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Original article

Synthesis, biological activity and resistance to proteolytic digestion of new cyclic dermorphin/deltorphin analogues

Krzysztof Bańkowski [a,*], Ewa Witkowska [b], Olga M. Michalak [a], Katarzyna Sidoryk [a], Ewa Szymanek [a], Bożena Antkowiak [c], Małgorzata Paluch [c], Katarzyna E. Filip [a], Marek Cebrat [d], Bartosz Setner [d], Zbigniew Szewczuk [d], Piotr Stefanowicz [d], Piotr Cmoch [a,e], Jan Izdebski [a,b]

[a] Pharmaceutical Research Institute, Rydygiera 8, Warsaw 01-793, Poland
[b] Warsaw University, Department of Chemistry, Pasteura 1, Warsaw 02-093, Poland
[c] Military Institute of Hygiene and Epidemiology (MIHE), Kozielska 4, Warsaw 01-163, Poland
[d] Faculty of Chemistry, University of Wrocław, 14 F. Joliot-Curie Str.,50-383 Wrocław, Poland
[e] Institute of Organic Chemistry Polish Academy of Sciences, M. Kasprzaka 44/52, Warsaw, Poland

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ABSTRACT

A series of novel cyclic ureidopeptides, analogues of dermorphine/deltorphine tetrapeptide, were synthesized by solid phase peptide synthesis and/or in solution. The antinociceptive activity of *N*-substituted amides **1**–**10** was evaluated using hot-plate and tail-flick tests. Analogue **1** showed significant, stronger than morphine, antinociceptive effect after systemic applications. All analogues were also tested for their *in vitro* resistance to proteolysis by means of mass spectroscopy and it was found that all substituted amides **1**–**10** showed full stability during incubation with large excess of chymotrypsin and pepsin. Compound **1** is a lead molecule for further evaluation.

1. Introduction

Dermorphin, Tyr–D–Ala–Phe–Gly–Tyr–Pro–Ser–NH_2, a heptapeptide isolated from the skin of the South American frog *Phyllomedusa sauvagei* [1] until recently was among the most potent opioid peptides with high μ receptor selectivity. On the other hand, deltorphin Tyr–D–Met–Phe–His–Leu–Met–Asp–NH_2, a heptapeptide isolated from the same source, is a very potent and highly specific agonist of the δ-opioid receptor [2–4].

In contrast to all mammalian opioid peptides, dermorphin and deltorphin contain a *D*-amino acid in position 2 and, therefore, they are relatively stable against enzymatic degradation [5].

It is known that *N*-terminal tetrapeptide of dermorphin is the minimum sequence required for the opioid activity [6] although this fragment shows lower potency than that of the parent heptapeptide [7]. Several synthetic peptides derived from the dermorphin/deltorphin tetrapeptide, of general sequence Tyr–D–AA–Phe–AA, have been reported to show a potent agonistic activity at the μ opioid receptors [8]. In a recent review [9] many dermorphin tetrapeptide analogues are presented as potent and long-lasting analgesics. Unfortunately, that review does not include peptides with cyclic structures.

High sensitivity of some linear peptides to proteolysis restricts their potential use as therapeutic agents. Therefore, proteolytic stability is an important factor in designing peptide-based drugs. Intramolecular cyclization is an interesting approach to increase the potency and duration of peptide action by introduction of conformational restriction in linear peptides. Cyclization through covalent linkage between two side chains or between a side chain and a terminal group has produced opioid peptide analogues with improved biological properties, such as metabolic stability, potency and receptor selectivity [10].

Schiller and co-workers synthesized a series of cyclic Leu-enkephalins analogues by cyclization of the ω-amino group of *D*-α,ω-diaminoacids in position 2 of Leu-enkephalin to the *C*-terminal

* Corresponding author. Tel.: +48 22 456 3921; fax: +48 22 456 3838.
E-mail addresses: k.bankowski@ifarm.eu (K. Bańkowski), b.antkowiak@wihe.waw.pl (B. Antkowiak), zbigniew.szewczuk@chem.uni.wroc.pl (Z. Szewczuk), jizdebski@chem.uw.edu.pl (J. Izdebski).

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carboxy group [11]. Subsequently, cyclic enkephalin analogues containing a disulphide bridge [12] or dithioether bridge [13] were prepared. Dermorphin-derived cyclic tetrapeptide analogues with disulphide bridge and their affinity to opioid receptors were also reported [13,14].

Izdebski and co-authors in a series of papers [15–23] described the synthesis, biological activity and conformation of enkephalin and dermorphin analogues restricted *via* the urea bridge. These analogues contained a carbonyl bridge, which linked two side-chain amino groups of dibasic amino acids in position 2 and 5 (enkephalin analogues) or 2 and 4 (dermorphin analogues) to form an ureido moiety. Most of these peptides showed very high agonistic potency in the guinea-pig ileum (GPI) and in the mouse vas deferens (MVD) *in vitro* receptor binding assays.

For example, ureido-analogue cyclo(N^{ε},N^{β}-carbonyl-D-Lys^2,-Dap^5]enkephalin-amide is one of the most potent agonists among the enkephalin analogues reported, as determined in the GPI and MVD assays [15,16]. Moreover, this analogue shown not only its high potency *in vitro* but also strong and long-lasting antinociception in both hot-plate and tail-immersion tests in rats after central administration [22]. Its cardiovascular and renal effects were also reported and discussed [23].

All of the previously synthesized cyclic ureidopeptides were simple unsubstituted amides of enkephalins or dermorphin/deltorphin analogues. The methodology for the synthesis of (*N*-ureidoethyl)amides of cyclic ureido-enkephalins using solid-phase approach was elaborated, and a series of such analogues were prepared [19,21] .The *N*-substituted ureido-enkephalins were found to be agonists in GPI and MVD tests, however their activities were slightly lower than for non-substituted counterparts. On the other hand, it was shown that cyclization *via* the carbonyl bridge increased the resistance to proteolytic digestion of the cyclic ureidopeptides incubated with pepsin and chymotrypsin, and the cyclic (*N*-ureidoethyl)amides were especially promising from this point of view [24].

The current studies were undertaken with the aim of developing new opioid peptides exhibiting potent antinociception upon systemic administration. The investigations assumed the elaboration of the synthesis of (*N*-substituted) amides of cyclic ureido-peptides, especially *N*-alkyl- and *N*-(ureidoethyl)amides of new dermorphin/deltorphin tetrapeptide analogues, using both solid-phase technique and the classical method in solution and estimation their *in vivo* antinociceptive activity in mouse using hot-plate and tail-flick tests as well as their resistance to proteolytic digestion.

2. Results and discussion

2.1. Chemistry

A series of novel analogues of *N*-substituted or unsubstituted amides of dermorphin/deltorphin tetrapeptide of general structure (Fig. 1) were prepared.

2.1.1. Solid phase synthesis

Analogues **1**–**8** and **11**–**14** were synthesized by solid phase peptide synthesis. For the synthesis of *N*-ureidoethylamides **1**–**8**, the method elaborated earlier [19,21] was used. This method consists in the functionalization of MBHA-resin by coupling with *p*-nitrophenoxycarbonyl derivative of Boc-1,2-diaminoethane to give Boc–NH–CH_2–CH_2–NH–CO–MBHA-resin. Following removal of the Boc group by treatment with 15% HCl/dioxane, four Boc amino acids were successively attached in the required sequence using TBTU/HOBt methodology. The amino groups in side chains of dibasic amino acids were protected by Fmoc. The protected peptide

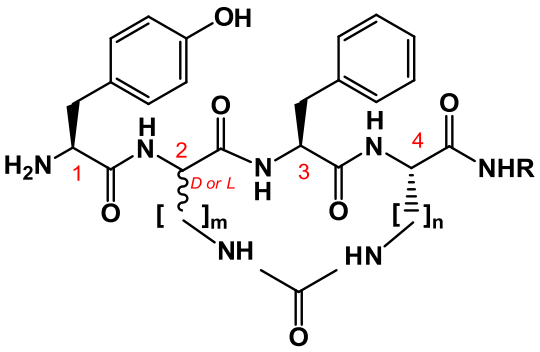


Fig. 1. Structure formulae of *N*-substituted amides of dermorphin/deltorphin tetrapeptide analogues. (details in Table 1).

resins were treated with 55% piperidine in DMF to remove Fmoc groups, and the cyclization on the resin was performed using *bis*(*p*-nitrophenyl)carbonate in slightly basic conditions (DIPEA) in DMF. The reaction was allowed to continue until free amino groups could no longer be detected on the resin (5–7 days). The protected cyclic peptides containing *N*-terminal Boc–Tyr(OBu^t) residue were cleaved from the resin by treatment with TFA cocktail (TFA:phenol:water; 13.5:1:1 mL/g/g) with simultaneous removal of Boc and Bu^t protecting groups. An interesting feature of the synthesis of peptides **1**–**10** was the use of 15% HCl/dioxane reagent for the selective removal of Boc groups without cleavage of peptide-MBHA-resin bond, which was later performed by treatment with TFA (or TFA-containing cleavage cocktail). Peptides **11**–**14** were synthesized on MBHA-resin with the final cleavage of the peptide by treatment of the peptide-resin with TFA:TFMSA:tioanizole: ethanedithiol mixture (10:1:1:0.5).

For the preliminary purification of the crude analogues silica gel column chromatography or preparative TLC were used. Final purification was accomplished by semipreparative reversed-phase high performance liquid chromatography (RP-HPLC). Homogeneity of the purified analogues was assessed by analytical HPLC. The purity of all analogues synthesized by SPPS was in the range 93.2–98.3%. Structure identification was achieved by HR-MS (high resolution mass spectrometry). Analytical data of the peptides are presented in Table 1.

2.1.2. Synthesis in solution

The above functionalized MBHA resin was designed for the specific synthesis of (2-ureido)ethylamides of peptides. The synthesis in solution is more versatile as makes the possibility to synthesize a series of differently substituted amides from one common synthon. This approach was used for the synthesis of analogues **1** (ureidoethylamide), **9** (ethylamide) and **10** (2-aminoethyl)amide from a common intermediate **20** as was illustrated in Scheme 1.

The synthesis was carried out according to the Boc/Z strategy (phenolic group of Tyr was protected as Bu^t ether). The starting ester **15** was prepared from Boc–Dab(Z)–OH by treatment with HCl/MeOH. The successive Boc-amino acids were coupled using TBTU/HOBt or HATU reagents. The linear protected peptide **18** was hydrogenolized using 10% Pd/C as a catalyst to remove both Z groups and to afford diamino compound **19**. Cyclization of **19** was performed with bis(*p*-nitrophenyl) carbonate (1.1 eq.) in DMF in the presence of DIPEA (2 eq.). The carbonyl bridge formation was completed after 6 days, than the crude cyclic ester **20** was purified by silica gel chromatography.

