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

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1. Materials and methods

Bull pancreatic chymotrypsin (ChT, EC 3.4.21.1, Sigma–Aldrich), *N*-benzoyl-L-tyrosine *p*-nitroanilide (BTNA, Sigma–Aldrich), Tris–HCl buffer (Sigma–Aldrich) and Amberlite IRA-400 Cl^- form (Sigma–Aldrich) were used.

^{1}H NMR spectra were recorded using a Bruker Avance-400 spectrometer at 400 MHz, and ^{13}C spectra were obtained on a Bruker Avance-400 pulse mode spectrometer at 100 MHz. Chemical shifts were determined relative to the signals of DMSO-d_6 residual protons, the concentration of sample solutions was 3–5 %. Elemental analysis was performed using a Perkin Elmer 2400 Series II instrument. ESI mass spectra were recorded on an AmaZonX mass spectrometer (Bruker Daltonik GmbH, Germany) with nitrogen as a drying gas at 300 °C and the capillary voltage 4.5 kV. The samples were dissolved in acetonitrile at ~10^{-6} gml^{-1}. Melting points were determined using a Boetius Block apparatus.

Electronic absorption spectra were recorded on a Shimadzu UV-3600 spectrophotometer in 5 mM Tris–HCl buffer containing 2% methanol using quartz cuvettes with an optical path length 10 mm. Solutions of compound **1** (20 μM) and ChT (0.5 μM), as well as their mixtures, were used immediately after preparation, the spectra of the mixtures were recorded additionally after 5, 10, 15, and 20 min. The experiment was carried out at 25 °C.

Fluorescence spectra were recorded using a Fluorolog 3 fluorescence spectrometer (Horiba Jobin Yvon). The excitation wavelength was chosen as 285 nm, the emission wavelength scanning range was 310–510 nm and spectral slit was 4 nm. Quartz cells with an optical path length 10 mm were used. For the compensation of self-absorption, the frontal recording mode was chosen. The emission spectra were corrected automatically by the 'Fluorescence' program. The solutions of ChT (0.5 μM), macrocycle **1** (20 μM) and their mixtures were prepared. Then, the spectra of the mixtures were recorded immediately after mixing as well as after 10 min, 1 h and 1 day. The experiment was carried out at 25 °C in 5 mM Tris–HCl buffer containing 2% methanol.

Particle size was determined on a Zetasizer Nano ZS nanoparticle size analyzer (Malvern) in polystyrene cuvettes at 25 °C in 5 mM Tris–HCl buffer containing 2% methanol at concentrations of compound **1** 20 μM and ChT 0.5 μM at 5, 30 and 60 min after mixing. For the preparation of solutions, deionized water with a resistance of >18.0 MΩ cm was obtained using a Millipore-Q purification system.

