Polish J. Chem., **76**, 519–523 (2002) **ORGANIC CHEMISTRY**

New Analogues of *Cucurbita maxima* Trypsin Inhibitor III (CMTI-III) Substituted with D-Arg or D-Lys in Position 5 (P_1)*

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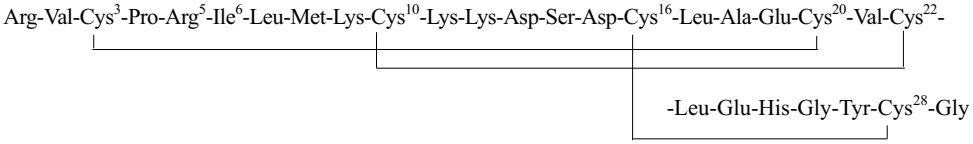
Two new analogues of trypsin inhibitor CMTI-III substituted with D-Arg or D-Lys in position 5 (P_1) were synthesized by the solid-phase method. The first analogue ([D-Arg^5] CMTI-III) displayed association equilibrium constants (K_a) with bovine β-trypsin by about three orders of magnitude lower than did wild CMTI-III. The second analogue ([D-Lys^5] CMTI-III) displayed K_a by about four orders of magnitude lower than [Lys^5] CMTI-III. The configuration of basic amino acid residue (Arg or Lys) in the reactive site (position P_1) of CMTI-III and its analogues played an important role for the stabilization of the inhibitors active structure.

Key words: peptidic trypsin inhibitors, solid-phase peptide synthesis

In the seeds of plants from the squash family (*Cucurbitaceae*), polypeptide serine proteinase inhibitors occur, molecules of which consist of 27–30 amino acid residues, each including six L-cysteine residues forming three disulfide bridges [1,2]. The representative of this inhibitors group is CMTI-III (*Cucurbita maxima* trypsin inhibitor III).



This inhibitor (and also other members of this family) interacts with the enzyme according to the canonical mechanism [3]. The binding loop of CMTI-III molecule is a segment P_3–P_3' of the peptide chain (Cys^3 – Met^8) with the reactive site peptide bond P_1–P_1' (Arg^5 – Ile^6) [4] exposed to the solvent. During the interaction with trypsin, this peptide bond is very slowly cleaved. The main force of inhibitor–trypsin or trypsin-like enzymes interaction comes from the ionic interactions network formed

*Abbreviations: The symbols of amino acids, peptides and their derivatives are in accordance with the 1983 Recommendations of the IUPAC-IUB Joint Commission on Biochemical Nomenclature [*Eur. J. Biochem.*, **138**, 9 (1984)] and European Peptide Society [*J. Peptide Sci.*, **5**, 465 (1999)].

between guanidine or ε-amino groups of the inhibitor (in position P_1) and β-carboxyl group of enzyme Asp^{189} residue [5]. Nevertheless, the inhibitory activity [6] or substrate specificity [7] can be achieved (although being significantly lower), when position P_1 is occupied by less positive charged amino acid residues.

Taking into the consideration the above mentioned facts, we decided to replace Arg^5 (P_1 position) by its enantiomer (D-Arg) or the D-Lys residue. The presence of D-amino acid residues should protect the reactive site P_1–P_1' against proteolysis. Both residues contain positive charged side groups, but the reverse configuration might decrease their interaction with the enzyme. We hope that this could be compensated by other contacts between the enzyme and the inhibitor. Two analogues: [D-Arg^5] CMTI-III (**1**) and [D-Lys^5] CMTI-III (**2**) were synthesized by the solid-phase method. The Fmoc/Bu^t procedure was used. Association equilibrium constants (K_a) with bovine β-trypsin were determined.

EXPERIMENTAL

Starting materials: All amino acid derivatives were obtained from Bachem AG (Bubendorf, Switzerland). Tenta Gel S AC Fmoc-Gly resin with capacity 0.22 mmol/g was purchased from Rapp Polymere (Tübingen, Germany). Sephadex G-10 was obtained from Pharmacia (Uppsala, Sweden). Tris(hydroxymethyl)aminomethane (Tris) was purchased from Serva Fine Chemicals (Heidelberg, Germany). Acetic acid (AcOH), dichloromethane (DCM), diisopropylcarbodiimide (DIPCDI), diethyl ether (Et_2O), diisopropylethylamine (DIPEA), dimethylformamide (DMF), dimethylsulfoxide (DMSO), N-hydroxybenzotriazole (HOBt), methanol (MeOH), N-methylpyrrolidone (NMP), piperidine, trifluoroacetic acid (TFA) and triisopropylsilane were obtained from Flucka AG (Buchs, Switzerland). Acetonitrile HPLC-grade and bovine β-trypsin (EC3.4.21.4) were purchased from Merck AG (Darmstadt, Germany). Bz-D,L-Arg-4-nitroanilide (BAPNA), Tos-Val-Gly-Arg-4-nitroanilide, Triton X-100, oxidized (GSSG) and reduced (GSH) forms of glutathion were obtained from Sigma Chemicals Co. (St. Louis, U.S.A.).