Hydrolysis of this key intermediate **20** using NaOH in aq. dioxane to the corresponding acid **21**, followed by coupling with 2-(ureido)ethylamine using HATU, or with *N*-Boc-etylenediamine

Table 1
Analytical data for new analogues of dermorphin/deltorphin tetrapeptide.

No	Abbreviated name[a]	HR–MS			HPLC, %
			Calcd.	Found	
1	{[H–Tyr–*D*–Lys(&^{1})–Phe–Dab(&^{2})–$CH_2CH_2NHCONH_2$][&^{1}CO&^{2}]}	$[M + H]^+$	668.353	668.353	98.3
		$[M + Na]^+$	690.333	690.333	
2	{[H–Tyr–*D*–Lys(&^{1})–Phe–Orn(&^{2})–$CH_2CH_2NHCONH_2$][&^{1}CO&^{2}]}	$[M + Na]^+$	704.349	704.347	96.9
		$[M + H]^+$	682.367	682.372	
3	{[H–Tyr–*D*–Lys(&^{1})–Phe–Dap(&^{2})–$CH_2CH_2NHCONH_2$][&^{1}CO&^{2}]}	$[M + Na]^+$	676.318	676.320	97.6
		$[M + H]^+$	654.336	654.358	
4	{[H–Tyr–*D*–Orn(&^{1})–Phe–Lys(&^{2})–$CH_2CH_2NHCONH_2$][&^{1}CO&^{2}]}	$[M + H]^+$	682.367	676.320	95.8
5	{[H–Tyr–*D*–Orn(&^{1})–Phe–Orn(&^{2})–$CH_2CH_2NHCONH_2$][&^{1}CO&^{2}]}	$[M + H]^+$	668.352	668.354	98.2
6	{[H–Tyr–*D*–Orn(&^{1})–Phe–Dab(&^{2})–$CH_2CH_2NHCONH_2$][&^{1}CO&^{2}]}	$[M + Na]^+$	676.318	676.319	97.2
		$[M + H]^+$	654.336	654.360	
7	{[H–Tyr–*D*–Orn(&^{1})–Phe–Dap(&^{2})–$CH_2CH_2NHCONH_2$][&^{1}CO&^{2}]}	$[M + Na]^+$	662.302	662.302	93.2
		$[M + H]^+$	640.320	640.344	
8	{[H–Tyr–*D*–Lys(&^{1})–Phe–Lys(&^{2})–$CH_2CH_2NHCONH_2$][&^{1}CO&^{2}]}	$[M + H]^+$	696.383	696.386	97.1
		$M + Na]^+$	718.369	718.362	
9	{[H–Tyr–*D*–Lys(&^{1})–Phe–Orn(&^{2})–CH_2CH_3][&^{1}CO&^{2}]}	$[M + H]^+$	610.344	610.344	98.9
10	{[H–Tyr–*D*–Lys(&^{1})–Phe–Dap(&^{2})–$CH_2CH_2NH_2$][&^{1}CO&^{2}]}	$[M + H]^+$	625.345	625.345	99.4
11	{[H–Tyr–Orn(&^{1})–Phe–Lys(&^{2})–NH_2][&^{1}CO&^{2}]}	$[M + H]^+$	596.319	596.331	97.3
		$M + Na]^+$	618.301	618.314	
12	{[H–Tyr–Dap(&^{1})–Phe–Lys(&^{2})–NH_2][&$_1$CO&$_2$]}	$[M + H]^+$	568.287	568.298	98.1
		$M + Na]^+$	590.269	590.281	
13	{[H–Tyr–Lys(&^{1})–Phe–Dap(&^{2})–NH_2][&^{1}CO&^{2}]}	$[M + H]^+$	568.287	568.298	98.3
		$M + Na]^+$	590.269	590.274	
14	{[H–Tyr–Orn(&^{1})–Phe–Dap(&^{2})–NH_2][&^{1}CO&^{2}]}	$[M + H]^+$	554.272	554.275	97.7
		$M + Na]^+$	567.254	567.252	

[a] The structures of cyclic peptides are described using abbreviated nomenclature for cyclic peptides [25].

using DCC/HOBt, provided compounds **23** and **24**, precursors of analogues **1** and **10** containing Boc/OBu^t protected *N*-terminal tyrosine. On the other hand, direct aminolysis of ester **20** with ethylamine in MeOH gave amide **22**, an analogous precursor of **9**.

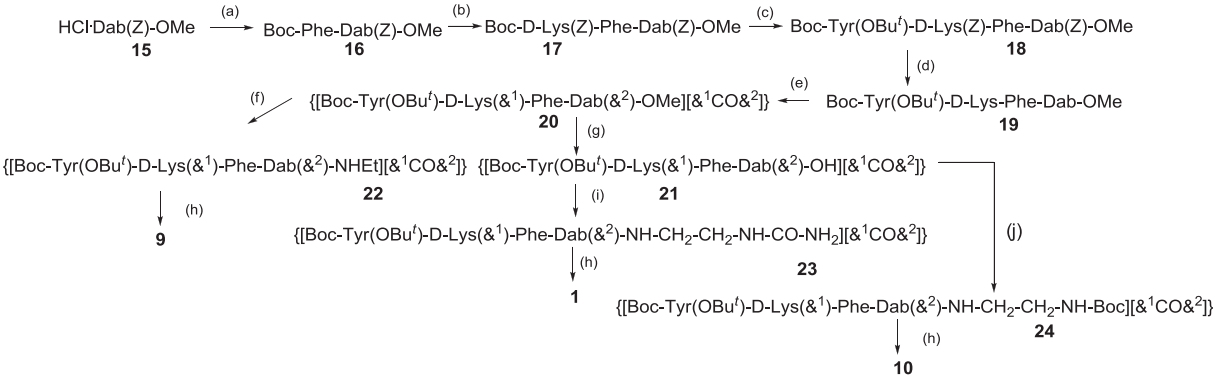
The crude products **22**, **23** and **24** were treated by TFA at RT (50 min) to remove Boc/Bu^t groups, and crude peptides were purified by semipreparative RP-HPLC (in the case of larger scale synthesis of **1** semipreparative RP-HPLC was preceded by flash chromatography purification on silica gel column). The appropriate peaks containing the main products were collected, reexamined by analytical RP-HPLC and lyophilized. The purity of analogues **1**, **9** and **10** was in the range 98.8–99.4%. The structure was confirmed by HR-MS (high resolution mass spectroscopy). Analytical data of peptides are presented in Table 1.

The structure of analogue **1** was also confirmed by detailed analysis of ^{1}H, ^{13}C and ^{15}N NMR spectra; the full assignments are given in Fig. 2.

We could observe some drawbacks of the synthetic route presented above. We found that product **19** prepared by the catalytic hydrogenolysis of di-Z-protected peptide **18** had a tendency to intramolecular cyclization to the corresponding lactam **25** (Fig. 3) which made isolation of **19** from the reaction mixture and storage difficult and decreased the yield of the following cyclization. When hydrogenolysis was carried out in MeOH/DMF mixture larger amount of **25** appeared; this impurity was isolated and characterized on the base of mass spectrometry and NMR spectra. When this hydrogenation reaction was performed in MeOH/AcOH mixture fresh ester **19** was pure, however during its storage at RT content of the lactam increased.

Moreover, we could also notice the moderate yield for condensation of acid **21** with (2-ureido)ethylamine. As a consequence, the removal of protecting groups from crude amide **23** led to the crude analogue **1** containing considerably high amount of acid {[H–Tyr–D–Lys(&^{1})–Phe–Dab(&^{2})–OH][&^{1}CO&^{2}]}, which had to be removed during semipreparative HPLC purification.

These disadvantages were overcome by changing the sequence in transformation of **18** into **23**. At first, hydrolysis of ester **18** to acid **26**, followed by coupling of **26** with (2-ureido)ethylamine gave



Scheme 1. Synthetic strategy to analogues **1**, **9** and **10**. Reagents and conditions: (a) Boc–Phe–OH, TBTU/HOBt, DMF; (b) 1. TFA, 2. Boc–*D*–Lys(Z)–OH, HATU, DMF; (c) 1. TFA, 2. Boc–Tyr(OBu^t)–OH,HATU; (d) H_2/Pd–10%C; (e) $CO(ONp)_2$/DMF/DIPEA; (f) H_2NEt, MeOH; (g) NaOH, dioxane/water; (h) TFA cocktail; (i) $H_2N–CH_2–CH_2–NH–CO–NH_2$/HATU/DMF; (j) $H_2N–CH_2–CH_2–NH$-Boc/DCC/HOBt, DMF.

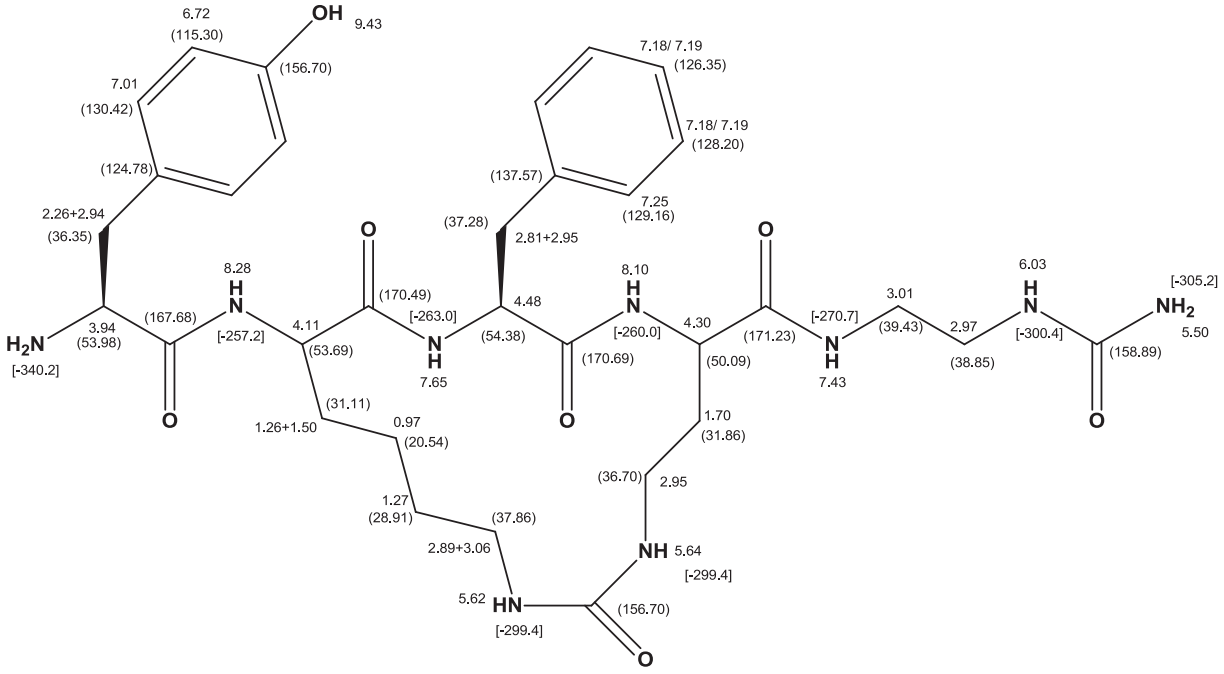


Fig. 2. The ^{1}H, (^{13}C) and [^{15}N] NMR signals assignments for analogue **1** (in DMSO-d_6).

linear amide **27**. Than, removal of Z protecting groups by catalytic hydrogenolysis gave **28** and following cyclization with the use of bis(p-nitrophenyl)carbonate provided the intermediate **23** (Scheme 2).

