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V. M. Buldenko[1], L. A. Kononets[1], O. L. Kobzar[1], A. B. Drapailo[2], S. G. Vyshnevsky[2], V. I. Kalchenko[2], A. I. Vovk[1]

[1]Institute of Bioorganic Chemistry and Petrochemistry of the National Academy of Sciences of Ukraine
1, Murmanska str., 02094, Kyiv-94, Ukraine. E-mail: vovk@bpci.kiev.ua
[2]Institute of Organic Chemistry of the National Academy of Sciences of Ukraine

The inhibitory potential of calixarenes against nucleotide pyrophosphatase/phosphodiesterase 1

It has been previously shown that phosphonic acids covalently attached to the macrocyclic platform of calix[4]arenes are capable of inhibiting alkaline phosphatases. In this paper the effects of the upper-rim functionalized calix[4]arenes on the activity of nucleotide pyrophosphatase/phosphodiesterase 1 (NPP1) have been examined.

Aim. To assess the inhibitory potential of calix[4]arene, thiacalix[4]arene and sulfonylcalix[4]arene derivatives against NPP1.

Results and discussion. It has been found that calix[4]arene, thiacalix[4]arene, and sulfonylcalix[4]arene tetrakismethylphosphonic acids inhibit NPP1 with the IC_{50} values in the micromolar range. The derivatives of sulfonylcalix[4]arene demonstrated the selectivity of inhibition of NPP1 over alkaline phosphatases. In addition, sulfonylcalix[4]arene tetrakismethylphosphonic acid was able to inhibit the nucleotide pyrophosphatase/phosphodiesterase activity of the human serum. The possible mechanism of the inhibition has been discussed.

Experimental part. The activity of NPP1 was monitored by spectrophotometry measuring the rate of hydrolysis of bis-*p*-nitrophenyl phosphate. The phosphodiesterase activity of the human serum was assessed in the presence of *p*-nitrophenyl ester of thymidine-5-monophosphate as a substrate. The homology model of the human NPP1 was generated based on the crystal structure of the murine enzyme. The molecular docking was performed using AutoDock 4.2.

Conclusions. The results obtained have shown the ability of sulfonylcalix[4]arene derivatives to inhibit the activity of NPP1 *in vitro,* including the nucleotide pyrophosphatase/phosphodiesterase activity in the human blood serum.

Key words: calix[4]arene; thiacalix[4]arene; sulfonylcalix[4]arene; nucleotide pyrophosphatase/phosphodiesterase 1; inhibition; molecular docking

В. М. Булденко, Л. А. Кононець, О. Л. Кобзар, А. Б. Драпайло, С. Г. Вишневський, В. І. Кальченко, А. І. Вовк

Інгібіторний потенціал каліксаренів відносно нуклеотидопірофосфатази/фосфодіестерази 1

Раніше було показано, що фосфонові кислоти, ковалентно приєднані до макроциклічної платформи калікс[4]аренів, здатні інгібувати лужні фосфатази. В цій роботі вивчено вплив похідних калікс[4]арену на активність нуклеотидопірофосфатази /фосфодіестерази 1 (NPP1).

Мета роботи – оцінити інгібіторний потенціал похідних калікс[4]арену, тіакалікс[4]арену та сульфонілкалікс[4]арену відносно NPP1.

Результати та їх обговорення. Встановлено, що калікс[4]арен-, тіакалікс[4]арен- та сульфонілкалікс[4]арен-тетракіс-метилфосфонові кислоти інгібують NPP1 зі значеннями IC_{50} в мікромолярному діапазоні. Інгібування NPP1 похідними сульфонілкалікс[4]арену було селективним відносно лужних фосфатаз. Крім того, сульфонілкалікс[4]арен-тетракіс-метилфосфонова кислота здатна інгібувати нуклеотидопірофосфатазну/фосфодіестеразну активність людської сироватки. Обговорюється можливий механізм інгібування.

Експерименальна частина. Активність NPP1 контролювали спектрофотометрично за швидкістю гідролізу біс-*п*-нітрофенілфосфату. Нуклеотидопірофосфатазну/фосфодіестеразну активність людської сироватки оцінювали з використанням *п*-нітрофенілового естеру тимідин-5-монофосфату як субстрату. Гомологічна модель людської NPP1 була згенерована на основі кристалічної структури мишачого ферменту. Молекулярний докінг проводили за допомогою програми AutoDock 4.2.

