyl group(s) within the hydrophobic pocket (S_1' subsite) of human DPP III are presumably two important components of inactivation mechanism.

Interestingly, the irreversible inhibition of another enzyme, nitric oxide synthase, by an amidine compound has been reported most recently, but inactivation mechanism has not been clarified [43].

Multiple interactions in several enzyme-non-peptidic inhibitor complexes are well documented through the resolution of their 3-D structures. The 2.9 Å structure determination of the catalytic domain of human tissue-type plasminogen activator in a complex with the bis-benzamidine inhibitor 2,7-bis-(4-amidinobenzylidene)-cycloheptan-1-one reveals three-site interaction where amidino groups are involved in the salt bridge formation and in a weak electrostatic interaction with the enzyme [44]. The crystal structures of novel inhibitors bound to the active sites of bacterial metallo-β-lactamase and Zn-dependent peptidase neprilysin show the complexity of the binding of heterocyclic compounds within the active site of metallo-hydrolases, and the presence of polar and stacking interactions [45,46].

4. Conclusion

Specific inhibitors of (metallo)peptidases have a potential in identifying the role of these enzymes. In order to find a potent antagonist of zinc-exopeptidase DPP III (enkephalinase B) we synthesized and screened the effect of 12 benzimidazole derivatives. Of them, 11 compounds inhibited human erythrocyte DPP III. The examination revealed that the structural feature important for strong inhibitory activity of these compounds towards human enzyme is amidino group (preferably cyclized) coupled to benzimidazole moiety and the presence of an additional aromatic ring (phenyl), which presumably interacts with DPP III hydrophobic pocket S_1'. This approach led to two compounds (**1′** and **4′**), both from the group of non-planar branched compounds with cyclobutane core and with amidino-substituted benzimidazole functionalities, prepared by photochemical [2 + 2] cyclization in water, which potently inhibited human DPP III, with binding affinities in submicromolar range.

In contrast, another mammalian dipeptidyl peptidase, DPP IV which belongs to the serine-catalytic type of peptidases, was not affected by any of the screened benzimidazole derivatives.

The inhibition of human DPP III with compound **1′** was studied in more detail. It was shown that its mode of action is not via non-specific chelation and that it inactivated DPP III time-dependently. The peptide substrate valorphin protected the enzyme from inactivation by **1′**.

This investigation provided information on new non-peptidic inhibitors of human proteolytic enzyme dipeptidyl peptidase III.

Acknowledgments

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References

[1] J.K. McDonald, A.J. Barrett (Eds.), Mammalian Proteases: A Glossary and Bibliography, Exopeptidases, vol. 2, Academic Press, London, 1986, pp. 127–131.
[2] N. Yokotani, K. Doi, R.J. Wenthold, K. Wada, Hum. Mol. Genet. 2 (1993) 1037–1039.
[3] C.A. Abbott, D.M.T. Yu, E. Woollatt, G.R. Sutherland, G.W. McCaughan, M.D. Gorell, Eur. J. Biochem. 267 (2000) 6140–6150.
[4] C. Olsen, N. Wagtmann, Gene 299 (2002) 185–193.
[5] S.Y. Qi, P.J. Riviere, J. Trojnar, J.-L. Junien, K.O. Akinsanya, Biochem. J. 373 (2003) 179–189.
[6] P. Bušek, R. Malík, A. Šedo, Int. J. Biochem. Cell Biol. 36 (2004) 408–421.
[7] S. Ellis, J.M. Nuenke, J. Biol. Chem. 242 (1967) 4623–4629.
[8] J.K. McDonald, in: A.J. Barrett, N.D. Rawlings, J.F. Woessner (Eds.), Handbook of Proteolytic Enzymes, Academic Press, San Diego, 1998, pp. 536–538.
[9] M. Abramić, M. Zubanović, Lj. Vitale, Biol. Chem. Hoppe-Seyler 369 (1988) 29–38.
[10] C.-M. Lee, S.H. Snyder, J. Biol. Chem. 257 (1982) 12043–12050.
[11] H. Zhang, Y. Yamamoto, S. Shumiya, M. Kunimatsu, K. Nishi, I. Ohkubo, K. Kani, Histochem. J. 33 (2001) 511–521.
[12] C. Mazzocco, K.M. Fukasawa, P. Auguste, J. Puiroux, Eur. J. Biochem. 270 (2003) 3074–3082.
[13] S. de la Baume, C.C. Yi, J.C. Schwartz, P. Chaillet, H. Marcais-Callado, J. Costentin, Neuroscience 8 (1983) 143–151.
[14] J.G.C. van Amsterdam, K.J.H. van Buuren, M.J. Krielaart, O.P. Zuiderveld, R.P.J.H. Tijms, Life Sci. 43 (1988) 1529–1536.
[15] Š. Šimaga, D. Babić, M. Osmak, J. Ilić-Forko, Lj. Vitale, D. Miličić, M. Abramić, Eur. J. Cancer 34 (1998) 399–405.
[16] Š. Šimaga, D. Babić, M. Osmak, M. Šprem, M. Abramić, Gynecol. Oncol. 91 (2003) 194–200.
[17] K. Fukasawa, K.M. Fukasawa, M. Kanai, S. Fujii, J. Hirose, M. Harada, Biochem. J. 329 (1998) 275–282.
[18] J.M. Chen, A.J. Barrett, in: A.J. Barrett, N.D. Rawlings, J.F. Woessner (Eds.), Handbook of Proteolytic Enzymes, second ed., Academic Press, San Diego, 2004, pp. 809–812.
[19] K. Fukasawa, K.M. Fukasawa, H. Iwamoto, J. Hirose, M. Harada, Biochemistry 38 (1999) 8299–8303.
[20] M. Abramić, D. Schleuder, Lj. Dolovčak, W. Schröder, K. Strupat, D. Šagi, J. Peter-Katalinić, Lj. Vitale, Biol. Chem. 381 (2000) 1233–1243.
[21] T. Nishikiori, F. Kawahara, H. Naganawa, Y. Muraoka, T. Aoyagi, H. Umezawa, J. Antibiot. 37 (1984) 680–681.
[22] T. Akiyama, S. Harada, F. Kojima, Y. Takahashi, C. Imada, Y. Okami, Y. Muraoka, T. Aoyagi, T. Takeuchi, J. Antibiot. 51 (1998) 553–559.
[23] K. Nishimura, T. Hazato, Biochem. Biophys. Res. Commun. 194 (1993) 713–719.
[24] Y. Yamamoto, H. Kanazawa, M. Shimamura, M. Ueki, T. Hazato, Life Sci. 62 (1998) 1767–1773.
[25] Y. Yamamoto, J.-I. Hashimoto, M. Shimamura, T. Yamaguchi, T. Hazato, Peptides 21 (2000) 503–508.

