VILNIUS UNIVERSITY
CPST INSTITUTE OF CHEMISTRY

Edita Čapkauskaitė

SYNTHESIS OF CARBONIC ANHYDRASE INHIBITORS AND ANALYSIS OF THEIR STRUCTURE - ACTIVITY RELATIONSHIP

Summary of doctoral dissertation
Physical sciences, chemistry (03P)

Vilnius 2012

The work was carried out in a period of 2008-2012 at Vilnius University

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Thesis defence will take place at 1 p.m. on December 7, 2012, at the Auditorium of Inorganic Chemistry of the Faculty of Chemistry of Vilnius University.
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The summary of the thesis was distributed on November 7, 2012.
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VILNIAUS UNIVERSITETAS
FTMC CHEMIJOS INSTITUTAS

Edita Čapkauskaitė

KARBOANHIDRAZIŲ SLOPIKLIŲ SINTEZĖ IR JŲ STRUKTŪROS – AKTYVUMO TYRIMAS

Daktaro disertacijos santrauka
Fiziniai mokslai, chemija (03P)

Vilnius 2012

Disertacija parengta 2008 – 2012 m. Vilniaus universitete

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Disertacija bus ginama viešame chemijos mokslo krypties tarybos posėdyje 2012 m. gruodžio 7 d. 13 val. Vilniaus universiteto Chemijos fakulteto Neorganinės chemijos auditorijoje.

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Disertacijos santrauka išsiųsta 2012 m. lapkričio 7 d.
Disertaciją galima peržiūrėti Vilniaus universiteto ir Chemijos instituto bibliotekose

Abbreviations

Ac – acetyl;
AZA – acetazolamide;
Bn – benzyl;
Bu – butyl;
t-Bu – *tert*-butyl;
CA – carbonic anhydrase (EC 4.2.1.1);
DMSO – dimethylsulfoxide;
Et – ethyl;
EZA – ethoxzolamide;
IND – indapamide;
i-Pr – izopropyl;
i-Bu – izobutyl;
Me – methyl;
MTZ – methazolamide;
PDB – Protein Data Bank (http://www.rcsb.org);
Ph – phenyl;
Pr – propyl;
THF – tetrahydrofuran;
TPR – topiramate.

Introduction

Carbonic anhydrases (CAs, EC 4.2.1.1) are ubiquitous zinc-containing metalloenzymes present in prokaryotes and eukaryotes that catalyze the reversible hydration of carbon dioxide to bicarbonate. There are 15 different isozymes of human carbonic anhydrases (CA), with different tissue distributions, subcellular localizations, and expression levels. CAs are involved in numerous important biological processes related to respiration, pH balance, CO_2 homeostasis, electrolyte secretion in a variety of tissues/organs, gluconeogenesis, lipogenesis, ureagenesis, bone resorption, calcification, tumorigenicity, and many other physiological or pathological processes.

Many of these isozymes have shown promise as druggable targets-with CAs now implicated in areas where human therapies are much needed, for example, in cancer-specific (CA IX, XII) and obesity-specific (CA V) pathways and in brain function (CA XIV). CA II and XII are the targets for the development of antiglaucoma drugs. Diffuse localization of CA isoforms in many tissues/organs limits potential pharmacological applications. Presently clinically used CA inhibitors, when acting non-specifically, cause a number of side-effects. Especially toxic are systemic inhibitors. They cause electrolyte disbalance, drowsiness, headache, depression, apathy, malaise, irritability, nervousness, fatigue, gut irritability, anorexia, nausea, thirst, obstruction, muscle weakness, tremor, hyper- and hypoglycaemia, kidney pain, disuria, bone marrow depression, metabolic acidosis and other.

Hence, the design of isozyme-specific inhibitors is the current challenge in the development of new therapeutic agents.

In view of this, **the aim of this work** was formulated - the synthesis of potent human carbonic anhydrase inhibitors, and their structure - activity study.

In order to achieve this aim it was decided:

- to synthesize benzenesulfonamides containing various heterocyclic/aromatic moieties. Modifications of inhibitors by the variation of benzenesulfonamide (“head”) and heterocyclic/aromatic (“tail”) fragments with the introduction of various substituents at these fragments were also important.

- to study CA-inhibitory activity of the synthesized compounds, to investigate cocrystal structures of selected compounds with CA using X-ray crystallography and analyse the structure - activity relationship .

Scientific novelty of the dissertation

Simple and effective procedures for *N*-and *S*-alkylation of heterocyclic compounds (benzimidazoles), *S*-alkylation of heterocyclic and aromatic compounds (imidazole, benzothiazole, benzimidazothiadiazole, pyrimidine, phenylalkylthiol) with bromoacetylbenzenesulfonamides were developed. It was found that [(6-oxopyrimidine-2-yl)sulfanyl]acetylbenzenesulfonamides exist in an open and cyclic forms in solution. For the first time, the influence of benzenesulfonamide and hetero/aromatic fragments on CA I, II, VII, XII and XIII inhibition was examined and found that the influence of benzenesulfonamide moietiy is higher than that of heterocyclic/aromatic part of molecules. It was found that some of the synthesized compounds have better or similar CA-inhibiting properties and the selectivity to some CAs than currently used drugs - acetazolamide, ethoxzolamide, methazolamide, topiramate, and indapamide. On the basis of X-ray analysis of CA II, XII and XIII complexes with several inhibitors, the reduced affinity to CA XII compared with CA II, and decreased 5-(substituted)-2-chlorobenzenesulfonamide affinity to CA I was explained.

Main statements of the dissertation

- Effective *N*-and *S*-alkylation methods of benzimidazole, imidazole, benzothiazole, benzimidazothiadiazole, pyrimidine, phenylalkylthiol derivatives with bromoacetylbenzenesulfonamides were developed.
- 4-Oxopyrimidines with *S*-methylcarbonylbenzenesulfonamide moiety exist in two forms - open and cyclic in solution.
- The changes in the structure of the "head" (benzenesulfonamide) of the molecule on CA inhibitory activity are greater than the changes in the "tail" (heterocycle/phenyl) of the molecule.
- The comparison of CA II active site with CA I, XII and XIII active sites in complexes of CA II, XII, XIII-synthesized compounds, has shown that the interaction between the inhibitor and CA is determined by the sulfonamide group interaction with the catalytic zinc ion and the "tail" (heterocycle/phenyl) cohesion with secondary binding site. Decreased 5-(substituted)-2-chlorobenzenesulfonamide binding affinity to CA I may be explained by the steric hindrance on the secondary binding site shifted compared to CA II. Decreased affinity of compounds to CA XII, compared with CA II can be explained by environmental change in secondary binding site from hydrophobic to a more hydrophilic.