Висновки. Отримані результати показали здатність похідних сульфонілкалікс[4]арену інгібувати активність NPP1 *in vitro*, в тому числі нуклеотидопірофосфатазну/фосфодіестеразну активність сироватки крові людини.

Ключові слова: калікс[4]арен; тіакалікс[4]арен; сульфонілкалікс[4]арен; нуклеотидопірофосфатаза/фосфодіестераза 1; інгібування; молекулярний докінг

В. Н. Булденко, Л. А. Кононец, А. Л. Кобзарь, А. Б. Драпайло, С. Г. Вишневский, В. И. Кальченко, А. И. Вовк

Ингибиторный потенциал каликсаренов относительно нуклеотидпирофосфатазы/фосфодиэстеразы 1

Ранее было показано, что фосфоновые кислоты, ковалентно присоединенные к макроциклической платформе каликс[4]аренов, способны ингибировать щелочные фосфатазы. В настоящей работе исследовано влияние производных каликс[4]арена на активность нуклеотидпирофосфатазы /фосфодиэстеразы 1 (NPP1).

Цель работы – оценить ингибиторный потенциал производных каликс[4]арена, тиакаликс[4]арена и сульфонилкаликс[4] арена относительно NPP1.

Результаты и их обсуждение. Установлено, что каликс[4]арен-, тиакаликс[4]арен- и сульфонилкаликс[4]арен-тетракис-метилфосфоновые кислоты ингибируют NPP1 со значениями IC_{50} в микромолярном диапазоне. Ингибирование NPP1 производными сульфонилкаликс[4]арена было селективным относительно щелочных фосфатаз. Кроме того, сульфонилкаликс[4]арен-тетракис-метилфосфоновая кислота способна ингибировать фосфодиэстеразную активность человеческой сыворотки. Обсуждается возможный механизм ингибирования.

Экспериментальная часть. Активность NPP1 контролировали спектрофотометрически по скорости гидролиза бис-*п*-нитрофенилфосфата. Нуклеотидпирофосфатазную/фосфодиэстеразную активность человеческой сыворотки оценивали в присутствии *п*-нитрофенилового эфира тимидин-5-монофосфата в качестве субстрата. Гомологическая модель человеческой NPP1 была сгенерирована на основе кристаллической структуры мышиного фермента. Молекулярный докинг проводился с помощью программы AutoDock 4.2.

Выводы. Полученные результаты показали способность производных сульфонилкаликс[4]арена ингибировать *in vitro* NPP1, в том числе нуклеотидпирофосфатазную/фосфодиэстеразную активность сыворотки крови человека.

***Ключевые слова*:** каликс[4]арен; тиакаликс[4]арен; сульфонилкаликс[4]арен; нуклеотидпирофосфатаза/фосфодиэстераза 1; ингибирование; молекулярный докинг

Nucleotide pyrophosphatase/phosphodiesterase 1 (NPP1, EC 3.1.4.1) represents a group of enzymes, which catalyze the hydrolysis of phosphodiester bonds of various substrates, including nucleotides [1]. NPP1 is a membrane bound glycoprotein presented in many tissues and involved in the regulation of cell differentiation, bone and tissue mineralization, and cellular signaling [2]. The abberant activity of NPP1 can result in chrondrocalcinosis, pathological calcification, osteoarthritis and other diseases [3]. In this connection, NPP1 is considered to be a possible pharmacological target for novel therapeutics.

Several natural and synthetic compounds are described as inhibitors of NPP1 [1, 4]. As an example, potent non-nucleotide inhibitors of NPP1 were represented by polyoxometalates [5], polysaccharides [6], glycosides [7], as well heterocyclic compounds [8, 9].

Taking into account the fact that NPP1 belongs to the alkaline phosphatase superfamily, and there is certain similarity in the structure of active sites of these enzymes [10], we assumed that the strategy of phosphatase inhibition by calix[4]arenes [11-13] might be used for searching for inhibitors of NPP1. The present study was undertaken in order to assess the inhibitory potential of calix[4]arenes, thiacalix[4]arenes, and sulfonylcalix[4]arenes **1-6** against NPP1 (Fig. 1).

