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# Analogues of *Ecballium elaterium* Trypsin Inhibitor II (EETI-II) with L-Cysteine Residues Substituted by L-Penicillamine (Pen) and L-Homocysteine (Hcy) in Positions 19, 21 and 27*

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Two analogues of peptidic trypsin inhibitor isolated from seeds of *Ecballium elaterium* (EETI-II): [$Pen^{19,21,27}$] EETI-II (**1**) and [$Hcy^{19,21,27}$] EETI-II (**2**) were synthesized by the solid-phase method using the Fmoc/$Bu^t$ procedure. Their inhibitory activity was determined by the calculation of association equilibrium constants ($K_a$) with bovine β-trypsin. In comparison with the parent compound, both analogues showed reduced trypsin inhibitory activity more than 7 and 18 times, respectively. We postulate that the observed differences may reflect the role of disulfide bridges in the interaction of inhibitors with trypsin or the introduced modifications change the conformational equilibrium of the analogues synthesized towards conformation(s) less favorable for the interaction with the enzyme.

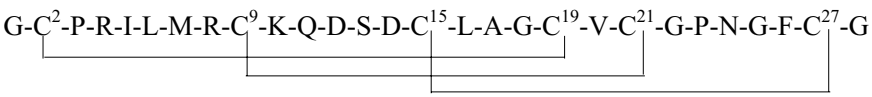
**Key words**: peptidic trypsin inhibitors, solid-phase peptide synthesis, analogues of EETI-II

In the 80-ties, a new family of peptidic serine proteinase inhibitors was established, which occurred mainly in seeds of *Cucurbitaceae.* The molecules of the inhibitors consist of 27–30 amino acid residues, each including six L-cysteine residues forming three disulfide bridges. First trypsin inhibitors belonging to this family were isolated from the seeds of squash (*Cucurbita maxima).* The inhibitors were named CMTI-I and CMTI-III (*Cucurbita maxima* trypsin inhibitors I and III) [1–3]. In the last several years, over fifty analogues of CMTI-III were synthesized in our laboratory in order to study structure-activity relationships [4–6]. The most difficult problem, which occurred during the synthesis of CMTI analogues, was the very low yield of the final products. Several attempts made in the synthetic procedures did not give satisfactory results [7].

One of the trypsin inhibitors belonging to the *Cucurbitaceae* family is *Ecballium elaterium* trypsin inhibitor II (EETI-II) isolated and synthesized by the French group in 1989 from the jumping cucumber [8,9]. In fact, EETI-II is an analogue of CMTI-III:

*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)].

[des Arg$^{1}$, Gly$^{2}$, Arg$^{9}$, Gln$^{23}$, Pro$^{24}$, Asn$^{25}$, Phe$^{27}$]CMTI-III. Its amino acid sequence is given below:




It is worth noticing, that in spite of the high sequence homology between CMTI inhibitors and EETI-II, the chemical synthesis of the latter one is much easier. After the synthesis of the linear protected EETI-II precursor, we obtained the crude product with 73% yield and over 80% purity as judged by HPLC, whereas in the case of CMTI-III a crude product contained only a few per cent of CMTI-III protected precursor. Also the oxidation of EETI-II linear precursor leads to the final product with higher yield than oxidation of CMTI-III linear precursor (see Table 1). These results motivated us to use EETI-II inhibitor as a template for designing new serine proteinase inhibitors.

In our previous work we have shown that removing of one disulfide bridge from CMTI-III molecule dramatically reduced trypsin inhibitory activity of such modified analogues [5,6]. This gave a clear indication that all three disulfide bridges are crucial for the proper folding of the inhibitor. Both X-ray [10] and NMR [11] studies revealed that these three disulfide bridges bring an important contribution to the stability of well defined three-dimensional structure of CMTI-I. Recently, we have shown that the replacement of all six Cys residues by L-penicillamine (Pen) residues yielded a CMTI-III analogue with the inhibitory activity of the same order of magnitude as the wild CMTI-III [12].