Solid-phase peptides synthesis: 0.5 g portions of Fmoc-Gly resin were used for the automated solid-phase synthesis on an Applied Biosystem Model 430A peptide synthesizer. A standard machine programme was used. During the synthesis the following amino acids derivatives were used: Fmoc-Ala, Fmoc-Asp(OBu^t), Fmoc-D-Arg(Pbf), Fmoc-Cys(Trt), Fmoc-Gly, Fmoc-Leu, Fmoc-Ile, Fmoc-Glu(OBu^t), Fmoc-His(Boc), Fmoc-Lys(Boc), Fmoc-D-Lys(Boc), Fmoc-Ser(Bu^t), Fmoc-Tyr(Bu^t). Deblockings were performed with 20% piperidine in a mixture of DMF-NMP (1:1 v/v) with 1% Triton X-100. After each stage, the resin was washed with a mixture of DMF-DCM-NMP (1:1:1 v/v) with 1% Triton X-100. Following the completed synthesis and removal of Fmoc protection from the N-terminal α-amino group, peptidyl-resins were washed with DMF, MeOH, Et_2O and dried carefully *in vacuo*.

Cleavage from the resin and side chain protecting groups deblocking: Cleavage from 0.5 g of each of both peptidyl-resins and removal of side chain protecting groups were performed with a deoxidated mixture of 88% TFA, 5% phenol, 2% triisopropylsilane and 5% H_2O for 2 hours at room temperature [8]. Then the resins were filtered off and washed with cooled TFA. The peptides were precipitated with cooled Et_2O, filtered off, dissolved in water and lyophilized. The linear peptides were purified on a Sephadex G-10 column eluted with 5% AcOH with a flow rate 1 ml/min. The elutes were fractionated and monitored for absorbance at 254 nm. The fractions comprising the major peak were collected and lyophilized to obtain the purified, reduced form of inhibitors.

Oxidation (refolding) of peptides: The reduced, linear forms of the both peptides were dissolved in 20 mM Tris/HCl buffer (pH 8.3) containing 1 mM GSSG and 2 mM GSH, the peptides concentration were about 0.2 mg/ml. The mixtures were stirred at room temperature. After three days, the solutions

were concentrated *in vacuo* and desalted on a Sephadex G-10 column (3×100 cm) in 5% AcOH with a flow rate 1 ml/min. In the next step the fractions containing oxidized peptides were lyophilized.

Purification of the synthetic inhibitors: The HPLC purification was performed on a Beckman Model 338 chromatograph with a semipreparative reversed phase (RP) C_8 Kromasil-100 column, (10 × 250 mm, 15 μm, Knauer, Germany). Solvent system: (A) – 0.1% TFA, (B) – 80% acetonitrile in 0.1% TFA (linear gradient from 20 to 80% of B for 20 min. flow rate 3 ml/min, monitored at 226 nm). The collected fractions containing the active material were evaporated *in vacuo,* dissolved in water and lyophilized. Both inhibitors were 96% pure as determined by HPLC on an analytical RP C_8 Kromasil column (10 μm, 4.6 × 250 mm, Knauer) applying linear gradient from 20 to 80% of B for 20 min. (flow rate 1 ml/min). Retention times (R_T) and capacity factors (k′) are given in Table 1.

Table 1. Details of the synthetic steps, molecular ions, retention times of HPLC analysis and association equilibrium constants (K_a) with β-trypsin for CMTI-III and its analogues.

Analogue	Amount [mg] (yield %)[a]			RP C^8 HPLC[b]		K_a [M^{-1}]	Molecular ion MS-ES[c]	
	Reduced peptide	Oxidized peptide						
		Crude	*Purified*	R_T	k′		cal.	found
[D-Arg^5] CMTI-III	133 (37%)	52 (39%)	1.91 (3.6%)	7.96	4.56	8.1×10^8	3267.9	3268
[D-Lys^5] CMTI-III	114 (32%)	42 (37%)	1.33 (3.1%)	8.06	3.82	7.5×10^7	3239.4	3240
CMTI-III [13]	–	–	–	–	–	6.8×10^{11}	–	–
[Lys^5] CMTI-III [4]	–	–	–	–	–	5.8×10^{11}	–	–

[a]All results refer to 1 g of the peptide resin.
[b]HPLC was performed on a Beckman Gold System chromatograph with a C8 RP Kromasil – 100 column (5 μm particle size, 4.6 × 250 mm, Knauer). Solvent system: (A) 0.1% TFA, (B) 80% acetonitrile in A, linear gradient from 20 to 80 for 20 min, flow rate 1 ml/min, A_{226}.
[c]Mass spectra (MS-ES) were measured with a Finnigan MAT 95S (Finnigan, Germany) spectrometer. The instrument was equipped with an electrospray ionization source.