For assessment of functionalized calix[4]arenes as inhibitors of NPP1 and alkaline phosphatases, bis-*p*-nitrophenyl phosphate and *p*-nitrophenyl phosphate were used as substrates, respectively. As is seen from Tab. 1, calix[4]arene and thiacalix[4]arene tetrakismethylphosphonic acids **1** and **2** showed approximately the same inhibition effects on the activities of NPP1 and alkaline phosphatase from the bovine intestinal mucosa with the micromolar values of IC_{50}. The inhibition effects of these compounds on alkaline phosphatase from the human placenta were not significant. At the same time, methylphosphonic acid

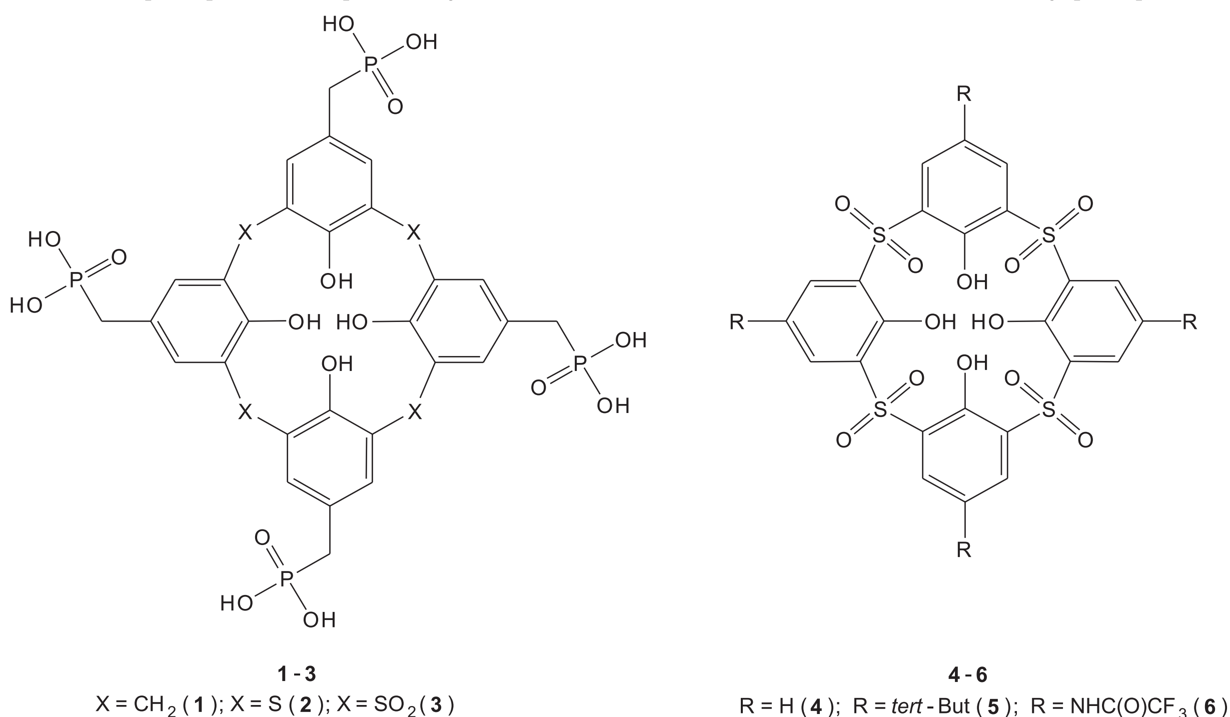

Fig. 1. Structures of calix[4]arene, thiacalix[4]arene, and sulfonylcalix[4]arene derivatives **1-6**

Table 1

Calix[4]arene, thiacalix[4]arene, and sulfonylcalix[4]arene phosphonic derivatives **1**-**6** as inhibitors NPP1 and alkaline phosphatases*