Structure-activity-relationships studies of angiotensin II performed by Zhang *et al.* [13] revealed that various disulfide bridges formed by Cys, Pen, L-homocysteine (Hcy) and 4-mercapto-cis- or –trans-prolins changed dramatically the biological activity of such modified analogues. Therefore, in this study we decided to investigate the influence on the inhibitory potency a mixed Cys-Pen and Cys-Hcy disulfide bridges introduced into EETI-II. In comparison with Cys, the presence of two methyl groups attached to the β carbon in Pen, introduced into the peptide structure additional steric hindrance, serving as additional conformational constrains. On the other hand, the additional methylene group in the side chain of Hcy expanded the length of the disulfide bridge and, as a consequence, should increase the conformational flexibility of such a modified EETI-II analogue. Both analogues [Pen$^{19,21,27}$]EETI-II (**1**) and [Hcy$^{19,21,27}$]EETI-II (**2**) were synthesized by the solid-phase method using the Fmoc/Bu$^{t}$ procedure.

## EXPERIMENTAL

Starting materials, the solid–phase peptide synthesis, cleavage from the resin and side chain protecting groups deblocking, air oxidation (refolding) of peptides, purification of the synthetic inhibitors**,** determination of trypsin inhibitory activity as well as determination of trypsin-inhibitor association equilibrium constant ($K_a$) were performed as described previously [14].

## RESULTS AND DISCUSSION

Two analogues of trypsin inhibitor EETI-II isolated from seeds of *Ecballium elaterium* [Pen$^{19,21,27}$] EETI-II (**1**) and [Hcy$^{19,21,27}$] EETI-II (**2**) were synthesized by the solid-phase method using the Fmoc/Bu$^t$ procedure. The yields of the syntheses and the purification steps, HPLS and MS analyses, as well as the values of association equilibrium constants ($K_a$) of the inhibitors synthesized with bovine β-trypsin, are summarized in Table 1. The inhibition of bovine β-trypsin by EETI-II analogues synthesized is shown in Fig. 1.

Despite high sequential homology between the CMTI-III and EETI-II inhibitors, the second one displays a 50-fold lower trypsin inhibitory activity as judged by the $K_a$ values. Nevertheless, it is still a potent inhibitor. The replacement of all three Cys residues, forming the disulfide bridges by mixed Cys-Pen or Cys-Hcy, reduces the inhibitory activity. The analogue with Hcy residues is more than 7 times and the one with Pen residues over 18 times less active than the parent EETI-II. Considering a high inhibitory activity of the CMTI-III analogue with all six Cys residues substituted by Pen, the results obtained are rather surprising. In order to verify these results, we decided to synthesize a CMTI-III analogue with mixed Cys-Pen disulfide bridges ([Pen$^{20,22,28}$]CMTI-III). It appears that this modification introduced into the CMTI-III inhibitor had the same impact on the inhibitory potency as had in EETI-II ($K_a$ = $1.2\times10^8$ M, unpublished results). Therefore, one can assume that the influence of mixed Cys-Pen (and probably Cys-Hcy) disulfide bridges on the inhibitory activity has similar impact on other members of *Cucurbitacea* family inhibitors.

Our results correspond well with similar studies carried out on a 72 amino acid residue protein, interleukin-8 (containing two disulfide bridges) [15]. Also in this case, the substitution of naturally occurring disulfides with mixed Cys-Hcy, Hcy-Cys, Cys-Pen and Pen-Cys decreased significantly the biological activity. On the other hand, the NMR studies of interleukin-8 and its analogues indicated that the introduced modification had practically the same tertiary fold. Therefore, the authors postulated that for the observed differences in both biological potency and receptor binding, the direct interaction between disulfide and the receptor are responsible. Unfortunately, among interleukin-8 analogues investigated, none of them contained all Pen-Pen or Hcy-Hcy disulfides. This would allow to make the direct comparison with our results.