Scientific approbation and publication of presented work

The results of this study have been published in 13 publications: 2 articles are published in journals included in the Thompson Reuters ISI database, 7 poster presentations at international conferences and 4 poster presentations at national conferences.

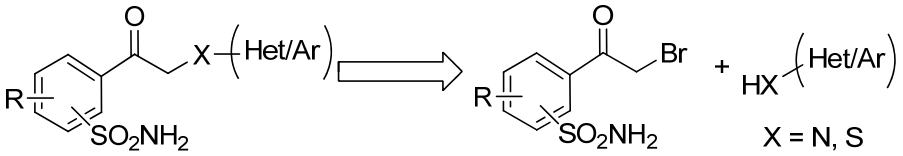
Contents of the dissertation

The dissertation is written in Lithuanian on 164 pages and includes 16 tables, 69 figures, 44 schemes, 108 references and 2 appendixes.

Results and Discussion

1. Synthesis

It was decided to synthesize compounds where the structure can be changed into two directions. Inhibitors can be divided into two parts - the benzenesulfonamide moiety ("head") and aromatic/heteroaromatic fragment ("tail") connected by a flexible linkage (Scheme 1-1).



Scheme 1-1. Retrosynthesis of benzenesulfonamides.

The position and nature of "head" substituents can change the acidity of the sulfonamide group and at the same time the strength of binding to the CA active center. Furthermore, the substituents on the benzene ring can also improve selectivity. Heterocyclic fragment connected by a flexible linkage to sulfonamide-substituted benzene ring can better adapt to the secondary recognition site of CA active center. In this study we investigated the structure-activity relationships by changing the structure of benzensulfonamide moiety and aromatic/heteroaromatic fragment.

Four benzenesulfonamides were chosen as fragments for inhibitor construction. They are shown in Fig. 1-1 as alkylating reagents **A - D**.

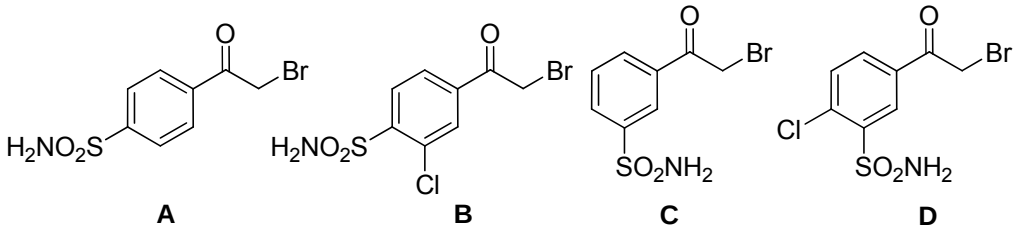


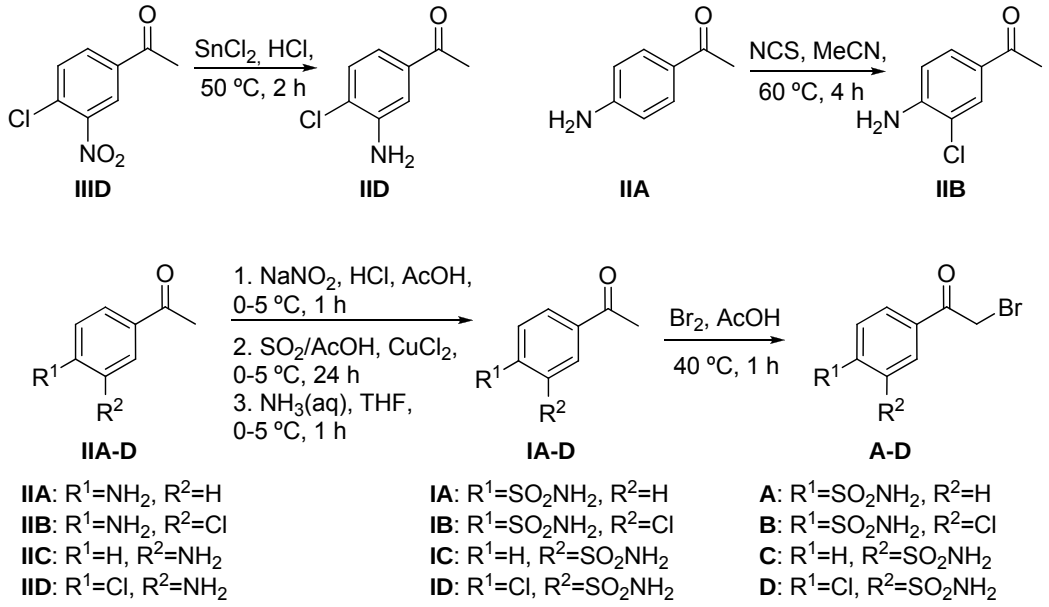
Fig. 1-1. Alkylating reagents **A-D**.

It formed four inhibitor classes, which will be generally referred as **A**, **B**, **C** and **D**. The second fragment contains a different heterocycles and benzene rings. Benzenesulfonamides with heteroaromatic fragment directly connected to the benzene ring were also synthesized.

Synthesis of alkylating reagents

Aminoacetophenone **IIB** was synthesized by chlorination of 1 - (3-aminophenyl)-*N* ethanone **II** with *N*-chlorosuccinimide in acetonitrile[1],[2] (Scheme 1-2). Aminoacetophenone **IID** was obtained by reduction of nitroacetophenone **IIID** with tin (II) chloride dihydrate[3]. Synthesis of sulfonamides **IA-D** was performed from aminoacetophenones **IIA-D** according to the literature method[4].

[1] T. E. Nickson, C. A. Roche-Dolson. *Synthesis* 6/7, **1985**, 669-670.
[2] A. Zanka, A. Kubota. *Synlett*. 12, **1999**, 1984-1986.
[3] H. Oelschlager, O. Schreiber. *Justus Liebigs Annalen der Chemie* 641 (1), **1961**, 81-94.
[4] T. Fujikura, K. Miigata, S. Hashimoto, K. Imai, T. Takenaka. *Chem. Pharm. Bull.* 30 (11), **1982**, 4092–4101.