The introduction of these changes to the synthetic protocol simplified and improved the process of the purification of the final analogue **1**

2.2. Proteolytic stability

The ureidopeptides **1**–**14** were tested to determine their enzymatic stability in the presence of pepsin and chymotrypsin. Peptide **1** was incubated with enzymes for 20 min, 150 min and 12 h. Since no evidence of the enzymatic hydrolysis have been noticed other peptides were incubated with enzyme solution for 12 h only. The details of experiments were given in Experimental protocols. Briefly, the solution of the peptide was incubated with the enzyme solution and after quenching the enzyme sample was diluted and directly studied by ESI-MS technique. The high resolution ESI-MS spectra of all samples before and after incubation with both pepsin and chymotrypsin were given in the Supplementary data.

It should be noted that the concentrations of enzymes used in these tests were much higher than those applied in standard biochemical and proteomic protocols. Performed tests indicated extremely high proteolytic stability of peptides **1**–**10** in respect to pepsin and chymotrypsin – in all cases no hydrolysis products were found. This series of ureidopeptides were compared with compounds of similar structure, but containing L-amino acid residues in position 2 of the peptide sequence (peptides **11**–**14**). These peptides were significantly less stable than their D-amino acid counterparts reported previously [17] and than the compounds **1**–**10** presented in the current paper. Peptides were degraded more easily in the presence of chymotrypsin than pepsin. ESI-MS analysis (see Supplementary data) indicates that less than 1% of the unhydrolyzed peptides were left after just 1.5 h of incubation with chymotrypsin. In case of the proteolysis with pepsin, the results were more diversified. Peptides **11** and **14** were rapidly hydrolyzed by this enzyme while **12** and **13** were significantly more stable. After 2.5 h of incubation about 98% of the molecules were still observed with the intact cyclic fragment. Both chymotrypsin and pepsin cleaved the peptide bond between Tyr[1] residue and the dibasic L-amino acid residue in position 2. Additional cleavage sites were observed within the cyclic fragment of the ureidopeptides, especially in case of the peptide **11** which had the biggest ring size in this series (17 atoms forming the cyclic structure) [B. Setner, M. Cebrat, Z. Szewczuk, K. Filip, M. Ciszewska, J. Izdebski, poster at 21

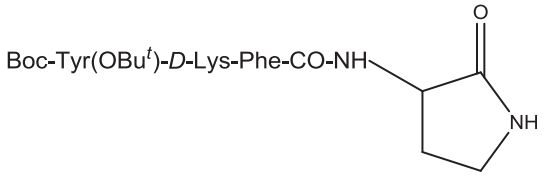
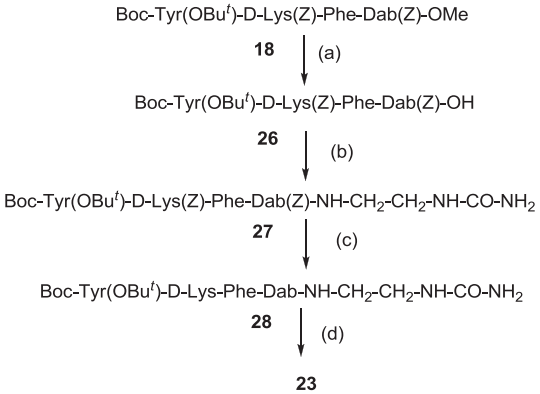


Fig. 3. Structure of lactam **21**.



Scheme 2. Improved transformation of **18** into **23**. Reagents and conditions: (a) NaOH, dioxane/water; (b) $H_2N-CH_2-CH_2-NH-CO-NH_2$/HATU/DMF; (c) H_2/Pd–10%C; (d) $CO(ONp)_2$/DMF/DIPEA.

Polish Peptide Symposium, 2011, Suprasl, Poland, unpublished results].

2.3. Biological results

Antinociceptive activity of new compounds was evaluated using hot-plate (HP) and tail-flick (TF) tests. Initially HP test was performed in order to select the most active compound after systemic administration and then its effect was additionally assessed in TF test and compared to that of morphine (MF).

Analysis of the results of HP test indicated that significant antinociceptive effect was observed after intravenous (iv) application of compound **1** in the 1 mg/kg dose (Fig. 4). Upon subcutaneous (sc) and intraperitoneal (ip) administration compound **1** was not active in both doses studied (0.1 and 1 mg/kg). None of the rest tested compounds **2**–**10** did significantly affect animals pain threshold to thermal stimulus in any of the applied routes of administration and dose regimen. Possibly, the use of higher doses would reveal their antinociceptive effect. MF given iv in the 1 mg/kg dose also did not significantly influence the latency of pain response to thermal stimulus as well. The nociceptive response of mice to iv administration of studied compounds in the 1 mg/kg dose is presented in Fig. 4.

Further evaluation of antinociceptive activity using TF test was performed only on **1** – the sole active compound in HP test and its effect was compared to that of MF. Iv injection of **1** elicited dose-dependant antinociceptive effect. As compared to vehicle group significant increase of TF latency was observed in the 0.5 mg/kg (max. 41.8 ± 13.3%MPE) and 1 mg/kg (max. 54.7 ± 13.3%MPE) lasted from 30 to 90 min after injection (Fig. 5). MF in the same doses was inactive (Fig. 6).

In the 2 mg/kg dose compound **1** elicited highly potent antinociception lasting 2 h (Fig. 5). The analgesic effect induced by MF in the same dose was significantly lower reaching the highest value of 63 0.1 ± 9.1 %MPE (at 30 min) (Figs. 6 and 7).

In the highest dose applied (4 mg/kg) compound **1** exerted the same potent analgesia as MF up to 60 min. However, during the longer action (90–120 min) the effect of **1** was significantly higher than MF (Figs. 5–7).

Summing up, the present studies revealed that significant antinociception in HP and TF tests after iv application of analogue **1**. Compounds **9** and **10** with the structures very similar to **1** but having less complex C-termini did not influence pain threshold to thermal stimulus in hot plate test. It may be suggested that besides the unique combination of amino acids, more expanded C-terminal structure could positively correlate with biological activity of compound **1**.

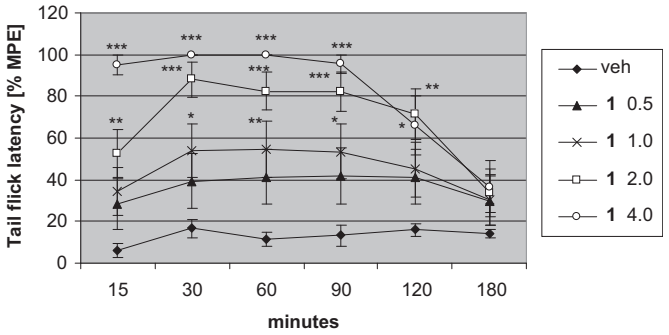


Fig. 5. Tail flick latency expressed as the percent of maximal possible effect (%MPE) in groups of mice ($n = 10$) at 15–180 min after i.v. injection of compound **1** in the 0.5, 1, 2 and 4 mg/kg dose or vehicle (veh). Data reported as mean ± SEM. * – $P < 0.05$; ** – $P < 0.01$; *** – $P < 0.001$ versus vehicle group.

As compared to MF, longer duration of antinociception and more potent effect of **1** in lower doses may be attributed to the higher than MF affinity for opioid receptors in the spinal cord. Data concerning mechanisms of the antinociceptive effects of dermorphin and its analogues suggest minor supraspinal action of these peptides after peripheral application and contrary to morphine they act mainly at the level of spinal cord having at the same time less pronounced adverse effects characteristic for opioids [26–28]. Relatively long-lasting antinociception of compound **1** may also arise from enhanced metabolic stability as the evaluation of the resistance to hydrolysis by digestive enzymes indicated that all newly synthesized cyclopeptides, particularly analogues **1**–**10**, established long term and high resistance to enzymatic degradation. The lack of antinociceptive activity of the remaining compounds may probably be related to low opioid receptors affinity or/and pharmacokinetic factors. More detailed pharmacological and structure-activity studies would help to elucidate the results of the current investigations.

3. Conclusion

A series of new analogues of dermorphin/delthorphin tetrapeptide **1**–**14** were synthesized using both SPPS technique and classical method in solution. SPPS synthesis of *N*-(2-ureidoethyl) amides **1**–**8** and unsubstituted amides **11**–**14** was carried out on functionalized, or respectively, on non-functionalized MBHA resin whereas classical method was found to be more convenient for preparation of series of differently substituted amides starting from a common synton (synthesis **1**, **9** and **10**). All new analogues were tested for their *in vitro* resistance to proteolysis by means of mass

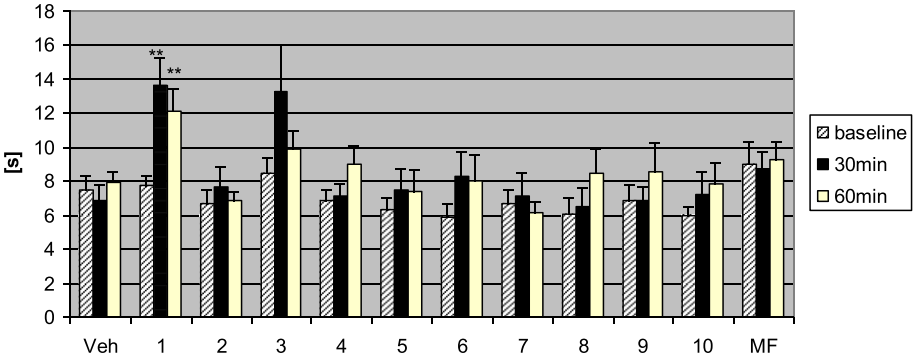


Fig. 4. Latency (in seconds) to nociceptive reaction to thermal stimulus in hot-plate test before (baseline) and 30 and 60 min after iv injection of agents **1**–**10**, morphine (MF) or vehicle. Data expressed as mean ± SEM. ** – $p < 0.01$ versus baseline.