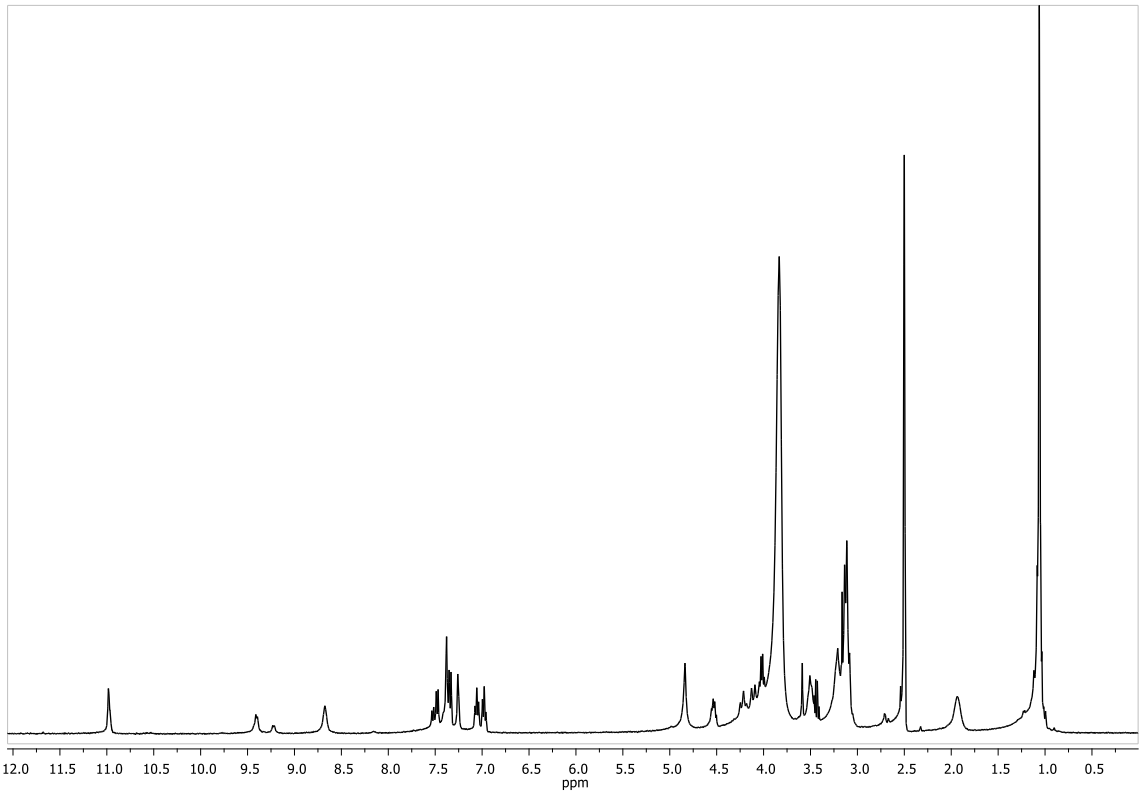
Kinetic measurements. ChT activity was measured in the hydrolysis reaction of N-benzoyl-L-tyrosine *p*-nitroanilide (BTNA) as a specific substrate. The enzyme was dissolved in 5 mM Tris–HCl buffer pH 7.8. The pH value is selected in accordance with the maximum activity of the enzyme. The substrate was dissolved in DMSO, the macrocycle was dissolved separately in DMSO and adjusted to the required concentration with a buffer, so that the concentration of DMSO in the matrix solution was 4%. The measurements were performed at 25 °C. Kinetic curves were recorded on a Shimadzu UV-3600 spectrophotometer. The enzyme solution containing compound **1** was incubated at a given temperature for 5 min. The reaction was initiated by the introduction of the substrate. The concentration of ChT in the reaction mixture was constant (1 μM). Measurements were performed in a buffer containing 2% DMSO. Reaction mixtures were prepared with seven fixed concentrations of the BTNA substrate, namely 5, 7, 10, 20, 30, 40 and 60 μM. Compound **1** concentrations were 0, 50, 80 and 100 μM. The rate of substrate hydrolysis was monitored by the change in optical absorption at 390 nm. The initial reaction rate v_0 was determined by the slope of the linear part of the product accumulation curve *vs*. time for 1 min after the start of the reaction and was calculated by the formula $v_0 = \Delta D/(\Delta\varepsilon \times l \times \Delta t)$.