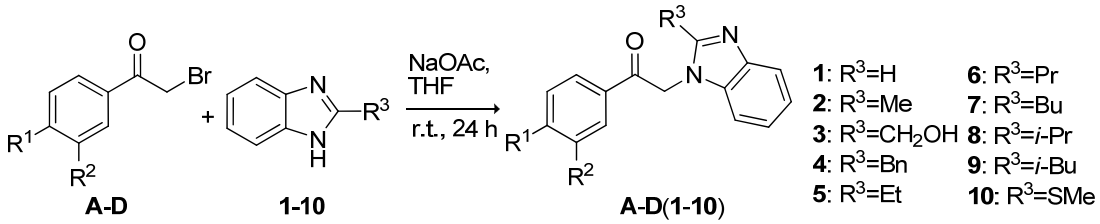


Scheme 1-2. Synthesis of compounds **A**-**D**.

Alkylating reagents **A**-**D** were obtained from sulfonamides **IA**-**D** via bromination[4].

Synthesis of *N*-alkylated benzimidazoles

N-alkylation of benzimidazoles **1**-**10** with **A**-**D** was carried out to give 40 *N*-substituted benzimidazole derivatives **A**-**D(1**-**10)** (Scheme 1-3).



Scheme 1-3. Synthesis of compounds **A**-**D**(**1**-**10**).

It was found that the reaction of **A**-**D** with benzimidazoles **1**-**10** in the presence of such bases as sodium methanolate in methanol, potassium carbonate in acetone led to the formation of various by-products, reducing the product yield (20-28%). Choosing a weaker base - sodium acetate and performing the reaction in tetrahydrofuran at room temperature led to avoid the formation of side compounds and products were obtained in higher - 30-92% yields. It should be also noted that better results in the *N*-alkylation reaction were obtained when a slight excess of the corresponding benzimidazole **1**-**10** was used. Conversely, employing an excess of alkylating reagent dialkylation of the benzimidazole ring took place. For example, in the reaction of **1** with **D** in a ratio 1:2 the main reaction product was compound **D1a** (Fig. 1-2).

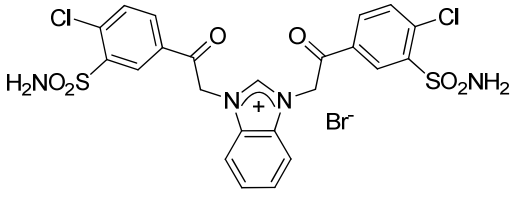
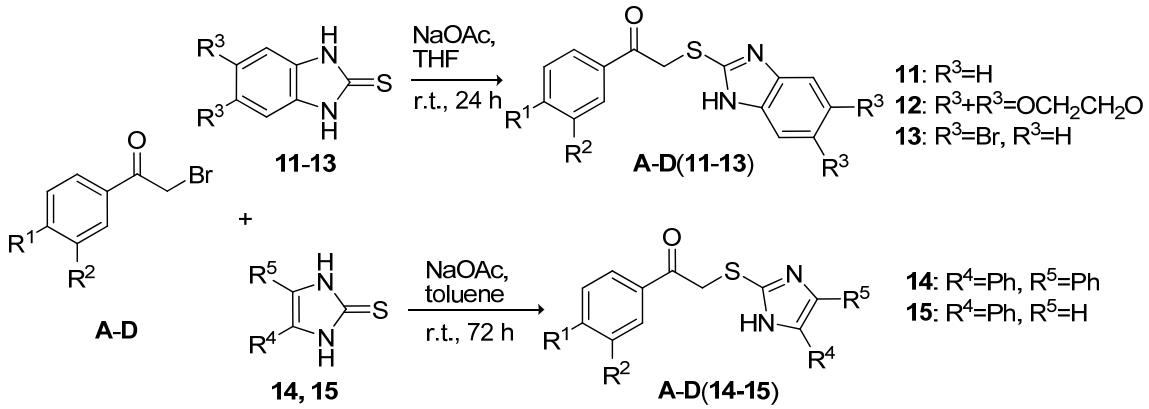


Fig. 1-2. The structure of compound **D1a**.

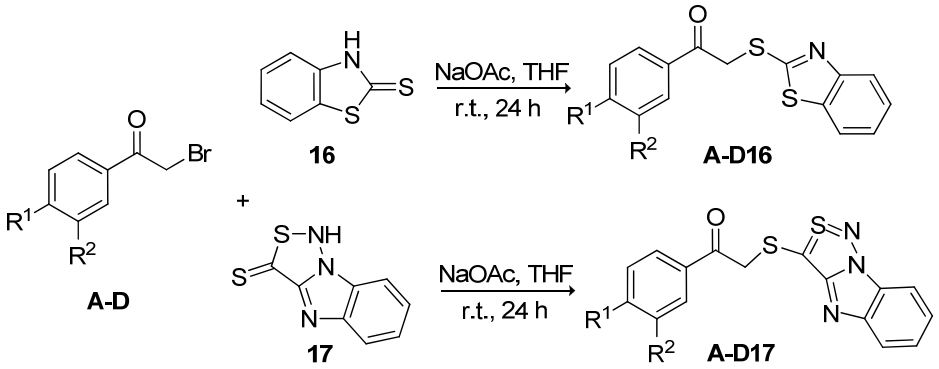
2-Substituted benzimidazoles **1-9** were prepared from 1,2-benzenediamine and the appropriate carboxylic acids according to a Phillips procedure[5]. 2-Methylsulfanylbenzimidazole (**10**) was purchased from Alfa Aesar and was used without further purification.

Synthesis of *S*-alkylated benzimidazoles, imidazoles, benzothiazole and benzimidazo[1,2-c]-1,2,3-thiadiazoles

Benzimidazoles **A-D**(**11-13**), imidazoles **A-D**(**14-15**), benzothiazoles **A-D16**, benzimidazo[1,2-c][1,2,3]thiadiazoles **A-D17** were synthesized by *S*-alkylation of thiones **11-13** and **16**, **17** with **A-D** in THF in the presence of sodium acetate as a base (Schemes 1-4, 1-5).



Scheme 1-4. Synthesis of compounds **A-D**(**11-15**).



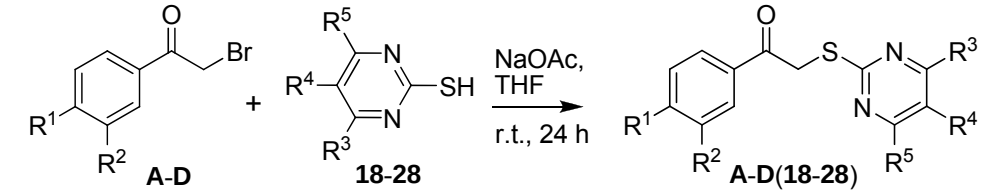
Scheme 1-5. Synthesis of compounds **A-D**(**16, 17**).