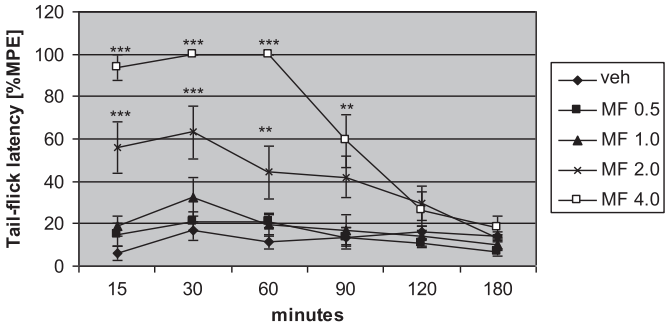


Fig. 6. Tail flick latency expressed as the percent of maximal possible effect (%MPE) in groups of mice ($n = 10$) at 15–180 min after i.v. injection of morphine (MF) in the 0.5, 1, 2 and 4 mg/kg dose or vehicle (veh). Data reported as mean ± SEM. * – $P < 0.05$; ** – $P < 0.01$; *** – $P < 0.001$ versus vehicle group.

spectroscopy (MS) method and it was found that all *N*-substituted amides containing D-amino acid in position 2 showed the full stability (no degradation products) during 12-h incubation with large excess of hydrolyzed, chymotrypsin and pepsin. This extremely high resistance to enzymatic degradation can be explained by additive effect of three modifications: the presence of D-amino acid in position 2 in peptide chain, cyclic structure of analogues with carbonyl bridge, and the substitution of amide at the *C*-termini especially by (2-ureido)ethyl group. Similar peptides with L-amino acid residues in position 2 were much less stable against proteolysis. The *in vivo* antinociceptive activity of the peptides was evaluated using hot-plate test and significant effect was observed after intravenous application of compound **1**. The analogues **9** and **10** with the structures having less complex than **1** *C*-termini did not influence pain threshold to thermal stimulus. This may suggest that besides the same combination of amino acids applied, more expanded and unique (2-ureido)ethyl substitution at *C*-termini could positively correlate with biological activity of compound **1**. Compound **1** was further evaluated using tail-flick assays in mouse. In both assays analogue **1** showed significant, stronger than morphine antinociceptive effect after systemic application. These results make compound **1** a lead molecule for further investigation.

4. Experimental protocols

4.1. Chemistry

4.1.1. General

Protected amino acids were purchased from IRIS Biotech GMBH and other chemicals from Sigma–Aldrich and were used without purification. Dioxane was distillated from Na, DMF from ninhydrin under reduced pressure; solvents were stored over molecular sieves (4 Å). Evaporation was performed under reduced pressure. Reactions were monitored and purity of compounds was substantiated by TLC on aluminium sheets coated with silica gel 60 F_{254} (Merck) in the following systems (v/v): $CHCl_3$/AcOH/MeOH (85:10:15), (II) $CHCl_3$/AcOEt (4:1), (III) $CHCl_3$/AcOEt (2:1), (IV) $CHCl_3$/MeOH (15:1), (V) $CHCl_3$/MeOH/AcOH/H_2O = 6:1.8:0.2:0.3, (VI) $CHCl_3$/MeOH = 9:1, (VII) $CHCl_3$/MeOH = 15:4, (VIII) $CHCl_3$/MeOH = 15:3, (IX) MeOH/$CHCl_3$ = 9:1, (X) (DCM/MeOH = 15:2).

Purification of crude analogues by preparative TLC chromatography was performed on silica gel GF plates, prep. layer 2000 microns with UV 254, (Analtech, Inc.) using $CHCl_3$:MeOH:AcOH:H_2O (6:4.5:0.6:1.4) system for elution. The band corresponding to appropriate product was separated, eluted with the same solvent system, evaporated and finally dissolved in water or in water/AcOH, optionally filtered, and lyophilized. Purification by flash chromatography was carried out on silica gel column using the same solvent system as for preparative TLC purification. The fractions corresponding to appropriate product were combined, dissolved in water or in water/AcOH and lyophilized. The purity of peptides at this stage were in the range of 70–80% (HPLC). The semi-preparative RP-HPLC purification was accomplished on Vertex Nucleosil 300 C-18 column (250 mm × 8 mm, 30 μm) in gradient system, mobile phases: (A) 0.05% TFA in water, (B) 80% (A)/ACN, (C) 80% ACN/H_2O. Homogeneity of the purified analogues was assessed by analytical HPLC in the following conditions: YMC-Pack PROTEIN-RP column (250 mm × 4.6 mm, 5 μm), mobile phases: (A) 0.05% TFA/H_2O, (B) 60% ACN in (A), gradient system: 15%–30% (B) during 20 min, then 30%–80% (B) in 10 min, flow rate = 1 mL/min, PDA detector.

4.1.1.1. Analytical studies. High resolution mass spectra were obtained using the Mariner ESI/TOF mass spectrometer (Perseptive Biosystems).

The ^1H and ^{13}C NMR spectra of several compounds and main product **1** were measured in DMSO-d_6 solutions with Varian-NMR-vnmrs600 (at temperature 298 K) equipped with a 600 MHz PFG Auto XID ($^1H/^{15}N–^{31}P$ 5 mm) indirect probehead.

To assign the structures under consideration following 1D and 2D experiments were employed: ^1H selective TOCSY, COSY, $^1H–^{13}C$ gradient selected HSQC and HMBC for $^1J(C–H) = 140$ Hz and $^nJ(C–H) = 8$ Hz, respectively. In order to fully characterize compounds studied $^1H - ^{15}N$ gradient selected experiments (HSQC and HMBC) were also recorded. Standard experimental conditions and standard Varian programmes were used. The ^1H and ^{13}C NMR chemical shifts are given relative to the TMS signal at 0.0 ppm, whereas nitrogen signal of nitromethane ($\delta = 0.0$ ppm) was used to calibrate ^{15}N NMR spectra. Concentration of all solutions used for measurements was about 20–30 mg of compounds in 0.6 cm^3 of solvent.

4.1.2. Solid phase synthesis

The p-nitrophenoxycarbonyl derivative of Boc-1,2-diaminoethane was obtained as described earlier [19]. This compound was coupled to the MBHA resin (0.75 meq./g, 1% crosslink, 100–200 mesh, 6 eq.) in DMF at 60 °C for 48 h to afford Boc–NH–CH_2–CH_2–NH–CO–MBHA-resin. The Boc group was removed by treatment with 15% HCl/dioxane (5 and 15 min), resin was neutralized with DIPEA, then protected amino acids were successively attached in the required sequence, by dividing resin into smaller portions, and using TBTU/HOBt methodology (3 fold excess of coupling reagent). Completeness of couplings was monitored by Kaiser test. The protected peptide resins were treated with 55% piperidine in DMF with stirring for 50 min to remove Fmoc groups.

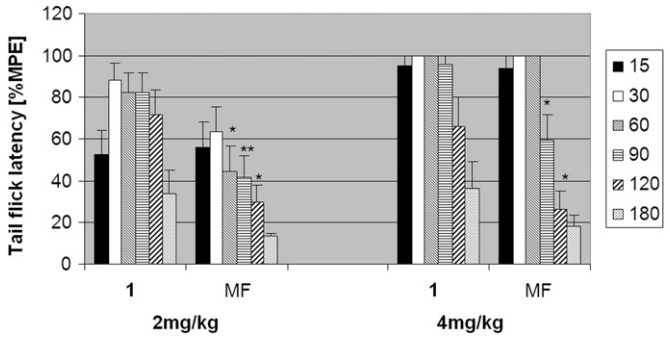


Fig. 7. Comparison between compound **1** and morphine (MF) in tail flick test. Results expressed as the percent of maximal possible effect (%MPE) in groups of mice ($n = 10$) at 15–180 min after i.v. injection in the 2 and 4 mg/kg dose. Data reported as mean ± SEM. * – $P < 0.05$; ** – $P < 0.01$ versus **1** at relevant time point.

To the suspension of each resin in 350 mL DMF (for 1 mmol scale) containing DIPEA (1.2–1.3 eq.) the solution of bis(p-nitrophenyl) carbonate (1.2–1.3 eq.) in 150 mL DMF was added during 4 h. The reaction was allowed to continue until free amino groups could no longer be detected on the resin (5–7 days), then the solvent was removed under reduced pressure. The peptides were cleaved from the resin by treatment with TFA/cocktail (TFA:phenol:water, 13.5:1:1, mL/g/g) for 10 min at −5 °C, and 3 h at RT under argon. The resin was filtered off, washed with TFA, AcOH and DCM. Filtrate and washings were combined, evaporated to oily residue. This residue was dissolved in AcOH and dropped into 200 mL of stirred MTBE. After cooling in the refrigerator; fluffy precipitate was filtered and dried under reduced pressure over KOH pellets. Starting from 0.88 mmol of substituted resin *ca.* 400 mg of crude peptide was obtained. In case of peptides 11–14 TFA:TFMSA:tioanisole:ethandithiole (10:1:1:0.5 mL) mixture was used to cleave the peptides from the resin.

All crude analogues synthesized by the SPPS method were subjected to two-steps purification, first by flash column chromatography or by preparative TLC, and final purification which was accomplished by semi-preparative RP-HPLC in conditions described above. Homogeneity of the purified analogues was assessed by TLC and analytical HPLC. Analytical data of the peptides are presented in Table 1.

4.1.3. Synthesis in solution

4.1.3.1. HCl Dab(Z)–OMe, ***15***. To Boc–Dab(Z)–OH (15.3 g, 43.6 mmol) 150 mL 20% HCl in MeOH was added in three portions and reaction mixture was stirred at RT overnight. HCl and solvent were removed under reduced pressure, treated with 50 mL portions of methanol and evaporated. This procedure was repeated three times. Drying under reduced pressure over KOH gave 11.25 g **15** (85%) as a white solid; TLC: R_f(I) 0.52, R_f; . MS for $C_{13}H_{18}N_2O_4$, M calcd. 266.13, found $[M + H]^+ = 267.2$ (no peaks corresponding to a product without Z group was observed). ^{1}H NMR (DMSO-d_6, 600 MHz) δ [ppm]: 8.80 (s, 3H, NH_2 HCl), 7.43 (t, 1H, $J = 5.3$ Hz, NH), 7.38–7.28 (aromatic protons), 5.01 (s, 2H, OCH_2), 4.00 (t, 1H, $J = 6.4$ Hz, C_αH), 3.70 (s, 3H, OCH_3), 3.16 (m, 2H, $C_\gamma H_2$), 2.00 (m, 2H, $C_\delta H_2$); ^{13}C NMR (DMSO-d_6, 150 MHz) δ [ppm]: 169.8 (COCH_3), 156.1 (PhCH$_2$OCO), 137.1 (quaternary carbon of aromatic ring), 128.4, 127.8, 127.7 (carbons of aromatic ring), 65.4 (OCH_2), 52.8 (OCH_3), 49.8 (C_α Dab), 36.4 (C_γ Dab), 30.1 (C_β Dab); ^{15}N NMR (DMSO-d_6, 60 MHz) δ [ppm]: −298.0 (C_γNH Dab), −338.0 (NH_2).