Inhibitor	NPP1			Alkaline phosphatase from the bovine mucosa	Alkaline phosphatase from the human placenta
	IC_{50}, μM	K_i, μM	$K_i^{´}$, μM	IC_{50}, μM	IC_{50}, μM
1	0.37 ± 0.06	0.34 ± 0.03	0.63 ± 0.23	0.43 ± 0.04	> 100
2	0.36 ± 0.08	0.21 ± 0.07	0.30 ± 0.06	0.27 ± 0.02	> 100
3	0.44 ± 0.08	0.39 ± 0.12	–	n.a**	n.a
4	2.3 ± 0.03			> 50	n.a
5	8.4 ± 1.8			n.a	n.a
6	0.2 ± 0.07			n.a	n.a

Notes: * – IC_{50} values are the means of 2-3 assays ± standard deviations; ** – not active at 20 μM.

derivative **3** bearing the sulfonylcalix[4]arene skeleton (Fig. 1) demonstrated the inhibitory activity only against NPP1 and did not affect the activity of alkaline phosphatases from the bovine mucosa and from the human placenta. The results obtained indicates that selectivity of sulfonylcalix[4]arene derivative **3** as an inhibitor of NPP1 can be attributed to the modified macrocyclic scaffold.

According to Lineweaver–Burk plots the effect of inhibitors **1** and **2** on the activity of NPP1 are in agreement with a mixed-type inhibition. The apparent K_i and K_i values are of 0.34 μM and 0.63 μM (compound **1**) and 0.21 μM and 0.30 μM (compound **2**), respectively. The mechanism of NPP1 inhibition by methylphosphonic acids **1** and **2** involves the binding of the inhibitor to the enzyme with formation of enzyme-inhibitor and enzyme-substrate-inhibitor complexes. On the contrary, inhibition of NPP1 by sulfonylcalix[4]arene tetrakismethylphosphonic acids **3** was found to be of competitive type with the K_i value of 0.39 μM (Fig. 2, C). This type of inhibition suggests that the sulfonylcalix[4]arene inhibitor binds to the enzyme only in the substrate binding site.

Our further efforts for designing inhibitors of NPP1 were directed towards sulfonylcalix[4]arene **4** and compounds **5** and **6** with non-ionogenic substituents (Fig. 1). Among them, tetrakis-*tert*-butyl sulfonylcalix[4]arene **5** showed the lowest inhibition effect, while tetrakis-3-fluormethylacetamide sulfonylcalix[4]arene **6** had the IC_{50} value of 0.2 μM with selectivity over alkaline phosphatases from the bovine mucosa and from the human placenta (Tab. 2).

The nucleotide pyrophosphatases/phosphodiesterase activity of the human serum was assessed in the presence of sulfonylcalix[4]arene **1**-**3** using thymidine-5-monophosphate-*p*-nitrophenyl ester as a substrate [14]. To the best our knowledge, sulfonylcalix[4]arene tetrakismethylphosphonic acid **3** exhibited the inhibitory effect on the NPP1 activity of the human serum preventing the hydrolysis of the