In the reaction of 2-mercaptoimidazoles **14**, **15** with **A-D** the best yields of **A-D16** were achieved using toluene as a solvent. Starting materials - 1,3,6,7-tetrahydro-2*H*-[1,4]dioxin-[2,3-*f*]benzimidazol-2-thione (**12**)[6], 5-bromo-1,3-dihydro-2*H*-benzimidazol-2-thione (**13**)[7], 4-phenyl-1,3-dihydro-2*H*-imidazole-2-thione (**15**)[8], imidazo-2-thione **14**[9] and benzimidazo-[1,2-*c*][1,2,3]thiadiazol-3-thione (**17**)[10] were synthesized according to the described procedures. 2-Mercaptobenzothiazole (**16**) was purchased from Alfa Aesar and was used without further purification. .

[5] M. A. Phillips. *J. Chem. Soc.* **1928**, 2393-2399.
[6] H. Krasso, A. Ramuz. *U.S. Patent* 4,599,347, **1986**.
[7] Z. I. Ermakova, A. N. Gricenko, S. V. Zhuravlov. *Khim. Geterotsikl. Soedin.* 2, **1974**, 202-203.
[8] G. R. Clemo, T. Holmes, G. C. Leitch. *J. Chem. Soc.* **1938**, 753-755.
[9] P. M. Kochergin. *Zh. Obshch. Khim.* 31, **1961**, 1093-1096.
[10] M. V. Dudutiene, L. Baranauskiene, D. Matulis. *Bioorg. Med. Chem. Lett.* 17 (12), **2007**, 3335 – 3338.

Synthesis of *S*-alkylated pyrimidines

The alkylation of 2-mercaptopyrimidines **18-28** with bromoacetophenones **A-D** was carried out in tetrahydrofuran using sodium acetate as a base to give *S*-substituted pyrimidine derivatives **A-D**(**18-28**) in 68-93 % yields (Scheme 1-6).

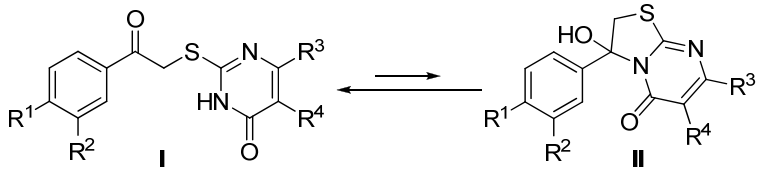


18: R^3=OH, R^4=H, R^5=Me
19: R^3=OH, R^4=Bn, R^5=Me
20: R^3=OH, R^4=H, R^5=Pr
21: R^3=OH, R^4=H, R^5=*t*-Bu
22: R^3=OH, R^4=H, R^5=Ph
23: R^3=OH, R^4=CO_2Et, R^5=H
24: R^3=Me, R^4=H, R^5=Me
25: R^3=H, R^4=Et, R^5=H
26: R^3=H, R^4=Pr, R^5=H
27: R^3=H, R^4=Bu, R^5=H
28: R^3=H, R^4=H, R^5=H

Scheme 1-6. Synthesis of compounds **A-D**(**18-28**).

Initial 6-alkyl-2-thioxo-2,3-dihydro-4(1*H*)-pyrimidinones **19**[11], **20-22**[12] were synthesized by condensation of the appropriate substituted *β*-keto esters with thiourea. 5-Alkyl-2(1*H*)-pyrimidinethiones **25** and **27** were synthesized from thiourea and 2-alkyl-1,1,3,3-tetraethoxy-propane according to[13]. 2- Mercaptopyrimidines **18**, **23**, **24**, **26** and **28** were purchased from Alfa Aesar and were used without further purification.

An investigation of the compounds **A-D**(**18-28**) by NMR spectroscopy showed that the ^{1}H and ^{13}C NMR spectra of pyrimidinones **A-D**(**18-23**) in DMSO-d_6 solution contained two sets of signals. These results led to a suggestion that this phenomenon could be due to the existence of compounds **A-D**(**18-23**) in two forms: open chain **I** and cyclic **II** (Scheme 1-7).



Scheme 1-7. Open chain **I** and cyclic **II** forms of compounds **A-D**(**18-23**).

Especially large differences were observed for signals of SCH_2 group protons in the ^{1}H NMR spectra. This signal of open chain form **I** was observed as a singlet in the 4.72-4.97 ppm region, while SCH_2 group protons of cyclic form **II** appeared as doublets with J = 12-12.9 Hz at 3.54-3.79 ppm. ^{13}C NMR spectra of compounds **A-D**(**18-23**) gave additional evidence for the existence of equilibria between open chain and cyclic forms of compounds **A-D**(**18-23**) in solution. As expected, the main differences in the ^{13}C NMR spectra were observed for carbon atoms taking part in the transformation. Thus, the signal for carbon of the SCH_2 group in open chain form **I** was observed at 37.3-39.3 ppm. In the ^{13}C NMR spectra of cyclic form **II**, a signal for this carbon was observed in the 42.1-43.0 ppm region. Moreover, the open chain isomers **A-D**(**18-23**) were characterized by the C=O group carbon signal ranging from 192.2-194.1 ppm. The C-OH group carbon signal of cyclic form **II** was observed in the 95.5-97.8 ppm range.

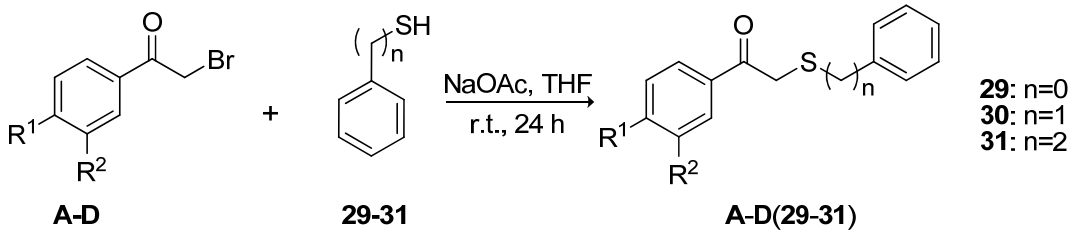
Thus, the obtained NMR spectral data showed the presence of two forms of compounds **A-D**(**18-23**) in DMSO-d_6 solution. It was found that the **B**-type compounds are more involved in cyclic form (the ratio **I**: **II** was 1:0.83 as calculated from integral intensities of SCH_2 signals

[11] B. R. Baker, R. E. Schaub, J. P. Joseph, F. J. McEvoy, J. H. Williams. *J. Org. Chem.* 18 (2), **1953**, 133–137.
[12] G. W. Anderson, I. F. Halverstadt. *J. Am. Chem. Soc.* 67, **1945**, 2197–2200.
[13] V. R. Kruse, E. Breitmaier. *Chemiker-Zeitung* 101, **1977**, 305–306.

of ^{1}H NMR spectra), when compared with the others (type **D** - 1:0.30, type **A** - 1:0.29, type **C** - 1:0.21). These differences can be explained by the influence of electron withdrawing substituents in benzene ring on the carbonyl group.

