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Synthetic modulator of chymotrypsin activity based on *p-tert*-butylthiacalix[4]arene

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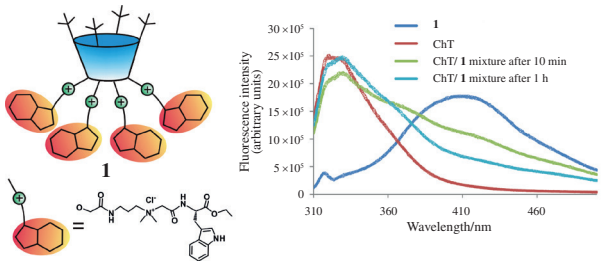
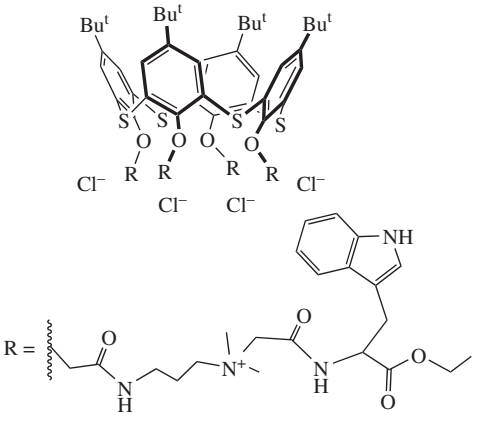
A *p-tert*-butylthiacalix[4]arene derivative containing tryptophan moieties on the lower rim was synthesized and proposed as a chymotrypsin inhibitor. Its ability to competitively inhibit the enzyme was revealed by kinetic methods. A mechanism of the inhibitory action has been established, which consists in the binding of macrocycle tryptophan moieties to the enzyme active site, resulting in its blocking.



Modulation of the catalytic activity of enzymes is widely used in various fields of human activity, such as medicine,[1–3] agriculture and food industry.[4,5] Typically, the synthetic enzyme inhibitors intended for medical applications should meet, in addition to the absence of toxicity, some definite requirements, such as low molecular weight, strictly defined hydrophobic–hydrophilic balance as well as small number of hydrogen bond donor and acceptor groups.[6,7] However, considering inhibitors for non-medical applications, the number of limitations can be reduced, the non-toxicity being the main requirement. Currently, much attention is paid to the search for modulators of enzyme activity based on polyfunctional building blocks, such as fullerenes, nanotubes[8,9] or macrocyclic compounds.[1] In particular, the polyfunctional macrocycles allow one to arrange on their backbone the numerous binding moieties, capable of interacting with diverse guests, which makes it possible to design ligands for various types of biomolecules, including enzymes.[10] Chymotrypsin (ChT, EC 3.4.21.1) is a protease catalyzing the cleavage of peptide bonds, and one of the most investigated enzymes. Its inhibitors based on calix[4]arene[11] and resorcin[4]arene[12] scaffolds are known, whereas there is no data on the similar application for derivatives of thiacalixarene as their closest structure analogue. It is known, that thiacalixarene, contrary to its calixarene progenitor, readily forms various spatial isomers,[13,14] which are widely used to recognize different substrates.[15–17] In this work, we investigated *p-tert*-butylthiacalix[4]arene derivative **1** in *cone* conformation, containing tryptophan moieties and chloride anions on the lower rim, as a ChT ligand and a modulator of its catalytic activity. Macrocycle **1** was obtained by replacing bromide anions in the compound described (see Online Supplementary Materials).[18]



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It is known, that ChT cleaves peptide bonds formed by aromatic amino acids,[19] therefore the tryptophan residues of compound **1** make it structurally similar to the specific substrates of this enzyme.