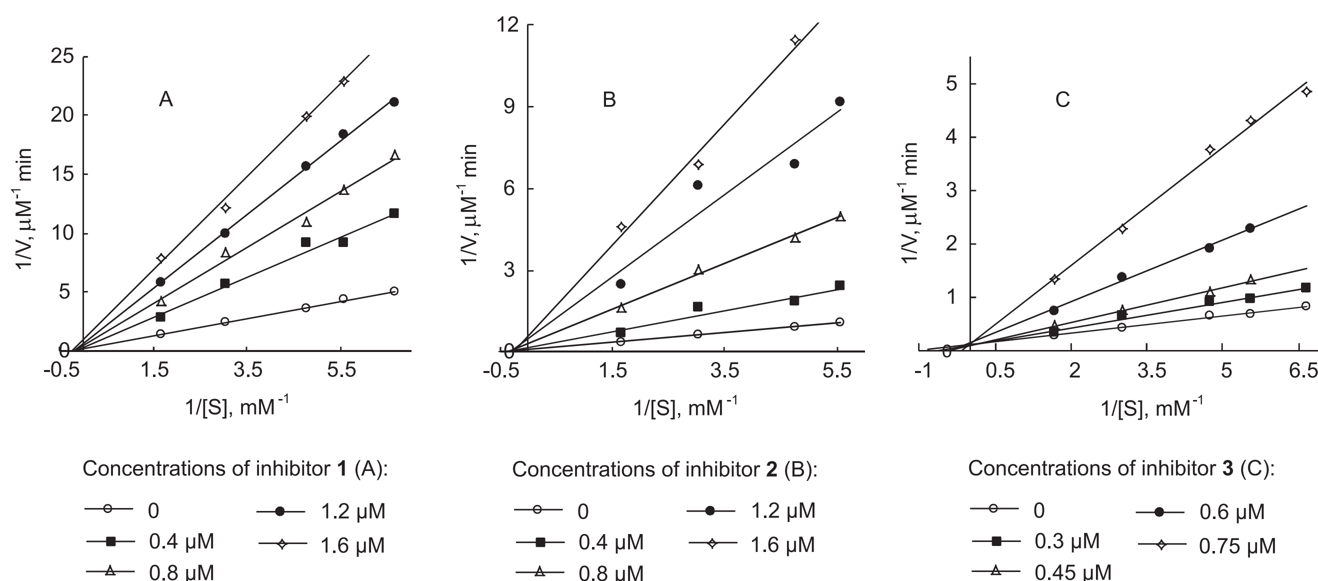

Fig. 2. Lineweaver-Burk plots for inhibition of snake venom NPP1

Table 2

Free energy of binding of the conformers of calix[4] arene derivatives **1-3** into the active site of the homology model of the human NPP1

Conformer	ΔG (kcal/mol)		
	1	**2**	**3**
cone	-6.53	-7.25	-9.73
partial cone	-6.66	-9.86	-10.27
1,2-alternate	-6.18	-8.9	-8.71
1,3-alternate	-8.38	-8.48	-10.29

substrate. At the same time, compounds **1**, **2**, **4-6** did not show sufficient effects (Fig. 3).

To get insights into a possible binding mechanism of compounds **1-3** the molecular docking studies were performed using the homology model of the human NPP1 based on the X-ray structure of its murine ortholog. Four conformers [15] for compounds **1-3** were docked to the homology model of the human NPP1 using Autodock 4.2. The docking results suggest that the most preferable for binding are 1,3-alternate of calix[4]arene derivative **1**, the partial cone of thiacalix[4]arene derivative **2**, and both partial cone and 1,3-alternate of sulphonylcalix[4]arene derivative **3** (Tab. 2).

Further examination of the docking results showed that compounds **1-3** were oriented towards two zinc ions of the enzyme. The differences in the inhibitory activity (Fig. 3) might be explained by formation of hydrogen bonds between SO_2-bridged groups of the sulfonylcalix[4]arene platform of compound **3** and amino acid residues at the catalytic center of NPP1 (Fig. 4). One of SO_2-groups of sulfonylcalix[4]arene formed hydrogen bonds with amino acid residues of His535, His380, Thr256, Asn277 located near metal ions. Other SO_2-groups had H-bonds with Ser377, Lys291, and Tyr451. The OH-group on the lower rim of the macrocycle provides the H-bond with Lys528. The phosphonic acid groups interact with His535, Gln519, Leu290, Lys291, Lys291, and Ser377. The oxygen of the phosphonate group of the inhibitor and two aromatic rings of the sulfonylcalix[4]arene platform were involved in the interaction with His535, Tyr340, and Lys528, respectively.

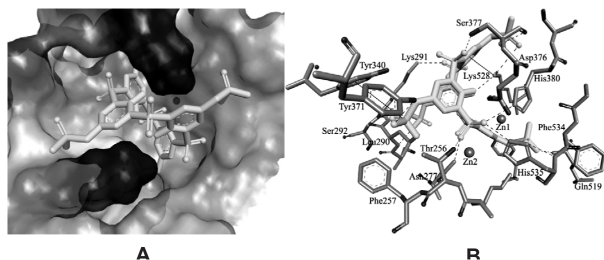

Fig.4. The posible binding mode of compound **3** at the active site of the homology model of human NPP1: general view (A) and amino acids involved in the complex formation (B)