Synthesis of *S*-alkylated phenylalkylthiols

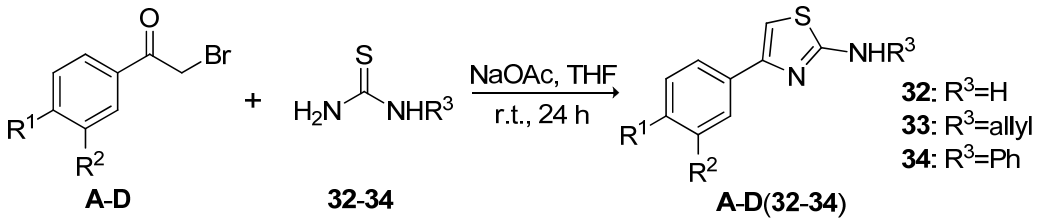
Synthesis of sulfonamides **A-D**(**29-31**) was carried out by the reaction of bromoacetophenones **A-D** with the corresponding thiols **29-31** using the same reaction conditions as for the synthesis of heterocyclic moiety containing sulfonamides (Scheme 1-8).



Scheme 1-8. Synthesis of compounds **A-D**(**29-31**).

Synthesis of 1,3-thiazole derivatives

Benzenesulfonamides containing thiazole moiety, directly attached to the benzenesulfonamide fragments, were synthesized from thioureas **32-34** and **A-D** according to the Hantzsch thiazole synthesis (Scheme 1-9).



Scheme 1-9. Synthesis of compounds **A-D**(**32-34**).

The reaction conditions used were the same as in the alkylation reaction.

2. Analysis of CA inhibitory activity of the synthesized compounds

The equilibrium dissociation constant (Kd) and the inhibitory constant (Ki) are among the most commonly used measures for evaluation the in vitro efficacies of the binding of small-molecule-based ligands to their enzyme targets. The binding affinities of compounds towards CA I, II, VII, XII and XIII were determined by the thermal shift assay (TSA) at the Institute of Biotechnology, Vilnius University. The observed binding constants (Kb) are inversely proportional to the dissociation constants (Kd).

2.1. Brief analysis. Influence of "head" (benzenesulfonamide fragment) and "tail" (aromatic/heteroaromatic fragment) of the molecule to CA-inhibiting properties.

According to the analysis of all compound inhibitory activity towards CA, the CAs can be sorted in ascending order - CA XII<VII<I<XIII≤ II by inhibition potency (Fig. 2.1-1).

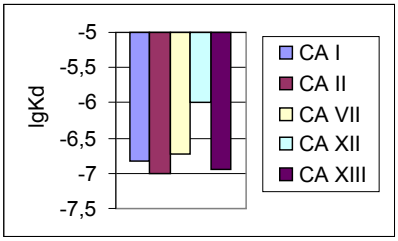


Fig. 2.1-1. The averages of Kd logarithms of all compounds for each CA.

The compounds can be divided into two parts, "head" and "tail". According to the "head" (benzenesulfonamide fragment), the compounds can be divided into four classes: **A**, **B**, **C**, **D**. According to the tail (aromatic/heteroaromatic fragment) - into five groups: *N*-alkylated benzimidazoles, *S*-alkylated benzimidazoles (also imidazoles, benzothiazoles, benzimidazothiadiazoles), *S*-alkylated pyrimidines, *S*-alkylated phenylalkylthiols, 1,3-thiazole derivatives.

According to the averages of Kd logarithms compound classes can be sorted by activity in ascending order: **C**<**D**<**A**<**B** (Fig. 2.1-2).

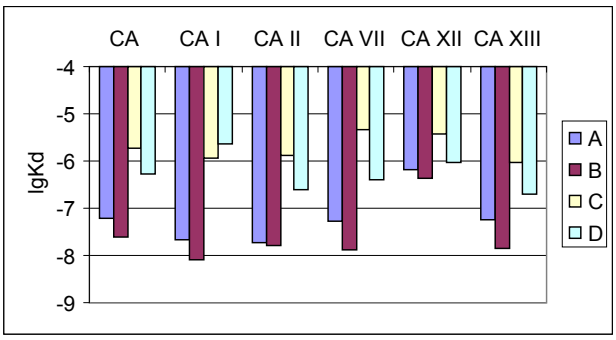


Fig. 2.1-2. The averages of Kd logarithms of classes of compounds for each CA.

Comparing the average of Kd logarithms for compounds **A**-**D** it can be seen that the compounds arrange in the order **C**<**D**<**A**<**B** of increase in activity for CAs: CA VII, XII and XIII, while for CA II the order is - **C**<**D**<**A**≤ **B** and for CA I - **D**<**C**<**A**<**B**.

Comparing to the averages of Kd logarithms for all CA compound groups, it can be sorted by activity in ascending order: 1,3-thiazole derivatives < *N*-alkylated benzimidazoles < *S*-alkylated benzimidazoles < *S*-alkylated pyrimidines < *S*-alkylated phenylalkylthiols (Figure 2.1-3). This order of activities is valid for all CAs, except for CA I. In this case an order of compound groups is 1,3-thiazole derivatives < *S*-alkylated benzimidazoles < *N*-alkylated benzimidazoles < *S*-alkylated pyrimidines < *S*-alkylated phenylalkylthiols.

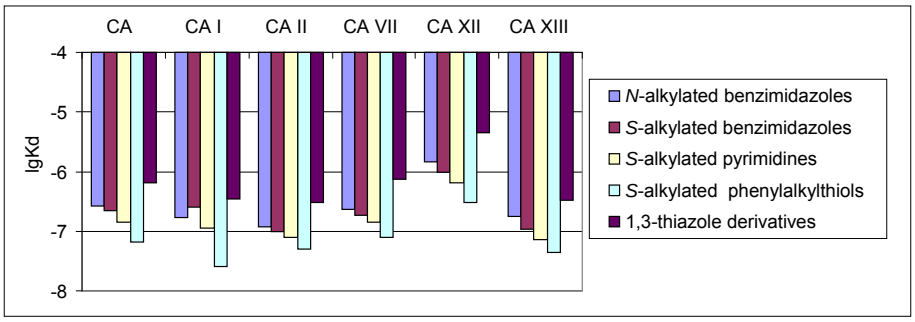


Fig. 2.1-3. The averages of Kd logarithms of groups of compounds.