2. Synthesis and spectral data of compound 1

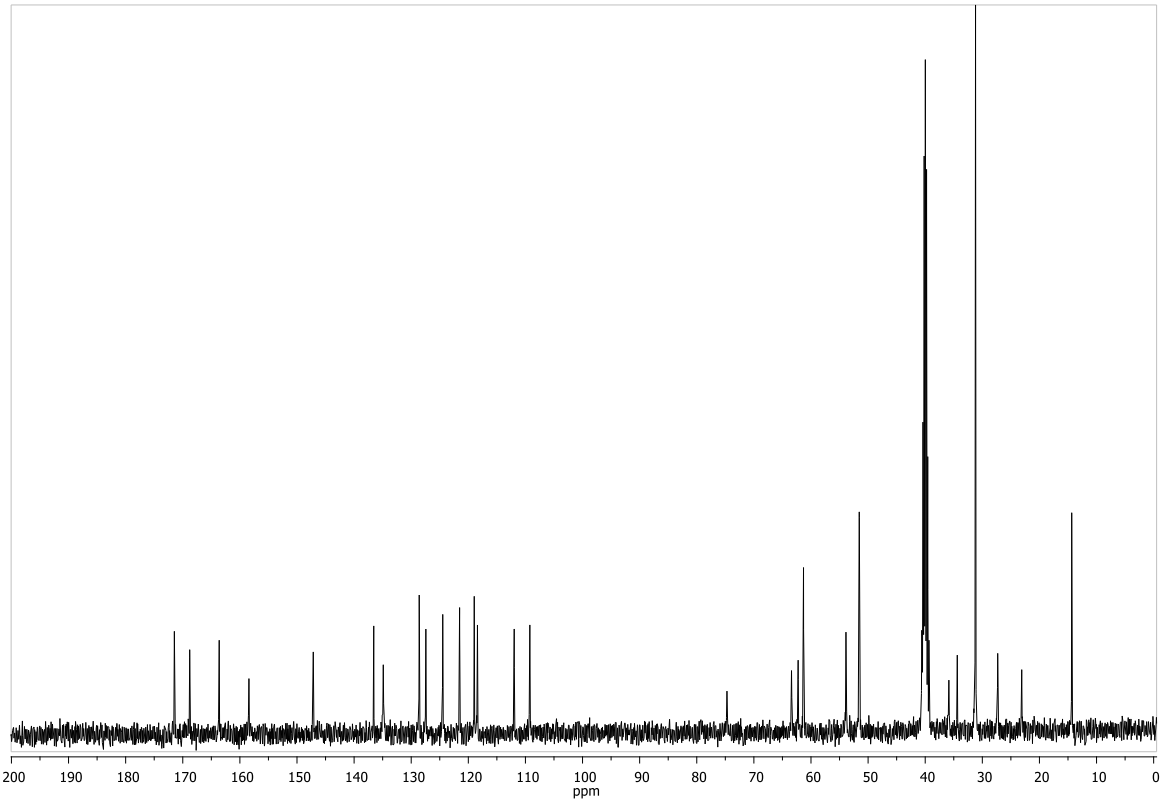
5,11,17,23-Tetra-*tert*-butyl-25,26,27,28-tetrakis{*N*-[3'-(dimethyl{[(*S*)-ethoxycarbonyl(1''*H*-indol-3''-ylmethyl)methyl]aminocarbonylmethyl}ammonio)propyl]aminocarbonylmethoxy}-2,8,14,20-tetrathiacalix[4]arene tetrachloride (*cone*) 1.

The corresponding bromide (0.10 g)[1] was dissolved in distilled water (5 ml). The solution was passed through the ion-exchange column filled with Amberlite IRA-400 Cl^- (2 g). The column was washed with additional distilled water (20 ml). The resulting solution was evaporated *in vacuo* to afford a crude product, which was dried over phosphorus pentoxide. Yield 0.093 g (99%). Mp: 130 °C. ^{1}H NMR (DMSO-d_6) δ: 1.06 (s, 36H, *Me*$_3$C), 1.11 (t, 12H, *Me*CH$_2$O, $^3J_{HH}$ 7.1 Hz), 1.94 (m, 8H, NHCH$_2$C*H*$_2$CH$_2$N$^+$), 3.09 (s, 24H, *Me*$_2$N$^+$), 3.12 (m, 8H, CHC*H*2–Trp), 3.22 (m, 8H, NHCH$_2$CH$_2$C*H*$_2$N$^+$), 3.50 (m, 8H, NHC*H*$_2$CH$_2$CH$_2$N$^+$), 4.02 (q, 8H, MeC*H*$_2$O, $^3J_{HH}$