The ability of macrocycle **1** to bind ChT was studied by UV spectroscopy (see Online Supplementary Materials). Compound **1** absorbs in the UV spectral range with maxima at 220 and 280 nm, the ChT spectrum has a similar appearance with an absorption maximum at 280 nm and a shoulder at 220 nm [Figure 1(*a*)]. After mixing macrocycle **1** and ChT, there was a pronounced hypochromic effect near 220 nm in the spectrum, which increased with time. An elevation of light scattering was also observed, manifested by a rise in the baseline of the absorption spectrum, which also indicated the possible aggregation of the substances investigated [see Figure 1(*a*) and Online Supplementary Materials].

Fluorescence spectroscopy allowed us to determine the mechanism of compound **1** interaction with ChT (see Online

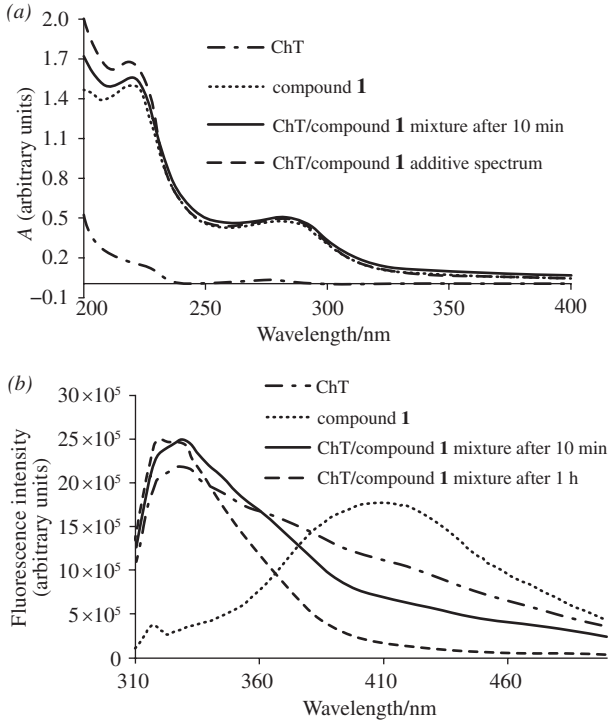

Figure 1 (*a*) Absorption and (*b*) fluorescence emission spectra of 0.5 μM ChT, 20 μM compound **1** and their mixture after 10 min and 1 h.

Supplementary Materials). In general, macrocycle **1** has the same prominent spectral features as its bromine-containing precursor,[18] namely, the maximum fluorescence emission due to tryptophan moieties at 415 nm and a large Stokes shift reaching 135 nm. This differs considerably from the ChT fluorescence with the maximum emission of tryptophan moieties at 320 nm[20] and makes it possible to track changes in the state of tryptophan parts of both compounds, depending on the nearest environment, polarity of the medium and other factors. Thus, the emission spectra were recorded for ChT and compound **1** as well as for their mixture after incubation for 10 min and 1 h [Figure 1(*b*)].

The fluorescence spectra demonstrated a decrease in the intensity of the emission band of compound **1** at 415 nm over time, with simultaneous broadening of the emission band of ChT. The prominent emission of macrocycle **1** near 415 nm was not observed after incubation of the mixture for one day. It may be concluded, that the tryptophan groups of compound **1** were bound to the active site of the enzyme, with the following breaking of their peptide bonds, resulting in a decrease in the intensity of the emission band at 415 nm. This corresponds to the known data noted above on the ChT-mediated cleavage of peptide, amide and ester bonds formed by residues of aromatic amino acids,[19] as well as the pronounced dependence of the wavelength of tryptophan maximal emission on its environment.[20] It was previously shown that a large Stokes shift for the bromide analogue of compound **1** originated from the formation of excimers as a result of the close proximity of tryptophan moieties.[18] Thus, it is reasonable to assume that macrocycle **1** functions as a substrate for ChT. The enzyme cleaves the amide bonds formed by tryptophan moieties in the macrocycle, which results in the destruction of the excimers, disappearance of the corresponding emission band at 415 nm and the increase in tryptophan fluorescence at 320 nm.