Moreover, the obtained results show that the influence of "head" on CA-inhibiting properties is greater than the "tail" one. *S*-alkylated phenylalkylthiols **B** appeared to be the best inhibitors, especially for CA I, while *N*-alkylated benzimidazoles **C** exhibited worst inhibitory properties, especially for CA VII.

Acording to the sulfonamide group proton acidity, evaluated on the basis of ^{1}H NMR spectra (Fig. 2.1-4) (the more acidic proton signal should be observed in the region of weaker field) classes of compounds can be sorted by sulfonamide group proton shift increase - **C**<**A**<**D**<**B**. Compounds **B** and **D** containing chlorine substituent in "head" can be characterized by a higher acidity of sulfonamide group than the **A** and **C**. The electron withdrawing influence of carbonyl group is much lower. A slight electron donating influence to sulfonamide group protons is observed in compounds **A-D**(**32-34**), containing a thiazole substituent instead of the carbonyl group.

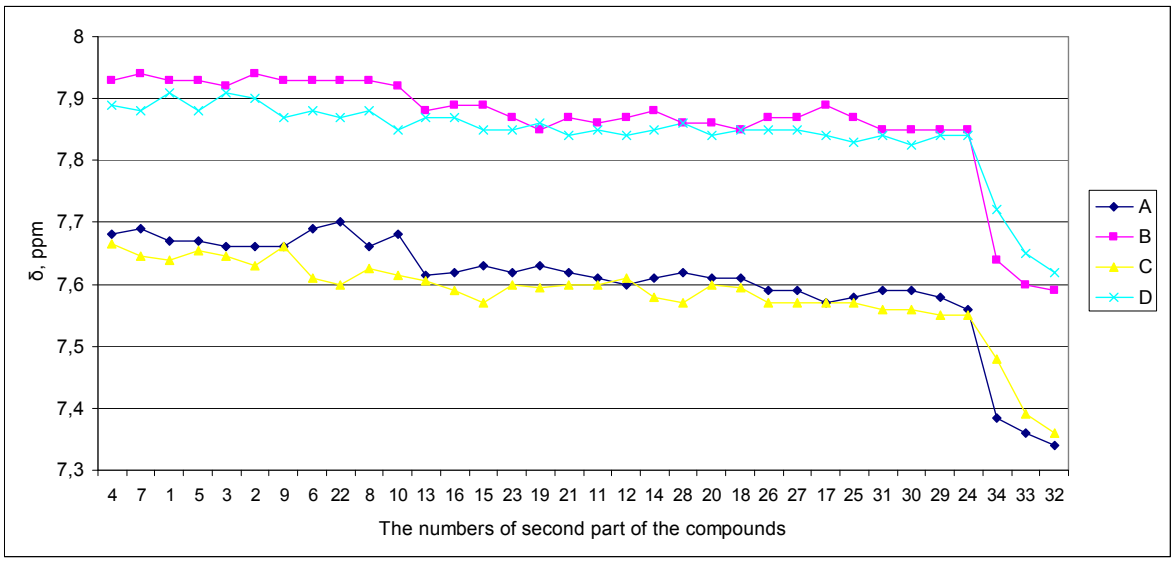


Fig. 2.1-4. Sulfonamide group proton chemical shifts in the ^{1}H NMR spectrum of compounds, grouped by classes (**A-D**). Compounds ordered in compound quartets with the same second molecular fragment ("tail"), according to the chemical shift averages in descending order.

According to the discrepancy between affinity to CA and sulfonamide group changes in acidity in these four classes, we assume that the inhibitor properties are affected by other factors, for example, steric, as well. Compounds **A** and **B** have better affinity to CA than **C** and **D** (Fig. 2.1-2).

2.2. Analysis of CA inhibitory activity of compounds according to the "head" (benzenesulfonamide fragment of the molecule

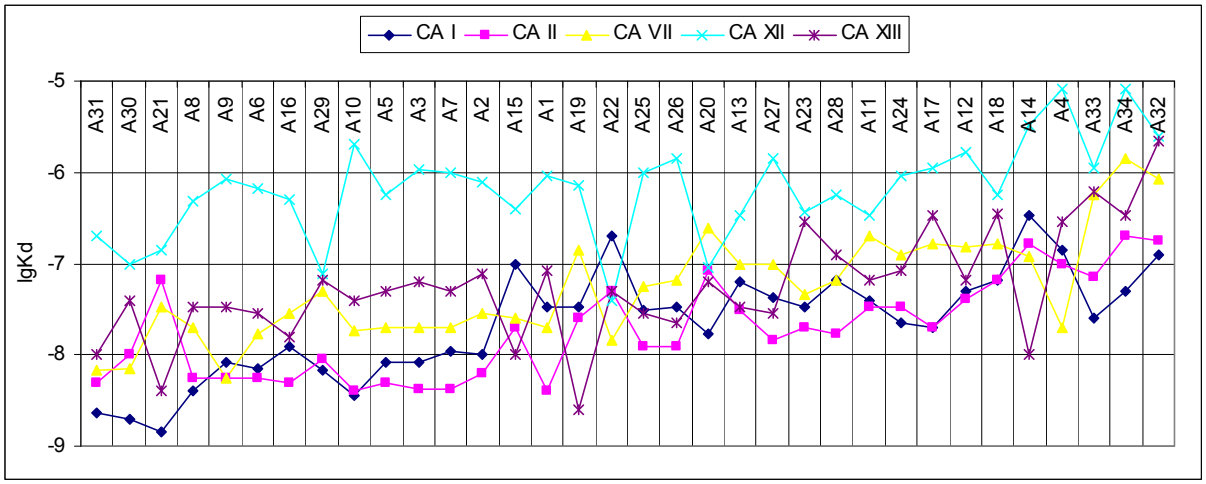


Fig. 2.2-1. The averages of Kd logarithms of compounds **A** sorted by CA. Compounds arranged by averages of Kd logarithms in ascending order.

Analysis of inhibitory activity data for compounds **A** (Fig. 2.2-1) shows that CA XII is the least inhibited isoform among the investigated CAs (32 compounds, Kd = 0.077 to 8.33 μM). It is rather difficult to observe general trends in compound inhibitory activity towards other CAs. Their inhibitory effects lie on a wide range (Kd = 1.43 nM – 8.33 μM). However Kd for CA I and II inhibition are in the range of 1.43 - 125 nM (11 compounds), and 4.00-40.0 nM (14 compounds) respectively.