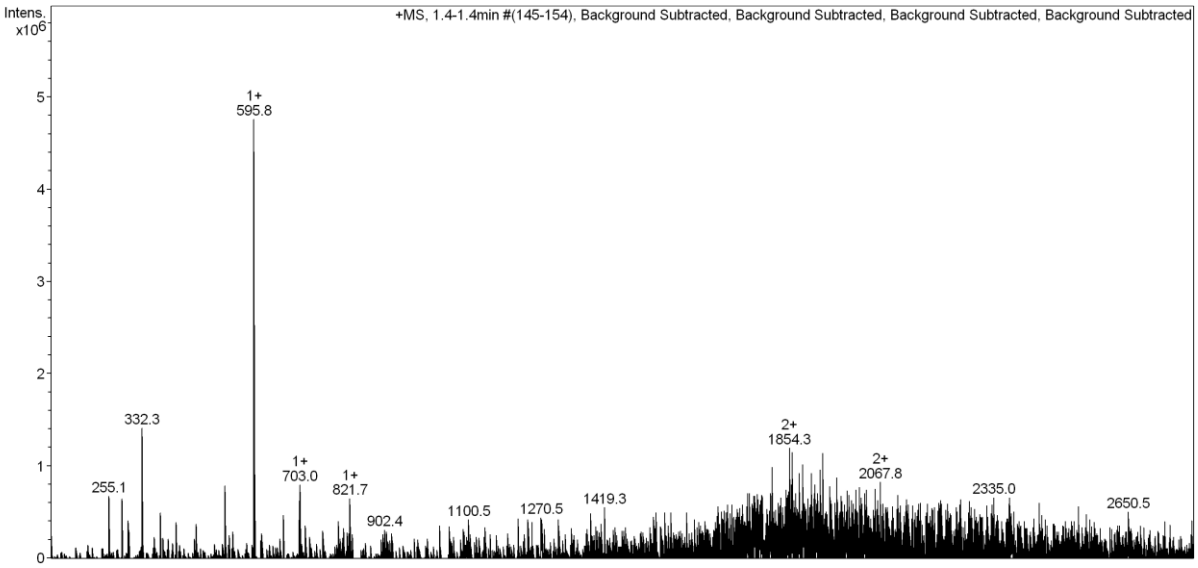
7.1 Hz), 4.13 (m, 8H, N^+CH_2CO), 4.54 (m, 4H, NHC*H*CO), 4.84 (s, 8H, OCH_2CO), 6.96–7.55 (m, 20H, ArH^{Trp}), 7.38 (s, 8H, Ar*H*), 8.57, (br.t, 4H, N*H*$CH_2CH_2CH_2N^+$), 9.41 (br.s., 4H, CON*H*CH), 10.98 (s, 4H, NH^{Trp}). ^{13}C NMR (DMSO-d_6) δ: 171.45, 168.77, 163.60, 158.39, 147.16, 136.55, 134.86, 128.63, 127.45, 124.48, 121.54, 118.98, 118.41, 111.96, 109.22, 74.72, 63.43, 62.25, 61.32, 53.91, 51.55, 35.86, 34.35, 31.12, 27.32, 23.10, 14.35. MS (ESI), *m*/*z*: 595.8 $[M - 4Cl^-]^{4+}$ (calc. for $[M]^+$, *m*/*z*: 595.8). Found (%) :C, 60.77; H, 6.95; Cl, 5.63; N, 8.71; S, 4.79. Calc. for $C_{128}H_{172}Cl_4N_{16}O_{20}S_4$ (%): C, 60.89; H, 6.87; Cl, 5.62; N, 8.88; S, 5.08.



^{1}H NMR spectrum of compound 1 (DMSO-d_6, 298 K, 400 MHz).



^{13}C NMR spectrum of compound 1 (DMSO-d_6, 298 K, 100 MHz).



ESI mass spectrum of compound 1.

3. Kinetics of BTNA hydrolysis by chymotrypsin

In the case of chymotrypsin, the mechanism of the classical enzymatic reaction represents a three-stage process with the corresponding rate constants for each stage: the formation of the enzyme-substrate complex k_1, the decomposition of the complex k_{-1}, the acylation process of the active center of the enzyme k_2 and its deacylation k_3:

$$E + S \underset{k_{-1}}{\overset{k_1}{\Leftrightarrow}} ES \xrightarrow{k_2} EP + P_1 \xrightarrow{k_3} E + P_2$$

where E is the enzyme, S is the substrate, ES is the enzyme–substrate complex and P_1, P_2 are the reaction products.

The Michaelis–Menten equation was used to calculate the kinetic parameters of the reaction:

$$v_0 = \frac{V_{\max}[S]_0}{K_M + [S]_0}$$

where v_0 is the initial reaction rate, $[S]_0$ is the initial substrate concentration, $V_{\max}$ is the maximum reaction rate and K_M is the Michaelis constant.

$$K_M = \frac{k_{-1} + k_2}{k_1}$$

$$K_S = \frac{k_{-1}}{k_1}$$

Most often, the maximum speed is not used, but the so-called catalytic constant k_{cat} is calculated, which, unlike V_{max}, does not depend on the concentration of the enzyme:

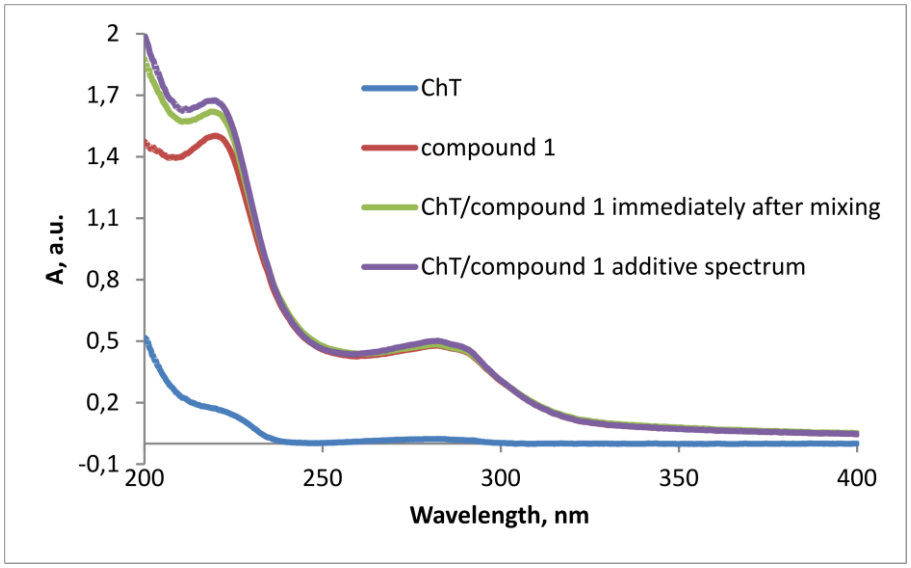
$$k_{cat} = \frac{V_{\max}}{[E]_0}$$

The efficiency of catalysis is calculated by the formula:

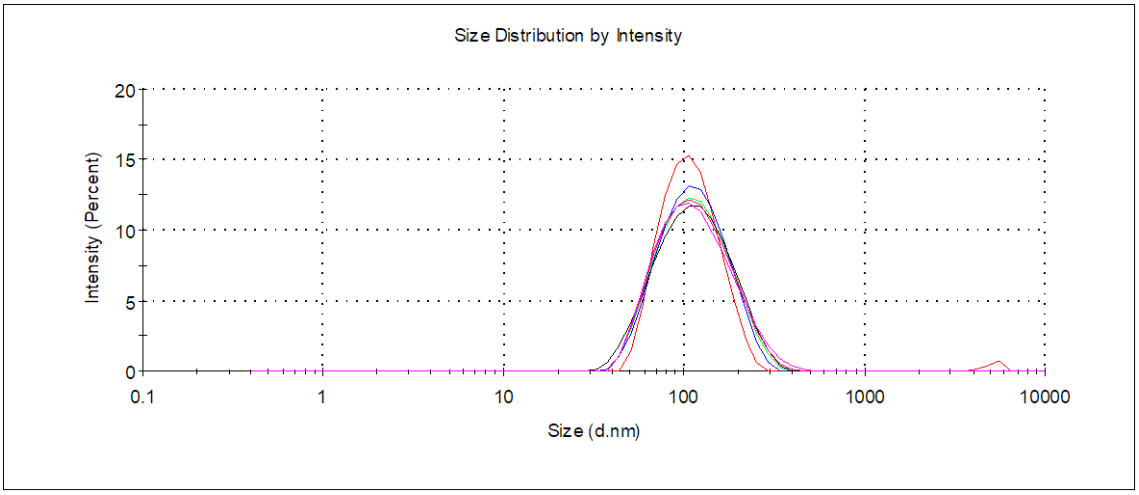
$$s = \frac{k_{cat}}{K_M}$$

The inhibition constant K_i at competitive inhibition was calculated by the Dixon method, according to which experimental data are deposited in the coordinates ($1/V$, $[I]$ as concentration of the inhibitor).[2] The constant K_i is then determined at the intersection point for the straight lines constructed at different concentrations of the substrate (BTNA).