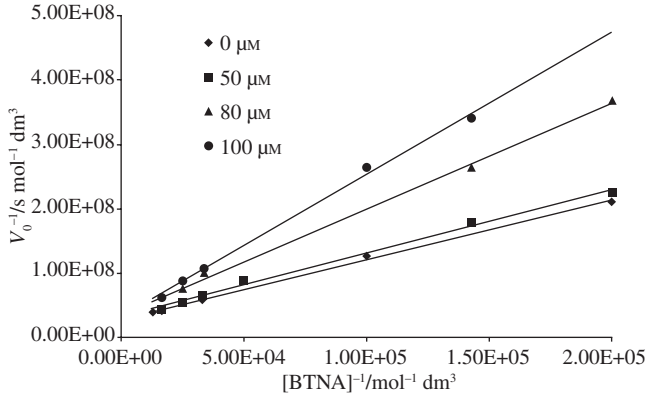

Figure 2 Lineweaver–Burk plot of BTNA hydrolysis by ChT (1 μM) with 0, 50, 80 and 100 μM macrocycle **1**.

Dynamic light scattering (DLS) confirmed the binding of macrocycle **1** to ChT (see Online Supplementary Materials). For a 20 μM solution of compound **1** in 5 mM Tris–HCl buffer at pH 7.8, we detected formation of monodispersed self-associated complexes with a diameter of 123.3 nm and polydispersity index (PDI) of 0.160. After the addition of 0.5 μM ChT, much larger aggregates with a diameter of 1032 nm were observed after 5 min, with simultaneous increase in the PDI value to 0.261. After 0.5 and 1 h, further coarsening of the particles was observed with their diameters up to 1151 and 1216 nm, as well as with PDI values of 0.254 and 0.272, respectively.

The investigation of ChT enzymatic activity in the presence of compound **1** (see Online Supplementary Materials) demonstrated a decrease in the hydrolysis rate for *N*-benzoyl-L-tyrosine *p*-nitroanilide (BTNA) as a substrate, as compared with the absence of macrocycle **1** (Figure 2). As well, there was a positive correlation between enzyme activity and compound **1** concentration in the reaction medium.

To make clear the molecular mechanism of the observed change in the enzyme activity, the kinetic parameters of BTNA enzymatic hydrolysis were determined according to the Michaelis–Menten model[21] (see Online Supplementary Materials and Table 1).

As follows from the data in Table 1, an elevation of K_M is observed with an increase in the compound **1** concentration. This change of K_M at a constant V_{max} value allowed us to make a conclusion that the presence of macrocycle **1** resulted in a decrease of ChT affinity to the substrate by the competitive type of inhibition. The inhibition constant K_i was determined to be 7.5 ± 0.6 μM by the Dixon method (see Online Supplementary Materials). We assume that compound **1** prevents the formation of an enzyme–substrate complex through the competition with the BTNA substrate for a place at the ChT active site and thus blocks the enzyme activity.

In summary, a quaternary ammonium salt based on *p-tert*-butylthiacalixarene, containing chloride anions and tryptophan moieties on the lower rim, has been obtained. Its ability to inhibit chymotrypsin has been demonstrated.

Table 1 Kinetic parameters of BTNA hydrolysis by ChT in the presence of compound **1**.[a]

Compound **1** concentration/μM	K_M/mol dm^{-3}	k_{cat}/K_M /dm^3 mol^{-1} s^{-1}
0	3.06×10^{-5}	1078
50	3.30×10^{-5}	1000
80	5.49×10^{-5}	601
100	7.32×10^{-5}	451

[a] K_M is the Michaelis constant; k_{cat}/K_M is efficiency of catalysis; ChT concentration is 1 μm; the maximum reaction rate $V_{max} = 3.33\times10^{-8}$ mol dm^{-3} s^{-1} and the catalytic constant $k_{cat} = 0.033$ s^{-1} for the whole range of compound **1** concentrations.

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Online Supplementary Materials

Supplementary data associated with this article can be found in the online version at doi: 10.1016/j.mencom.2019.09.014.

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