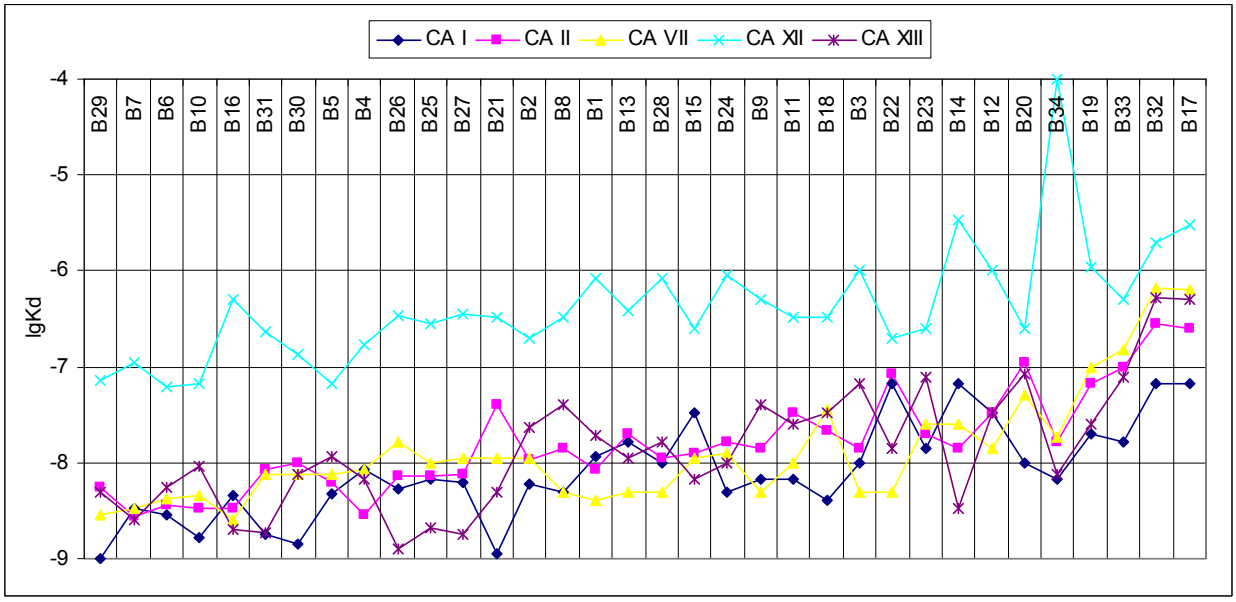


Fig. 2.2-2. The averages of Kd logarithms of compounds **B** sorted by CA. Compounds arranged by averages of Kd logarithms in ascending order.

The analogous analysis of data for compounds **B** (Fig. 2.2-2) shows that CA I is best inhibited in many cases (18 compounds, Kd = 1.00 to 66.7 nM).Other CAs were inhibited in the same order (CA II, Kd = 2.70 to 286 nM, CA VII, Kd = 2.50 to 667 nM, CA XIII, Kd = 1.25 to 526 nM). CA XII is inhibited worst (Kd = 0.063 to 100 μM) by these compounds of class **B**.

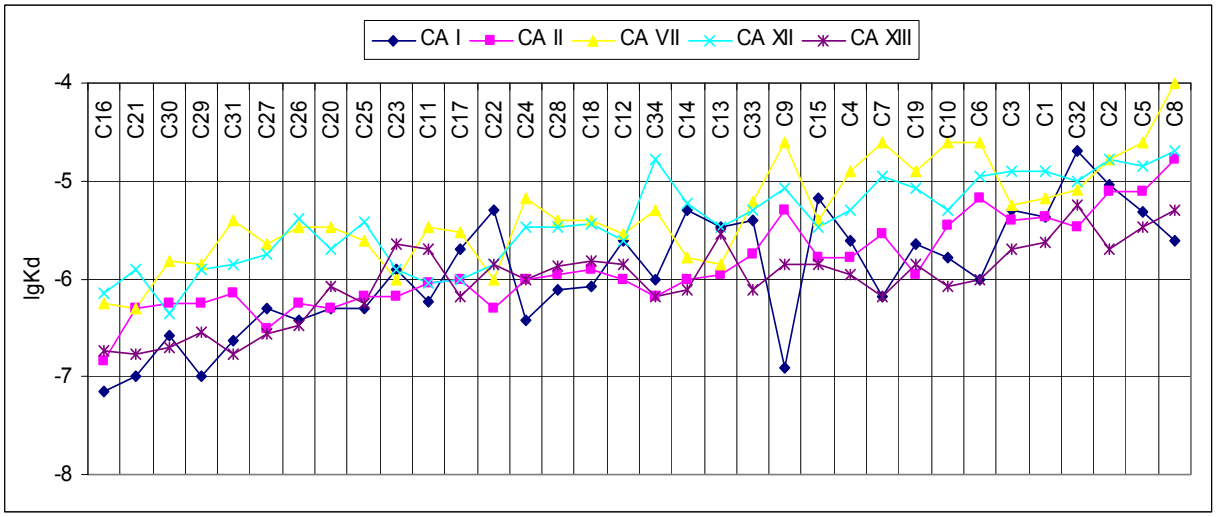


Fig. 2.2-3. The averages of Kd logarithms of compounds **C** sorted by CA. Compounds arranged by averages of Kd logarithms in ascending order.

The analysis of data obtained for compounds **C** (Fig. 2.2-3) shows that more than half of the studied compounds exhibit weakest inhibition of CA VII (Kd = 1.54 to 100 μM). CA XII is less inhibited than CA I, II and XIII – 25 compounds (Kd = 0.714 to 20.0 μM). The best inhibition has been achieved for CA I (10 compounds, Kd = 71.4 to 2500 nM) and CA XIII (14 compounds, Kd = 0.172 to 3.33 μM).