4.1.3.2. Boc–Phe–Dab(Z)–OMe, ***16***. To the cold (0 °C) solution of Boc–Phe–OH (8.33 g, 31.4 mmol), DIPEA (16.4 mL, 94.2 mmol) and 4.81 g (31.4 mmol) HOBt in 80 mL of DMF, 10.08 g (31.4 mmol) TBTU in 20 mL DMF were added. After 20 min stirring at 0 °C this mixture was added to a cold (0 °C) solution of **15** (7.92 g, 26.16 mmol) in 20 mL DMF containing DIPEA (4.49 mL, 26.16 mmol). The reaction mixture was stirred for 1 h at 0 °C and at RT overnight. DMF was removed under reduced pressure. Crude product was diluted with DCM (250 mL), washed with 10% citric acid (50 mL), 5% K_2CO_3 (3 × 50 mL) and brine (3 × 50 mL). DCM layer was dried over $MgSO_4$, filtered and concentrated under reduced pressure. The residue was crystallized from AcOEt/hexane to yield **16** after drying (14.7 g, 91%). TLC: R_f (II) 0.18, R_f (III) 0.66; ESI MS: for $C_{27}H_{35}O_7N_3$, calcd. M = 513.6; found $[M + H]^+ = 514.3$; $[M + Na]^+ = 536.3$. ^{1}H NMR (DMSO-d_6, 600 MHz) δ [ppm]: 8.30 (d, 1H, $J = 7.6$ Hz, C_αNH Dab), 7.36–7.22 (m, 10H, aromatic protons) 7.19 (m, 1H, C_γNH Dab), 6.83 (d, 1H, $J = 8.4$ Hz, Boc–NH), 4.97 (s, 2H, OCH_2), 4.28 (m, 1H, C_αH Dab), 4.16 (m, 1H, C_αH Phe), 3.57 (s, 3H, OCH_3), 3.06–3.00 (m, 2H, $C_\gamma H_2$ Dab), 2.92 and 2.68 (2m, 2H, $C_\beta H_2$ Phe), 1.87 and 1.73 (2m, 2H, $C_\beta H_2$ Dab), 1.24 (s, 9H, CH_3 of Boc); ^{13}C NMR (DMSO-d_6, 150 MHz) δ [ppm]: 172.2 (COOCH$_3$), 172.0 (CO Phe), 156.0 (PhCH$_2$OCO–NH Dab), 155.2 (t-Bu–O–CO–NH Phe) 138.1 (C_{ipso} Phe), 137.2 (C_{ipso} PhCH$_2$OCO), 129.2, 128.3, 128.0, 127.7, 126.1 (carbons of aromatic ring), 78.0 (quaternary carbon of t-Bu), 65.2 (PhCH$_2$O), 55.5 (C_α Phe), 51.9 (OCH_3), 49.8 (C_α Dab), 37.3 (C_β Phe), 37.2 (C_γ Dab), 30.9 (C_β Dab), 28.1 (CH_3 of t-Bu); ^{15}N NMR (DMSO-d_6, 60 MHz) δ [ppm]: −264.8 (C_αNH Dab), −291.2 (NH Phe), −298.2 (C_γNH Dab).

4.1.3.3. Boc–D–Lys(Z)–Phe–Dab(Z)–OMe, ***17***. **16** (4.67 g, 9 mmol) was treated with TFA (15 mL) and stirred at RT for 55 min; TFA was removed under reduced pressure; MeOH was added (30 mL) and evaporated. This procedure was repeated three times. The resulted trifluoroacetate TFA·Phe–Dab(Z)–OMe was dried over KOH under reduced pressure.

Coupling of Boc–D–Lys(Z)–OH with the above trifluoroacetate was performed by TBTU/HOBt method in similar manner as was described for the preparation of **16**; white solid (yield 79% after recrystallization from AcOEt). TLC: R_f (IV) = 0.43. ESI MS: for $C_{41}H_{53}O_{10}N_5$ calcd. M = 775.89, $[M + Na]^+ = 798.4$. ^{1}H NMR (DMSO-d_6, 600 MHz) δ [ppm]: 8.33 (d, 1H, $J = 7.5$ Hz, C_αNH Dab), 8.10 (d, 1H, $J = 8.6$ Hz, NH Phe), 7.36–7.15 (m, 17H, 15 aromatic protons and C_γNH Dab + C_εNH Lys), 6.72 (d, 1H, $J = 7.6$ Hz, Boc-NH Lys), 5.02 and 5.01 (2s, 4H, 2 × OCH_2), 4.57 (m, 1H, C_αH Phe), 4.30 (m, 1H, C_αH Dab), 3.82 (m, 1H, C_αH Lys), 3.61 (s, 3H, OCH_3), 3.13-3.06 (m, 3H, one of $C_\beta H_2$ Phe and $C_\gamma H_2$ Dab), 2.87 (m, 2H, $C_\varepsilon H_2$ Lys), 2.72 (m, 1H, one of $C_\beta H_2$ Phe), 1.94 (m, 1H, one of C_βH Dab), 1.81 (m, 1H, one of C_βH Dab), 1.34 (s, 9H, CH_3 of Boc), 1.30–1.20 (m, 4H, $C_\beta H_2$ Lys and $C_\delta H_2$ Lys), 1.06–0.90 (m, 2H, $C_\gamma H_2$ Lys); ^{13}C NMR (DMSO-d_6, 150 MHz) δ [ppm]: 172.0 (COOCH$_3$), 171.8 (CO Lys), 171.4 (CO Phe), 156.06 and 156.02 (PhCH$_2$OCO at C_εNH Lys and C_γNH Dab), 155.3 (t-Bu-O–CO), 137.7, 137.3, 137.2, 129.2, 128.3, 127.9, 127.7, 126.2 (carbons of aromatic rings), 78.0 (quaternary carbon of t-Bu), 65.2 and 65.1 (two carbons of PhCH$_2$OCO), 54.2 (C_α Lys), 53.3 (C_α Phe), 51.9 (OCH_3), 50.0 (C_α Dab), 39.9 (C_ε Lys), 37.4 (C_β Phe), 37.3 (C_γ Dab), 31.4 (C_β Lys), 30.8 (C_β Dab), 29.1 (C_δ Lys), 28.1 (CH_3 of t-Bu), 22.3 (C_γ Lys); ^{15}N NMR (DMSO-d_6, 60 MHz) δ [ppm]: −263.7 (NH Phe), −264.4 (C_αNH Dab), −290.0 (C_αNH Lys), −296.4 (C_εNH Lys), −298.3 (C_γNH Dab).

The same coupling was repeated substantially in the same manner (0.1 mole scale) using HATU as a coupling reagent (yield of **17**, 82%).

4.1.3.4. Boc–Tyr(OBu^t)–D–Lys(Z)–Phe–Dab(Z)–OMe, ***18***. The deprotection of **17** by TFA, and coupling of trifluoroacetate with Boc–Tyr(OBu^t)–OH was performed by TBTU/HOBt or HATU methods as described above. The crude **18** (94% yield, 85% purity according to HPLC) was purified by flash chromatography on silica gel column with MeOH/$CHCl_3$ (1.5–3% MeOH by vol.). The respective fractions were collected, evaporated and dried under reduced pressure to give pure **18** as a white solid. TLC: R_f (IV) = 0.59; MS: for $C_{54}H_{70}O_{12}N_6$ calcd. M = 995.19, found $[M + H]^+ = 996.4$, $[M + Na]^+ = 2017.40$. ^{1}H NMR (DMSO-d_6, 600 MHz) δ [ppm]: 8.45 (d, 1H, $J = 7.2$ Hz, C_αNH Dab), 8.32 (d, 1H, $J = 8.6$ Hz, C_αNH Phe), 7.90 (d, 1H, $J = 7.6$ Hz, C_αNH Lys), 7.38–7.28 (m, 11H, aromatic protons and C_γNH Dab), 7.27–7.21 (m, 4H, aromatic protons), 7.20–7.10 (m, 4H, aromatic protons and C_εNH Lys), 6.82 (d, 2H, $J = 8.4$ Hz, aromatic protons of Tyr ortho to O-t-Bu), 6.79 (d, 1H, $J = 8.6$ Hz, C_αNH Tyr), 5.02 (s, 2H, OCH_2 of PhCH$_2$OCO at C_γNH Dab), 4.99 (s, 2H, OCH_2 of PhCH$_2$OCO at C_εNH Lys), 4.60 (m, 1H, C_αH Phe), 4.31 (m, 1H, C_αH Dab), 4.20 (m, 2H, C_αH Lys, C_αH Tyr), 3.61 (s, 3H, OCH_3), 3.13 and 3.06 (2m, 2H, $C_\alpha H_2$ Dab), 3.09 (m, 1H, one of C_βH Phe), 2.87–2.82 (m, 3H, one of C_βH Tyr and $C_\varepsilon H_2$ Lys), 2.70 (m, 1H, one of C_βH Phe), 2.63 (m, 1H, one of C_βH Tyr), 1.93 and 1.83 (2m, 2H, $C_\beta H_2$ Dab), 1.30–1.16 (m and 2s, 13H, 2 t-Bu and $C_\beta H_2$ Lys, of $C_\delta H_2$ Lys), 0.83 (m, 2H, $C_\gamma H_2$ Lys); ^{13}C NMR (DMSO-d_6, 150 MHz) δ [ppm]: 172.1 (COOCH$_3$), 171.5 (CO Lys), 171.3 (CO Tyr), 171.1 (CO Lys), 156.1

($PhCH_2\underline{OCO}$ at C_γNH Dab), 156.0 ($PhCH_2\underline{OCO}$ at C_εNH Lys), 155.0 (t-BuOCO at C_αNH Tyr), 153.3 (C–O–t-Bu Tyr), 137.8 (C_{ipso} Phe), 137.3 and 137.1 (2 C_{ipso} of Ph at $PhCH_2OCO$), 132.6 (C_{ipso} Tyr), 129.7, 129.2, 128.3, 128.3, 127.9, 127.7, 127.7, 126.2 and 123.2 (carbons of aromatic rings), 77.9 and 77.5 (2 quaternary carbons of t-Bu), 65.2 and 65.1 (2 carbons of $Ph\underline{CH_2}OCO$), 55.6 (C_α Tyr), 53.5 (C_α Phe), 52.3 (C_α Lys), 51.9 (OCH_3), 50.0 (C_α Dab), 40.1 (C_ε Lys), 37.6 (C_β Phe), 37.3 (C_β Tyr and C_γ Dab), 31.9 (C_β Lys), 30.7 (C_β Dab), 29.1 (C_δ Lys), 28.5 and 28.1 (CH_3 of t-Bu), 21.8 (C_γ Lys); ^{15}N NMR (DMSO-d_6, 60 MHz) δ [ppm]: −262.4 (NH Phe), −262.7 (NH Lys), −264.2 (C_αNH Dab), −291.0 (NH Tyr), −296.2 (C_εNH Lys), −298.1 (C_γNH Dab).