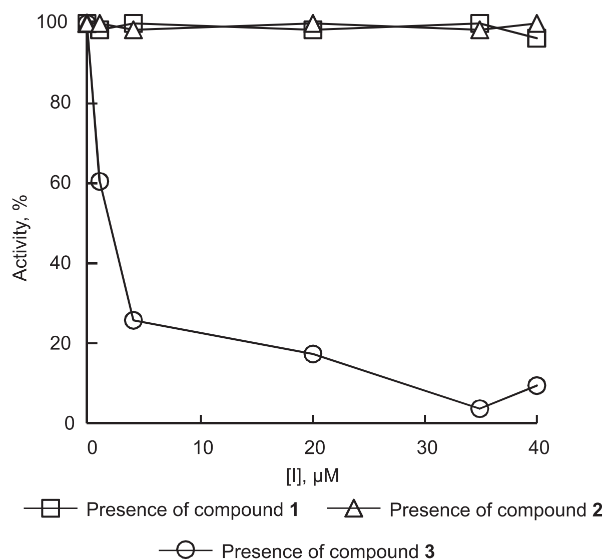

Fig. 3. The pyrophosphatase/phosphodiesterase activity of the human serum

Experimental part

In vitro study of NPP1 inhibition

Phosphodiesterase 1 from *Bothrops atrox* (type V), alkaline phosphatase from the bovine intestinal mucosa (type VII-L), alkaline phosphatase from the human placenta (type XXIV), human sera, bis-*p*-nitrophenyl phosphate, *p*-nitrophenyl phosphate, thymidine-5-monophosphate-*p*-nitrophenyl ester were purchased from Sigma-Aldrich.

The effect of calix[4]arene derivatives on the rate of hydrolysis of bis-*p*-nitrophenylphosphate catalyzed by NPP1 was assayed in 0.05 M tris-HCl buffer (pH 8.8) with 1 vol % of dimethyl sulfoxide. The mixture was incubated for 5 min at 25 °C in a buffer solution with the inhibitor. The reaction was initiated by addition of the enzyme. The total volume of the reaction mixture was 1.5 ml. The rate of hydrolysis of bis-*p*-nitrophenyl phosphate was measured by following the change in absorption of *p*-nitrophenol at 410 nm (the molar excitation coefficient of 18300 M^{-1} cm^{-1}). The value of IC_{50} was calculated as the concentration of the inhibitor, which reduced the rate of the enzymatic reaction by 50 %.

For the study of the alkaline phosphatase activity, the reaction mixture contained 0.1 M Tris–HCl buffer (pH 9), 0.5 mM *p*-nitrophenylphosphate as a substrate, and the inhibitor had been preliminarily thermostated for 5 min at 25 °C. The inhibitors **1-3** were preliminary dissolved in dimethyl sulfoxide (2 vol % in the reaction mixture).

The nucleotide pyrophosphatases/phosphodiesterase activity of the human serum was assayed in 0.05 M buffer solution (pH 9) containing 1 vol % of dimethyl sulfoxide, 0.2 mM thymidine-5-monophosphate-*p*-nitrophenyl ester, 4.5 mM $MgCl_2$. The mixture with the inhibitor was incubated for 5 min at 37 °C, and the reaction was initiated by addition of the human serum.

Molecular docking

The docking calculations were carried by AutoDock 4.2 [16]. For the preliminary preparation of docking files, the program AutoDockTools was used. Three dimensional structures of the inhibitors were optimized in the MMFF94s force field by the program Avogadro [17]. The homology model of the human NPP1 enzyme was generated based on the crystal structure of the mouse NPP1 (PDB code 4GTW) by the Swiss-Model server [18-20]. The sequence of the human NPP1 was retrieved from accession number P22413 of the UniProtKB server. The energy minimization of the model was performed by SPD viewer.

Synthesis of calixarenes

Calix[4]arene, thiacalix[4]arene, and sulfonylcalix [4]arene methylphosphonic acids **1-3** were synthesized according to the protocols previously developed [21-23]. For the synthesis of sulfonylcalix[4]arene **4** and its tetrakis-*tert*-butyl derivative **5** the procedures previously developed were used [24, 25]. Compound **6** was obtained by the oxidation reaction of thiacalix[4]arene tetrakis-3-fluormethylacetamide with $NaBO_3 \times 4H_2O$ in the presence of absolute trifluoroacetic acid.