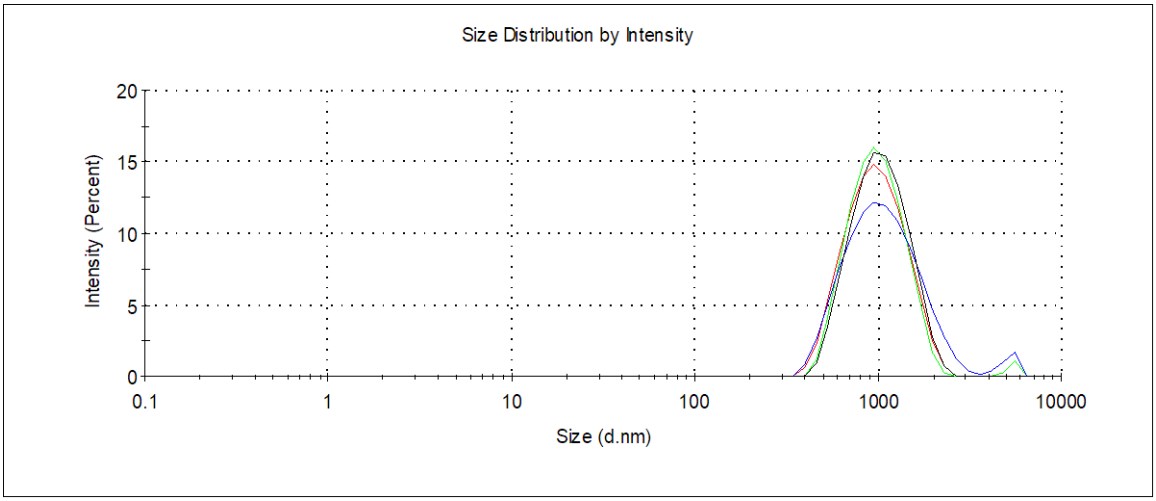
4. Spectral characteristics



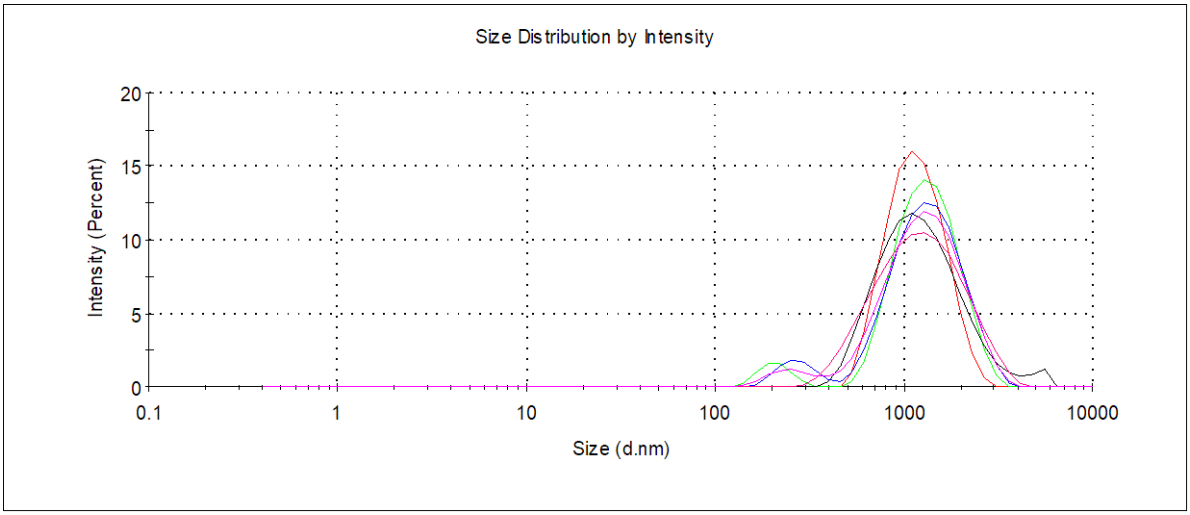
Absorption spectra of ChT (0.5 μM), compound 1 (20 μM) and their mixture immediately after mixing.



The size distribution of particles by intensity in the solution of compound 1 (20 μM) in 5 mM Tris–HCl buffer pH 7.8.



The size distribution of particles by intensity in a solution of compound 1 (20 μM) in the presence of 0.5 μM ChT in 5 mM Tris–HCl buffer pH 7.8 after 5 min.



The size distribution of particles by intensity in a solution of compound 1 (20 μM) in the presence of 0.5 μM ChT in 5 mM Tris–HCl buffer pH 7.8 after 60 min.

References

1. P. L. Padnya, I. A. Khripunova, O. A. Mostovaya, T. A. Mukhametzyanov, V. G. Evtugyn, V. V. Vorobev, Yu. N. Osin and I. I. Stoikov, *Beilstein J. Nanotechnol.*, 2017, **8**, 1825.

2. I. V. Berezin and K. Martinek, *Osnovy fizicheskoy khimii fermentativnogo kataliza (Fundamentals of the Physical Chemistry of an Enzymatic Catalysis)*, Vysshaya Shkola, Moscow, 1977 (in Russian).