4.1.3.5. Boc–Tyr(OBu^t)–D–Lys–Phe–Dab–OMe, ***19***. A solution of **18** (2.19 g, (2.2 mmol) in the mixture MeOH/AcOH (170/30 mL) was hydrogenated in the presence of 100 mg of 10% palladium on charcoal for 2 h. After filtration through a cellite pad, the solution was evaporated to 40 mL volume and dropped into 500 mL TBME. After cooling in the refrigerator, white precipitate was filtered, washed with TBME, and dried in vacuum to give 1.35 g of **19**. TLC: R_f (V) = 0.30; ESI MS: for $C_{54}H_{70}O_{12}N_6$ calcd. M = 726.60; found $[M + H^+] = 727.40$, $[M + Na^+] = 749.36$.

When hydrogenolysis was carried out in MeOH/DMF mixture large amount of lactam **25** was formed; this impurity could be isolated by column chromatography, ESI MS: for $C_{37}H_{54}O_7N_6$, calcd. M = 694.86; found $[M + H^+] = 695.4$, $[M + Na^+] = 717.3$. ^{1}H NMR (DMSO-d_6, 600 MHz) δ [ppm]: 8.74 (d, 1H, C_αNH Dab), 8.55 (d, 1H, C_αNH Phe), 8.19 (d, 1H, C_αNH Lys), 7.29–7.21 (m, 4H, aromatic protons), 7.18 (m, 1H, aromatic protons), 7.14(d, 2H, protons meta to t-Bu–O Tyr), 6.99 (d, 1H, C_αNH Tyr), 6.84 (d, 2H, protons ortho to t-Bu-O Tyr), 4.52 (m, 1H, C_αH Phe), 4.35 (m, 1H, C_αH Dab), 4.19 (m, 2H, C_αH Lys and C_αH Tyr), 3.62 (OCH_3), 3.09 (m, 1H, one of C_βH Phe), 2.87 (m, 1H, one of C_βH Tyr), 2.80–2.65 (m, 4H, $C_\gamma H_2$, one of C_βH Phe and one of C_βH Tyr), 2.57 (m, 2H, $C_\varepsilon H_2$ Lys), 1.98–1.92 (m, 2H, $C_\beta H_2$ Dab), 1.38–1.24 (m and 2 s, 22H, $C_\delta H_2$ Lys, $C_\beta H_2$ Lys and CH_3 of both t-Bu), 0.92 (m, 2H, $C_\gamma H_2$ Lys), ^{13}C NMR (DMSO-d_6, 150 MHz) δ [ppm]: 171.8 ($\underline{CO}OCH_3$), 171.6 (CO Phe), 171.5 (CO Tyr), 171.3 (CO Lys), 155.2 (t-Bu–$\underline{O}$–$\underline{CO}$–NH Tyr), 153.3 (C–O-t-Bu Tyr), 137.9 (C_{ipso} Phe), 132.7 (C_{ipso} Tyr), 129.7, 129.2, 128.0, 126.3 and 123.3 (aromatic carbons), 78.0 and 77.6 (2 quaternary carbons of t-Bu), 55.8 (C_α Tyr), 54.1 (C_α Phe), 52.2(C_α Lys), 52.0 (OCH_3), 50.1 (C_α Dab), 38.4 (C_ε Lys), 37.4 (C_β Phe), 37.3 (C_β Tyr), 36.2 (C_γ Dab), 31.4 (C_β Lys), 30.0 (C_β Dab), 28.5 and 28.1 (CH_3 of t-Bu), 27.0 (C_δ Lys), 21.7 (C_γ Lys); ^{15}N NMR: spectrum was not recorded due to fast decomposition of compound.

NMR spectrum for lactam **25**: ^{1}H NMR (DMSO-d_6, 600 MHz) δ [ppm]: 8.48 (d, 1H, J = 8.8 Hz, NH Phe), 8.34 (d, 1H, J = 8.2 Hz, NH C_αH Dab-lactam), 8.02 (d, 1H, J = 8.0 Hz, NH Lys), 7.87 (s, 1H, C_γNH Dab-lactam), 7.29–7.16 (m, 5H, aromatic protons), 7.13 (d, 2H, J = 8.4 Hz, protons meta to OCH_3 in substituted Tyr), 6.94 (d, 1H, J = 8.6 Hz, Boc–NH Tyr), 6.84 (d, 2H, J = 8.4 Hz, protons ortho to OCH_3 Tyr), 4.53 (m, 1H, C_αH Phe), 4.34 (m, 1H, C_αH Dab-lactam), 4.22–4.14 (2m, 2H, C_αH Lys and C_αH Tyr), 3.22–3.18 (m, 2H, C_γH Dab lactam), 3.09 (d, 1H, J = 13.5 and 3.6 Hz, C_βH Phe), 2.86 (dd, 1H, J = 13.5 and 9.0 Hz, C_βH Tyr), 2.75 (dd, 1H, J = 13.5 and 11.1 Hz, C_βH Phe), 2.65 (dd, 1H, J = 13.5 and 10.2 Hz, C_βH Tyr), 2.52 (m, 2H, $C_\varepsilon H_2$ Lys), 2.32 (m, 1H, C_βH Dab lactam), 1.86 (m, 2H, C_βH Dab lactam and C_γH Dab lactam), 1.35–1.18 (2s and m, 18H, CH_3 of both Boc and $C_\beta H_2$ Lys, C_δH Lys), 0.88 (m, 2H, $C_\gamma H_2$ Lys).^{13}C NMR (DMSO-d_6, 150 MHz) δ [ppm]: 174.2 (CO Dab lactam), 171.3 (CO Tyr), 171.2 (CO Phe), 171.1 (CO Lys), 155.1 (CO of NH-Boc Tyr), 153.3 (C(O-tBu) of Tyr), 137.9 (C_{ipso} Phe), 132.7 (C_{ipso} Tyr), 129.7 (C_{meta} to t-Bu-O of Tyr), 129.2 (C_{ortho} Phe), 127.9 (C_{meta} Phe), 126.2 (C_{para} Phe), 123.3 (C_{ortho} to t-Bu-O Tyr), 77.9 and 77.5 (quaternary carbons of both Boc), 55.8 (C_α Tyr), 53.9 (C_α Phe), 52.2 (C_α Lys), 49.6 (C_α Dab lactam), 38.9 (C_ε Lys), 38.0 (C_γ Dab lactam), 37.9 (C_β Phe), 37.2 (C_β Tyr), 31.6 (C_β Lys), 28.5 and 28.1(CH_3 of both Boc), 28.2 (2 C, C_β Dab lactam and C_δ Lys), 21.6 (C_γ Lys); ^{15}N NMR (DMSO-d_6, 150 MHz) δ [ppm]: −262.2 (NH Phe), −263.0 (NH Lys), −264.0 (NH between Phe and Dab lactam), −267.0 (NH Dab lactam), −290.7 (NH Tyr).

4.1.3.6. {[Boc–Tyr(OBu^t)–D–Lys(&^{1})–Phe–Dab(&^{2})–OMe] [&^{1}CO&^{2}]}, ***20***. To the solution of **19** (1.82 g, 2.5 mmol) and DIPEA (4.8 mmol) in 800 mL of DMF, a solution of *bis*(p-nitrophenyl)carbonate (760.5 mg, 2.5 milomol) in 300 mL of DMF was added with stirring during 4 h. The reaction mixture was stirred with keeping slightly basic conditions by addition of small amount of DIPEA (up to 1 mmol). Stirring was continued until free amino groups could no longer be detected (Kaiser test, 5–7 days). The solvent and DIPEA were removed by reduced pressure to an oil which was treated by addition of TBME (250 mL). The crude product was filtered and dried (1.25 g), and a sample (0.64 g) was purified by column chromatography (30 g SiO_2; elution with 3–7% mixture MeOH:$CHCl_3$) gave the title compound **20** (0.44 g) as a white solid. TLC: R_f (VI) = 0.34 ($CHCl_3$:MeOH = 9:1; MS: for $C_{39}H_{56}O_9N_6$, M calcd. 52.90, found $[M + H^+] = 753.50$, $[M + Na^+] = 775.50$.

Due to the similarity of structures (**20** and **21**) only the ^{1}H NMR chemical shifts were presented for **20.** ^{1}H NMR (DMSO-d_6, 600 MHz) δ [ppm]: 8.36 (d, 1H, C_αNH Dab), 8.00 (d, 1H, C_αNH Lys), 7.51 (d, 1H, C_αNH Phe), 7.26-7.10 (m, 7H, 5 aromatic protons of Phe and 2 aromatic protons (meta) to t-Bu–O Tyr), 6.92-6.86 (m, 3H, 2 aromatic protons (ortho) to t-Bu-O Tyr and C_αNH Tyr), 5.68 (t, 1H, C_γNH Dab), 5.57 (t, 1H, C_εNH Lys), 4.51 (m, 1H, C_αH Dab), 4.44 (m, 1H, C_αH Phe), 4.18 (m, 1H, C_αH Tyr), 4.07 (m, 1H, C_αH Lys), 3.59 (s, 3H, OCH_3), 3.06–3.00 (m, 2H, $C_\gamma H_2$ Dab), 2.98 (m, 2H, $C_\varepsilon H_2$ Lys), 2.93 (m, 1H, one of C_βH Phe), 2.85–2.80 (m, 2H, one of C_βH Phe and one of C_βH Tyr), 2.72 (m, 1H, one of C_βH Tyr), 1.90 and 1.72 (2m, 2H, $C_\beta H_2$ Dab), 1.54 (m, 1H, one of C_βH Lys), 1.38–1.24 (m and 2s within, 21H, 2 x t-Bu, one of C_βH Lys and $C_\delta H_2$ Lys), 1.17 and 1.08 (2m, 2H, $C_\gamma H_2$ Lys).