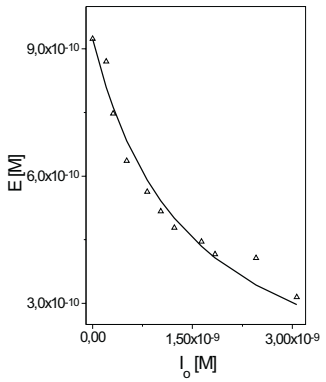
Taking into consideration the results mentioned above, two possible explanations for the observed lower inhibitory activity of the compounds studied can be postulated: as compared with the native Cys-Cys disulfides, the interactions of mixed disulfide bridges with the enzyme are less favorable, or the introduced modifications change the conformational equilibrium of the analogues synthesized towards conformation(s) less favorable for the interaction with the enzyme. At this stage it is impossible to predict which of the hypothesis is correct. To solve the problem, X-ray studies of the complexes between trypsin and these analogues are planned.

**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, EETI-II and its analogues.

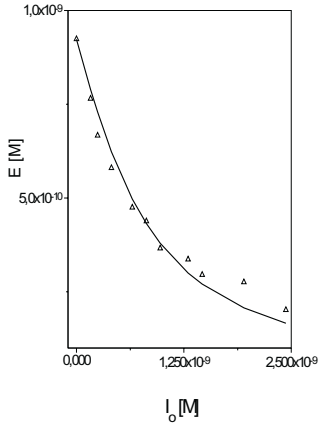
| Analogue | Amount [mg] (yield %)[a] | | | RP $C^8$ HPLC[b] | | $K_a$ [$M^{-1}$] | Molecular ion[c] | |
|---|---|---|---|---|---|---|---|---|
| | Reduced peptide | Oxidized peptide | | | | | | |
| | | *Crude* | *Purified* | $R_T$ [min] | k′ – | | cal. | found |
| [$Pen^{19,21,27}$]EETI-II (**1**) | 413 (63%) | 269 (65%) | 25.5 (9.5%) | 18.32 | 5.72 | $7.6 \times 10^8$ | 2982.6 | 2982.7 |
| [$Hcy^{19,21,27}$]EETI-II (**2**) | 433 (67%) | 307 (71%) | 10.7 (3.5%) | 26.55 | 4.12 | $1.9 \times 10^9$ | 2940.5 | 2940.5 |
| EETI-II | 465 (73%) | 386 (83%) | 23.9 (5.9%) | 20.33 | 4.38 | $1.4 \times 10^{10}$ | 2898.4 | 2898.5 |
| CMTI-III [2,4] | 54 (1.6%) | – | 1.35 (2.5%) | – | – | $6.8 \times 10^{11}$ | – | – |
| [$Pen^{3,10,16,20,22,28}$] CMTI-III [13] (**1**) | | | | | | $1.5 \times 10^{11}$ | | |

[a]All results refer to 1 g of the peptide resin.
[b]HPLC was performed on a Beckman Gold System chromatograph (Beckman, USA) with a C8 RP Kromasil – 100 column (5 μm particle size, 4.6 × 250 mm, Knauer, Germany). Solvent system: (A) 0.1% TFA, (B) 80% acetonitrile in A, linear gradient from 20 to 80 for 30 min, flow rate 1 ml/min, $A_{226}$; k′ – capacity factor.
[c]Mass spectra were measured in positive mode using Bruker Biflex III MALDI – TOF Mass Spectrometer (Bruker Daltonics, Germany).

**a)**




**b)**




**c)**

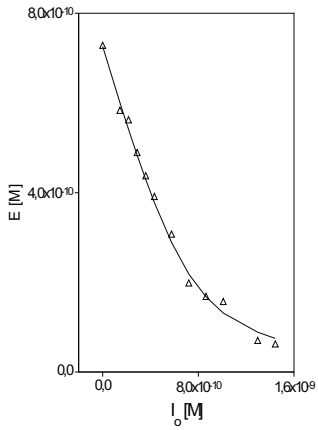



**Figure 1.** Inhibition of bovine β-trypsin with: a) [Pen$^{19,21,27}$]EETI-II (**1**), b) [Hcy$^{19,21,27}$]EETI-II (**2**), c) EETI-II; E – residual enzyme concentration, $I_0$ – initial inhibitor concentration.

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