[26] S. Sahli, B. Stump, T. Welti, D. Blum-Kaelin, J.D. Aebi, C. Oefner, H.-J. Böhm, F. Diederich, ChemBioChem. 5 (2004) 996–1000.
[27] J.J. Paul, S.R. Kircus, T.N. Sorrell, P.A. Ropp, H.H. Thorp, Inorg. Chem. 45 (2006) 5126–5135.
[28] B.A. Katz, J.M. Clark, J.S. Finer-Moore, T.E. Jenkins, C.R. Johnson, M.J. Ross, C. Luong, W.R. Moore, R.M. Stroud, Nature 391 (1998) 608–612.
[29] T.A. Fairley, R.R. Tidwell, I. Donkor, N.A. Naiman, K.A. Ohemeng, R.J. Lombardy, J.A. Bentley, M. Cory, J. Med. Chem. 36 (1993) 1746–1753.
[30] K. Starčević, D.W. Boykin, G. Karminski-Zamola, Het. Comm. 8 (2002) 221–226.
[31] K. Ramaiah, P.K. Dubey, D.E. Rao, J. Ramanatham, R. Kumar, J.S. Grossert, D.L. Hooper, Indian J. Chem. 39B (2000) 904–914.
[32] M. Abramić, Š. Šimaga, M. Osmak, L. Čičin-Šain, B. Vukelić, K. Vlahoviček, Lj. Dolovčak, Int. J. Biochem. Cell Biol. 36 (2004) 434–446.
[33] H. Barelli, J.-P. Vincent, F. Checler, Eur. J. Biochem. 211 (1993) 79–90.
[34] C. Gorenstein, S.H. Snyder, Life Sci. 25 (1979) 2065–2070.
[35] K. Tornheim, Anal. Biochem. 221 (1994) 53–56.
[36] F.R. Cibin, N. Di Bello, G. Doddi, V. Fares, P. Mencarelli, E. Ullucci, Tetrahedron 59 (2003) 9971–9978.
[37] M. D'Auria, R. Racioppi, J. Photochem. Photobiol. A-Chem. 112 (1998) 145–148.
[38] J.H. Kim, M. Matsuoka, K. Fukunishi, J. Chem. Res.-S 2 (1999) 132–133.
[39] A. Lalitha, K. Pitchumani, C. Srinivasan, J. Photochem. Photobiol. A-Chem. 134 (2000) 193–197.
[40] I. Škorić, Ž. Marinić, M. Šindler-Kulyk, Heterocycles 53 (2000) 55–68.
[41] N. Yayli, O. Üçüncü, A. Yaşar, Y. Gök, M. Küçük, S. Kolayli, Turk. J. Chem. 28 (2004) 515–521.
[42] S.P. Gromov, A.I. Vedernikov, Yu.V. Fedorov, O.A. Fedorova, E.N. Andryukhina, N.E. Shepel', Yu.A. Strelenko, D. Johnels, U. Edlund, J. Saltiel, M.V. Alfimov, Russ. Chem. Bull. 54 (2005) 1569–1579.
[43] E.A. Litzinger, P. Martásek, Lj. Roman, R.B. Silverman, Bioorg. Med. Chem. 14 (2006) 3185–3198.
[44] M. Renatus, W. Bode, R. Huber, J. Stürzebecher, D. Prasa, S. Fischer, U. Kohnert, M.T. Stubbs, J. Biol. Chem. 272 (1997) 21713–21719.
[45] D.J. Payne, J.A. Hueso-Rodríguez, H. Boyd, N.O. Concha, C.A. Janson, M. Gilpin, J.H. Bateson, C. Cheever, N.L. Niconovich, S. Pearson, et al., Antimicrob. Agents Chemother. 46 (2002) 1880–1886.
[46] S. Sahli, B. Frank, W.B. Schweizer, F. Diederich, F. Blum-Kaelin, J.D. Aebi, H.-J. Böhm, Helv. Chim. Acta 88 (2005) 731–750.