4.1.3.7. {[Boc–Tyr(OBu^t)–D–Lys(&^{1})–Phe–Dab(&^{2})–OH [[&^{1}CO&^{2}]}, ***21***. Ester **20** (2.04 g, 664.1 mg, 0.88 mmol) was treated with mixture dioxane:2 M aq. NaOH (6 mL:6 mL). The mixture was vigorously stirred being monitored by TLC ($CHCl_3$:MeOH, 9:1, v/v). After the substrate consumption (30–40 min), 10% aq. citric acid was added to pH 3; white precipitate was filtered off, washed with water and dried to yield the title acid **21** (1.96 g, 98%) as a white solid. MS: for $C_{38}H_{54}O_9N_6$, M calcd. 738,87, found $[M + Na]^+ = 761,4$. ^{1}H NMR (DMSO-d_6, 600 MHz) δ [ppm]: 8.31 (d, 1H, J = 8.1 Hz, C_αNH Dab), 8.00 (d, 1H, J = 8.5 Hz, C_αNH Lys), 7.51 (d, 1H, J = 8.2 Hz, C_αNH Phe), 7.25–7.10 (m, 7H, 5 aromatic protons of Phe and 2 aromatic protons (meta) to t-Bu–O Tyr), 6.92–6.86 (m, 3H, 2 aromatic protons (ortho) to t-Bu–O Tyr and C_αNH Tyr), 5.68 (t, 1H, C_γNH Dab), 5.57 (t, 1H, C_εNH Lys), 4.47 (m, 1H, C_αH Phe), 4.41 (m, 1H, C_αH Dab), 4.19 (m, 1H, C_αH Tyr), 4.07 (m, 1H, C_αH Lys), 3.06-3.00 (m, 2H, $C_\gamma H_2$ Dab), 2.98 (m, 2H, $C_\varepsilon H_2$ Lys), 2.93 (m, 1H, one of C_βH Phe), 2.85–2.80 (m, 2H, one of C_βH Phe and one of C_βH Tyr), 2.72 (m, 1H, one of C_βH Tyr), 1.90 and 1.72 (2m, 2H, $C_\beta H_2$ Dab), 1.54 (m, 1H, one of C_βH Lys), 1.38–1.24 (m and 2s within, 21H, 2 t-Bu groups, one of C_βH Lys, $C_\delta H_2$ Lys), 1.17 and 1.08 (2m, 2H, $C_\gamma H_2$ Lys); ^{13}C NMR (DMSO-d_6, 150 MHz) δ [ppm]: 173.5 (COOH), 171.0 (CO Phe and CO Lys), 170.9 (CO Tyr), 158.20 (CO between C_εNH Lys and C_γNH Dab), 155.1 (CO of Boc-NH at C_αNH Tyr), 153.5 (aromatic C(t-Bu–O-) Tyr), 137.6, 132.3, 129.7, 129.2, 128.0, 126.1, 123.3 (carbons of aromatic rings of Tyr and Phe), 78.2 and 77.6 (quaternary carbons of t-Bu), 56.0 (C_α Tyr), 53.9 (C_α Phe), 53.5 (C_α Lys), 49.1 (C_α Dab), 37.9 (C_ε Lys), 37.4 and 37.3 (C_β Tyr and C_β Phe), 36.7 (C_γ Dab), 31.2 (C_β Lys), 30.9 (C_β Dab), 29.3 (C_δ Lys), 28.5 and 28.1 (CH_3 of both t-Bu), 20.9 (C_γ Lys), ^{15}N NMR (DMSO-d_6, 60 MHz) δ [ppm]: -261.0 (C_αNH Dab), −261.5 (C_αNH Lys), −264.9 (C_αNH Phe), −290.2 (C_αNH Tyr), −299.1 (C_εNH Lys), −300.1 (C_γNH Dab).

*4.1.3.8. {[Boc–Tyr(OBu^t)–D–Lys(&^{1})–Phe–Dab(&^{2})–NHEt][&^{1}CO&^{2}]}, **22**.* Cyclic ester **20** (60 mg, 0.08 mmol) was added to 2 M solution of diethylamine in MeOH (12 mL) and reaction mixture was stirred at RT for 10 days (TLC control). After evaporation of solvent, crude amide **22** was dried under reduced pressure over P_2O_5 (62 mg), and used for further synthesis without purification. TLC: R_f (VII) 0.69, $R_f = 0.77$ for starting ester.

*4.1.3.9. {[Boc–Tyr(OBu^t)–D–Lys(&^{1})–Phe–Dab(&^{2})–NH–CH$_2$–CH$_2$–NH–CO–NH$_2$][&^{1}CO&^{2}]}, **23***

4.1.3.9.1. Boc–(2-ureido)-ethylamine. To the suspension of *p*-nitrophenoxycarbonyl derivative of Boc-1,2-diaminoethane [19] (11.71 g, 36 mmol) in 230 mL of MeOH, 11,5 mL of. 25 %aq. ammonia was added. The reaction mixture was stirred at RT for 48 h. After solvent evaporation the residue was precipitated by addition of ethyl ether (70 mL). White product was filtered, washed with ethyl ether (4 × 20 mL) and dried to give 6.05 g of Boc–NH–CH$_2$–CH$_2$–NH–CO–NH$_2$ (83%). TLC: R_f (VIII) 0.22; MS: for $C_8H_{17}O_3N_3$ calcd. M 203.24, found $[M + H]^+ = 204.12$, $[M + Na]^+ = 226.2$. ^{1}H NMR (DMSO-d_6, 600 MHz) δ [ppm]: 6.77 (t, 1H, NH–COO–t-Bu), 5.98 (t, 1H, NH–CONH$_2$), 5.48 (s, 2H, CO–NH$_2$), 3.00 and 2.92 (2 m, 4H, 2 × CH$_2$), 1.38 (s, 9H, CH$_3$), ^{13}C NMR (DMSO-d_6, 600 MHz) δ [ppm]: 158.9 (NH–CO–NH$_2$), 155.6 (t-Bu–OCO–NH), 77.6 (quaternary carbon of t-Bu), 40.8 and 39.1 (both CH$_2$), 28.2 (CH$_3$ of t-Bu); ^{15}N NMR (DMSO-d_6, 600 MHz) δ [ppm]: −298.0 (t-Bu–OCO–NH–CH$_2$), -300.0 (CH$_2$–NH–CO–NH$_2$), −305.2 (NH–CO–NH$_2$).

4.1.3.9.2. (2-Ureido)-ethylamine trifluoroacetate. Removal of Boc protection from the above Boc-ureidoethylamide was carried out by the TFA treatment using a procedure similar to that described for the deprotection of **16**. (2-Ureido)-ethylamine trifluoroacetate was carefully dried under reduced pressure over KOH pellets. Yield 100%, MS: for $C_3H_9ON_3$ calcd. M = 103.12, found $[M + H]^+ = 104.12$, $[M + Na]^+ = 126.10$. ^{1}H NMR (DMSO-d_6, 600 MHz) δ [ppm]: 7.90 (s, 3H, protonated NH$_2$–CH$_2$–CH$_2$), 6.43 (s, 1H, NH–CO–NH$_2$), 3.20 and 2.83 (2 m, 2H, CH$_2$–CH$_2$), ^{13}C NMR (DMSO-d_6, 600 MHz) δ [ppm]: 159.5 (NH–CO–NH$_2$), 39.9 and 37.5 (both CH$_2$), ^{15}N NMR (DMSO-d_6, 600 MHz) δ [ppm]: −301.6 (NH–CO–NH$_2$), −349.4 (protonated NH$_2$-CH$_2$–CH$_2$).

*4.1.3.9.3. Coupling of acid **21** with TFA (2-ureido)-ethylamine to **23** using HATU method.* To the solution of acid **21** (1.48 g, 2.0 mmol) in 15 DMF (15 mL), HATU (779.5 mg, 2.05 mmol) and DIPEA (0.87 mL, 5 mmol) were added at 0–5 °C. This acylating mixture was stirred for 30 min at 0–5 °C, and added to the solution of trifluoroacetate 2-(ureido)-ethylamine (1.08 g, 5 mmol) in 5 mL DMF neutralized with DIPEA (0.87 mL, 5 mmol). After overnight stirring DMF was evaporated under vacuo to the oil, which was triturated with TBME (40 mL). After few hours of cooling, TBME was decanted. Oily product was dissolved in water (50 mL), filtered and evaporated under reduced pressure to give crude **23** (white solid) which was used for further synthesis without purification, TLC: R_f (II) 0.81.

*4.1.3.9.4. Coupling of acid **21** with TFA (2-ureido)-ethylamine to **23** using DCC/HOBt method.* Acid **21** (170 mg, 0.23 mmol), TFA NH$_2$–CH$_2$–CH$_2$–NH–CO–NH$_2$ (49.9 mg, 0.23 mmol) and HOBt·H$_2$O (35.2 mg, 0.23 mmol) were dissolved in 5 mL of DMF, and after cooling to 0–5 °C DIPEA (39 μL, 0.23 mmol) and DCC (52.12 mg, 25.3 mmol) in DCM (3 mL) were added. The reaction mixture was stirred for 1 h at 0 °C and overnight in RT. DCM was evaporated and crude **23** was precipitated by addition of water, filtered dried under reduced pressure, and used for further synthesis without purification.

*4.1.3.10. {[Boc–Tyr(OBu^t)–D–Lys(&^{1})–Phe–Dab(&^{2})–NH–CH$_2$CH$_2$–NH–Boc][&^{1}CO&^{2}]}, **24**.* To the solution of acid **21** (98.7 mg, 0.134 mmol) in 2 mL DMF, Boc–NH–CH$_2$–CH$_2$–NH$_2$ (21 mg, 0.134 mmol) and HOBt (18.1 mg (0.134 mmol) were added. After the mixture was stirred at 0 °C, 0.023 mL of DIPEA and 27.6 mg (0.134 mmol) DCC in 2 mL DCM were added. The reaction mixture was stirred at 0 °C for 30 min and in RT for 4 days. The precipitated DCU was filtered off and washed with DCM. The filtrate and washing were combined and DCM was evaporated. To the residue 10% aq. AcOH (25 mL) was added and the mixture was kept in the refrigerator for few hours, than filtered, washed with water and dried under reduced pressure over P_2O_5 to yield crude **24** (118 mg). TLC: R_f (IX) = 0.15, which was used for further synthesis without purification.