The synthesis of 5,11,17,23-tetrakis(trifluoromethylacetamide)-25,26,27,28-tetrahydroxysulfonylcalix[4]arene 6. To the solution of 0.5 g (0.53 mM) of 5,11,17,23-tetrakis(trifluoromethylacetamide)-25,26,27,28-tetrahydroxythiacalix[4]arene in absolute trifluoroacetic acid (5 ml) add 1.0 g (6.5 mM) $NaBO_3 \times 4H_2O$. Stir the reaction mixture at 50 °C for 24 h, and then pour the reaction mass into a cooled solution of 5 N H_2SO_4 (20 ml). Filter the precipitate and wash with water (2 × 10 ml). Dry the resulting 5,11,17,23-tetrakis(trifluoromethylacetamide)-25,26,27,28-tetrahydroxysulfonylcalix[4]arene **6** (the crystalline substance of a white color) in vacuo (0.10 mm Hg) at 60 °C for 2 h.

The yield – 0.42 g (74 %). M. p. > 300 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 8.45 (s, 8H, H-arom.), 11.54 (s, 4H, NH); ^{19}F NMR (400 MHz, DMSO-d_6): δ -74.54; ^{13}C NMR (400 MHz, DMSO-d_6): 111.39; 114.26; 117.13; 119.99 (m, CF_3, $^{1}J_{FC}$ 288 Hz); 126.80; 127.07; 130.16 (C-arom); 154.79 (m, <u>C</u>OCF$_3$, $^{2}J_{CC}$ 37 Hz). Anal. calcd for $C_{32}H_{16}F_{12}N_4O_{16}S_4$, %: C, 35.96; H, 1.51; F, 21.33; N, 5.24; S, 12.00. Found, %: C, 36.16; H, 1.71; F, 21.05; N, 5.04; S, 11.85.

For the synthesis of 5,11,17,23-tetrakis(trifluoromethylacetamide)-25,26,27,28-tetrahydroxythiacalix[4]arene add 2.38 g (11.35 mmol) of trifluoromethylacetic anhydride and 1.2 g (11.86 mmol) of triethylamine to the suspension of 0.9 g (1.29 mM) of chlorhydrate aminothiacalix[4]arene [26] in absolute tetrahydrofuran (20 ml) at the room temperature with stirring. Stir the reaction mixture at room temperature for 24 h, filter the resulting precipitate, and evaporate the solution on a rotary evaporator. To the residue obtained add 10 ml of water, and filter the resulting precipitate. Dry the resulting 5,11,17,23-tetrakis (trifluoromethylacetamide)-25,26,27,28-tetrahydroxythiacalix[4]arene (the crystalline substance of a white color) in vacuo (0.05 mm Hg) at 60 °C for 2 h. The yield – 0.75 g (62.5 %). M. p. > 300 °C (decomp.). ^{1}H NMR (400 MHz, DMSO-d_6): δ 7.85 (s, 8H, H-arom.), 11.09 (s, 4H, NH); ^{19}F NMR (400 MHz, DMSO-d_6): δ 74.36; ^{13}C NMR (400 MHz, DMSO-d_6): 111.49; 114.35; 117.23; 120.09 (m, CF_3, $^{1}J_{FC}$ 288 Hz); 120.94; 127.89; 128.85 (C-arom.); 154.05 (m, <u>C</u>OCF$_3$, $^{2}J_{CC}$ 37 Hz). Anal. calcd for $C_{32}H_{16}F_{12}N_4O_8S_4$, %: C, 40.86; H, 1.71; F, 24.24; N, 5.96; S, 13.63. Found, %: C, 41.12; H, 1.83; F, 24.07; N, 6.15; S, 13.45.

Conclusions

1. Calix[4]arene and thiacalix[4]arene derivatives are found to be effective inhibitors of NPP1 with the micromolar IC_{50} values.

2. The results obtained have shown the ability of functionalized sulfonylcalix[4]arenes to selectively inhibit the activity of NPP1 *in vitro,* including the nucleotide pyrophosphatase/phosphodiesterase activity in the human blood serum.

Conflict of Interests: authors have no coflict of interests to declare.

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