Determination of trypsin inhibitory activity: The trypsin inhibitory activity of both analogues was determined according to Erlanger *et al.* [9] with BAPNA as the substrate. It was assayed after 10 minutes preincubations of the enzyme with the inhibitor. Both analogues revealed the antitrypsin activity.

Determination of trypsin-inhibitor association equilibrium constant (K_a): Association equilibrium constant was measured by the method developed in the laboratory of M. Laskowski Jr. [10,11]. Bovine β-trypsin-inhibitor interaction was determined in 0.1 M Tris-HCl, 20 mM $CaCl_2$, 0.005% Triton X-100, pH 8.3 at 22° C. The measurements were carried out at an enzyme concentration of 1×10^{-10} M. The inhibitor concentration varied from 0 to 3.8×10^{-8}M for analogue **1** and from 0 to 5.7×10^{-8} M for analogue **2**. The concentration of the active form of the inhibitor was determined by titration with the enzyme using BAPNA as a substrate. The residual enzyme concentration was developed with Tos-Val-Gly-Arg-4-nitroanilide as a substrate. The initial substrate concentration never exceeded 0.1 of the value of the substrate Michaelis constant Km. The experimental points were analyzed based on the plot of the residual enzyme concentration, [E], *versus* the initial inhibitor concentration [I_0] (Fig. 1). The K_a values are given in Table 1. The details of the non linear least square procedure were described elsewhere [11,12]. The inhibition of bovine β-trypsin by the analogues obtained is shown in Fig. 1.

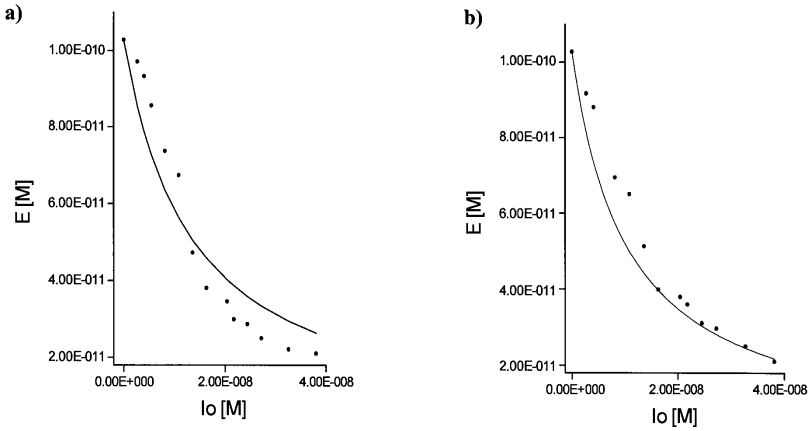


Figure 1. Inhibition of bovine β-trypsin with: a) [D-Arg^5] CMTI-III, b) [D-Lys^5] CMTI-III.

RESULTS AND DISCUSSION

Two analogues of trypsin inhibitor CMTI-III substituted with D-Arg or D-Lys in position 5 (P_1) were synthesized by the solid-phase method. Fmoc/Bu^t procedure was used for the synthesis. Products were purified on a Sephadex G10 column and semipreparative HPLC and characterized by MS-ES. Purity of the peptides was checked with RP HPLC (Table 1). The association equilibrium constants (K_a) with bovine β-trypsin were determined for both analogues (peptide **1** and **2**).

The K_a value obtained for peptide **1** with D-Arg in position P_1 is by about three orders of magnitude lower than that determined for the parent CMTI-III with L-Arg in position P_1. Likewise, K_a value for peptide **2** with D-Lys in position P_1 is by about four orders of magnitude lower than that for [Lys^5] CMTI-III.

The results obtained indicate that in this particular case the further increase of the stability of P_1–P_1' reactive site peptide bond is not an important factor for trypsin inhibitory activity. The change of the position (configuration) of the positive charged side chain, significantly reduced the association energy of such modified analogues with bovine β-trypsin. Unfortunately, other inhibitor-enzyme contacts, although important for the specificity were not able to compensate the proper interaction between guanidine or ε-amino group of the inhibitor and β-carboxyl group of the enzyme Asp^{189} residue.

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