*4.1.3.11. Boc–Tyr(OBu^t)–D–Lys(Z)–Phe–Dab(Z)–OH, **26**.* Ester **18** (4.98 g, 5.0 mmol) was dissolved in the mixture of MeOH (40 mL) and DMF (16 mL), and hydrolysis was performed by addition of 2 M KOH (10 mL) during 20 min at RT. After 35 min (TLC control) reaction mixture was diluted with water (80 mL), than acidified to pH 3 with 10% citric acid. After cooling in the refrigerator white precipitate was filtered, washed with water and dried under reduced pressure to give 4.71 g (96%) of **26**. TLC: R_f (X) = 0.36 MS: for $C_{53}H_{68}O_{12}N_6$ calcd. M = 981.17; found $[M + Na]^+ = 1003.48$. ^{1}H NMR (DMSO-d_6, 600 MHz) δ [ppm]: 8.35 (d, 1H, C$_\alpha$NH Dab), 8.29 (d, 1H, C$_\alpha$NH Phe), 7.85 (d, 1H, C$_\alpha$NH Dab), 7.38–7.10 (m, 19H, aromatic protons, C$_\gamma$NH Dab and C$_\varepsilon$NH Lys), 6.86–6.80 (m, 3H, 2 aromatic protons of Tyr (ortho to t-Bu-O) and C$_\alpha$NH Tyr), 5.01 and 4.99 (2 s, 4H, 2 × OCH$_2$), 4.61 (C$_\alpha$H Phe), 4.23-4.20 (C$_\alpha$H Dab and C$_\alpha$H Lys), 4.18 (C$_\alpha$H Tyr), 3.15–3.05 (m, 3H, C$_\gamma$H$_2$ Dab and one of C$_\beta$H Phe), 2.87–2.82 (m, 3H, one of C$_\beta$H Tyr and C$_\varepsilon$H$_2$), 2.69 (m, 1H, one of C$_\beta$H Phe), 2.62 (m, 1H, one of C$_\beta$H Tyr), 1.94 and 1.79 (C$_\beta$H$_2$ Dab), 1.30-1.15 (m and 2 s, 22 H, CH$_3$ of t-Bu, C$_\delta$H$_2$ Lys and C$_\beta$H$_2$ Lys), 0.82 (m, 2H, C$_\gamma$H$_2$ Lys), ^{13}C NMR (DMSO-d_6, 600 MHz) δ [ppm]: 173.2 (COOH), 171.4 (CO Phe), 171.3 (CO Tyr), 171.0 (CO Lys), 156.1 and 156.0 (PhCH$_2$OCO), 155.1 (t-BuOCO–NH), 153.3 (aromatic C(t-Bu–O–) Tyr), 137.8, 137.3, 137.2 (C$_{ipso}$ of Ph Phe and PhCH$_2$CO), 132.7 (C$_{ipso}$ Tyr), 129.7, 129.2, 128.3, 127.9, 127.7, 126.2, 123.2 (carbons of aromatic rings), 77.9 and 77.50 (2 quaternary carbons of Boc), 65.2 and 65.1 (OCH$_2$), 55.6 (C$_\alpha$ Tyr), 53.5 (C$_\alpha$ Phe), 52.1 (C$_\alpha$ Lys), 50.1 (C$_\alpha$ Dab), 40.1 (C$_\varepsilon$ Lys), 37.7 (C$_\beta$ Phe), 37.5 (C$_\gamma$ Dab), 37.2 (C$_\beta$ Tyr), 32.0 (C$_\beta$ Lys), 31.0 (C$_\beta$ Dab), 29.1 (C$_\delta$ Lys), 28.5 and 28.1 (CH$_3$ of both t-Bu), 21.8 (C$_\gamma$ Lys); ^{15}N NMR (DMSO-d_6, 600 MHz) δ [ppm]: −262.7 (NH Phe), −263.2 (NH Lys), −263.4 (C$_\alpha$NH Dab), −291.0 (NH Tyr), −296.1 (C$_\varepsilon$NH Lys), −297.7 (C$_\gamma$NH Dab).

*4.1.3.12. Boc–Tyr(OBu^t)–D–Lys(Z)–Phe–Dab(Z)–NH–CH$_2$–CH$_2$–NH–CO–NH$_2$, **27**.* Coupling of **26** with TFA·NH$_2$–CH$_2$–CH$_2$–NH–CO–NH$_2$ to **27** was performed by DCC/HOBt method similarly as described in Section 4.1.3.9. Crude **27** was purified by flash chromatography on silica gel column using DCM/MeOH as a solvent system (15:0.5, 15:1 and 15:1.5, v/v). The respective fractions were collected, evaporated and dried under reduced pressure to give **27** as a white solid. TLC: R_f (IV) = 0.59; MS: for $C_{55}H_{75}O_{12}N_9$ calcd. M = 1066.25; found $[M + H]^+ = 1066.69$ $[M + Na]^+ = 1088.62$.

NMR data suggests that even purified sample of **27** contains an impurity. Analysis of the ^{13}C NMR spectrum indicates the presence of eight C'$_\alpha$ carbons (60–50 ppm) and two quaternary urea carbons (*ca.* 158 ppm), what confirms an existence of two isomeric derivatives. First one is consistent with the proposed structure **27**, whereas second one is probably the result of coupling of **26** with H$_2$N–CH$_2$–CH$_2$–NH–CO–NH$_2$ through urea amino group.

*4.1.3.13. Boc–Tyr(OBu^t)–D–Lys–Phe–Dab–NH–CH$_2$–CH$_2$–NH–CO–NH$_2$, **28**.* Removal of Z groups from **27** by catalytic hydrogenation over Pd/C was performed in MeOH with few drops of AcOH substantially in the same manner as for reduction of **18** to **19**. From 670 mg of **28** after 2 h reduction 520 mg of **24** was obtained. MS: for

$C_{40}H_{63}O_8N_9$ calcd. M = 797.98; found $[M + H]^+$ = 798.50, $[M + Na]^+$ = 820.50.

*4.1.3.14. {[Boc–Tyr(OBu^t)–D–Lys(&^{1})–Phe–Dab(&^{2})–NH–CH$_2$–CH$_2$–NH–CO–NH$_2$] [&^{1}CO&^{2}]}, **23**.* The cyclization of **28** to **23** using *bis*(p-nitrophenyl)carbonate was performed in DMF by a procedure similar to that described for the preparation **20** from **19**. The crude product was purified by preparative TLC using for elution solvent system (II).

*4.1.3.15. Crude ureidopeptides **1**, **9** and **10**.* Crude protected ureidopeptide **23** or **22** or **24** (0.3–0.4 mmol) was treated with TFA (10–15 mL) and stirred at RT for 55 min, than TFA was removed under reduced pressure. MeOH was added (30 mL) and evaporated three times. The resulted trifluoroacetates of **1** or **9** or **10** were dried over KOH under reduced pressure, dissolved in water or water/AcOH and lyophilized.

Final purification of **1** included two-steps procedure: flash chromatography and semipreparative RP-HPLC whereas crude **9** and **10** were immediately applied on the semipreparative column.

4.2. Enzymatic degradation study

4.2.1. Hydrolysis of the peptides in the presence of chymotrypsin

Peptides were dissolved in 10 mM ammonium bicarbonate buffer (pH 8) to the final concentrations 10^{-4} M. The hydrolysis was initiated by addition of chymotrypsin (final concentration 50 μg/mL) at room temperature. Aliquots of the reaction solution were collected at intervals, and the reaction was quenched by changing the pH by addition of the equal volume of 10% formic acid. The solutions were diluted 20 times with water:acetonitrile (1:1) containing 0.1% formic acid and the samples were analyzed directly on the ESI-MS spectrometer.

4.2.2. Hydrolysis of the peptides in the presence of pepsin

Peptides were dissolved in 2% aqueous formic acid (pH 2) to the final concentrations 10–4 M and pepsin was added at room temperature (final concentration 50 μg/mL). Aliquots of the reaction solution were collected at intervals, and the reaction mixture was diluted 20 times with water:acetonitrile (1:1) containing 0.1% formic acid. The diluted samples were analyzed directly on the ESI-MS spectrometer.

4.2.3. ESI-MS analysis

The products of the enzymatic hydrolysis were studied on Bruker apex ultra 7T FT-ICR instrument (Bruker Daltonic) equipped with an ESI source using standard instrument settings. Positive ions were analyzed. The reaction mixture was infused to the ion source at flow rate 2 μl/min.

4.3. Biological activity

4.3.1. General

Experiments were performed on male BALB/c mice weighing 20–25 g, purchased from Center of Experimental Medicine (Medical University of Bialystok, Poland). Animals were housed in cages on a standard 12:12 h light/dark cycle. Water and food were available *ad libitum* until mice were transported to the laboratory approximately 1 h before experiments. All behavioural testing was performed between 9:00 am and 4:00 pm and the animals were used only once. Animal care and handling procedures were in accordance with the guidelines of the International Association for the Study of Pain (IASP) on the use of animals in pain research and the protocol was approved by the IV Local *Ethics Committee for Animal* Experimentation in Warsaw (decision No. 01/2010).

4.3.2. Hot-plate test

After habituation to the apparatus (Hot-Plate Analgesia Meter, IITC, Life Sci.,USA), individual mice were placed onto hot surface held at the constant temperature of 56 ± 0.1 °C. The latency to start licking the hind paws or the first jump as the nociceptive reaction to thermal stimulus was recorded before (baseline) and 30 and 60 min after drugs or vehicle (aqua pro injection) administration. Cut-off time was set on 30 s to avoid tissue damage. In particular groups of mice (n = 8) cyclopeptides (**1**–**10**) were given 30 min before testing in the 0.1 and 1 mg/kg dose by ip, sc and iv route of administration in the volume of 10 mL/kg (ip, sc) or 5 mL/kg (iv). Antinociceptive effect of studied compound was compared with that of MF (Morphini sulfas WZF, WZF Polfa S.A.) in the 1 mg/kg dose. Statistical evaluation of the results was performed using analysis of variance and Newman-Keul's or U Mann–Whitney's test where appropriate for post hoc comparisons. The level $P < 0.05$ was considered significant.

4.3.3. Tail-flick test

Analgesic effect of **1** in the TF test was determined using modified D'Amour and Smith method [29]. Pain was induced by radiant heat produced by focused light beam (IITC Life Science, USA) applied onto the mouse tail 2 cm away from the tip and the latency to flicking the tail was recorded. Animals were randomly assigned to ten tested groups (n = 10). Before drug assessment two baseline measurements were taken for each mouse at 15 min interval. Compound **1**, MF or vehicle were injected iv (into the tail vein) in the volume of 5 mL/kg and TF latency was recorded at 15, 30, 60, 90,120 and 180 min after the drugs administration. **1** or MF were given in the 0.5, 1, 2, and 4 mg/kg dose. Results were expressed as the percent of maximal possible effect (%MPE) according to the formula: %MPE = (test latency − baseline latency)/(cut off − baseline latency) × 100, where test latency denotes latency after drug or vehicle administration. Cut off value was adjusted as two and a half times the mean baseline latency for the group.

The data were reported as mean ± SEM. For statistical evaluation of the results analysis of variance (ANOVA) followed by Newman-Keul's test for *post hoc* comparisons was applied. Two-group comparisons were carried out using Student's *t*-test for independent samples. *P*-value level less than 0.05 was considered significant. ED50 dose with 95% confidence interval for 1 and MF was calculated by the method of Litchfield and Wilcoxon [30].

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Appendix A. Supplementary material

Supplementary material related to this article can be found at http://dx.doi.org/10.1016/j.ejmech.2013.02.019.

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