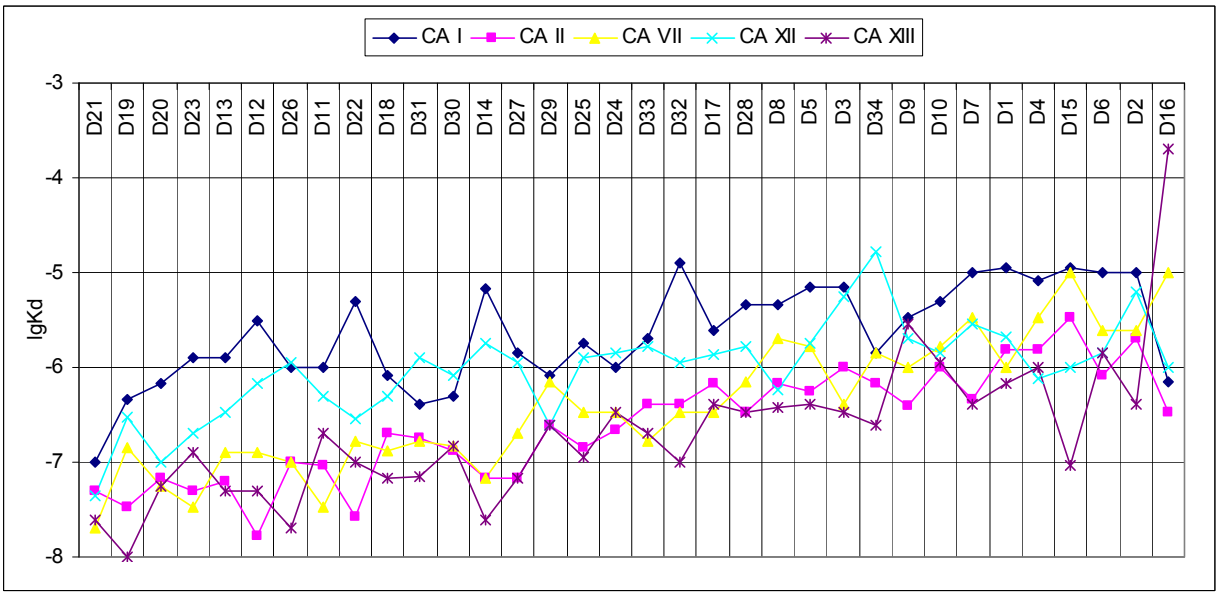


Fig. 2.2-4. The averages of Kd logarithms of compounds **D** sorted by CA. Compounds arranged by averages of Kd logarithms in ascending order.

The examination of compounds **D** (Fig. 2.2-4) shows that CA I is inhibited to the lowest extent (28 compounds, Kd = 0.100 to 12.5 μM).With the exception of CA I, CA XII is inhibited weakest (24 compounds, Kd = 0.100 to 16.7 μM). More often than the other CAs, the CA XIII is inhibited best (16 compounds with Kd = 10.0-667 nM).

In summary, compounds **A** well inhibit CA I and II, CA XII is inhibited worst. Compounds **B** well inhibit CA I, II, VII and XIII. Inhibition of CA XII by compounds **B** is weakest. Compounds **C** best inhibit CA II and XIII, CA VII and XII are poorly inhibited by this class of compounds. Compounds **D** best inhibit CA II and XIII, CA I and XII are being inhibited poorly.

2.3. Analysis of CA inhibitory activity of compounds according to the "tail" (heterocyclic/aromatic fragment of the molecule)

2.3.1. Brief analysis

Figure 2.3.1-1 shows that the difference of CA inhibitory activity between **A**, **B**, and **C**, **D** classes pairs of compounds containing the same "tail" is greater than among the same class of compounds with different "tails". The "head" is related not only to the acidity of the sulfonamide group, but also influences the orientation of the "tail" in the active site of CA. So, the influence of "tail" on binding to CA is lower than the "head".

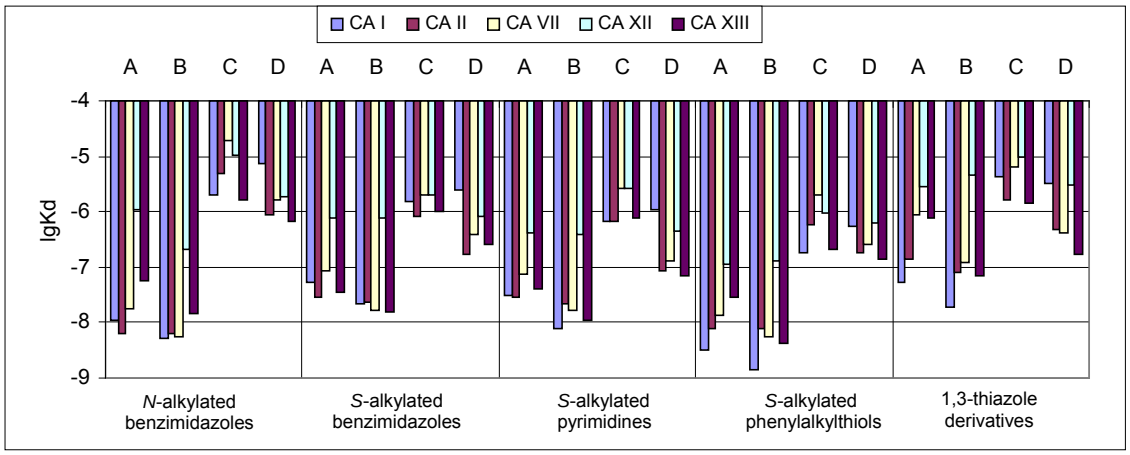
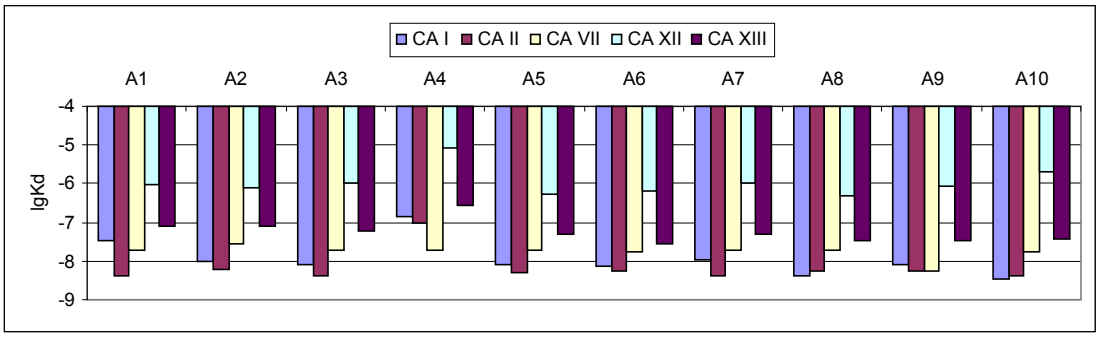


Fig. 2.3.1-1. The averages of Kd logarithms of class and groups of compounds for each CA.

In case of *N*-alkylated benzimidazoles, the greatest loss of inhibition of CA has been observed, compared to **A** and **B** with **C** and **D** classes. This perhaps can be explained by the fact that the molecule in which benzenesulfonamide moiety is connected through the the $COCH_2$ linker to the nitrogen atom of benzimidazole fragment has fewer degrees of freedom than, for instance, an analogous molecule with the benzimidazole fragment connected through a sulfur atom, which is no longer so constrained. Moreover, in *N*-alkylated benzimidazole derivatives the linker between the two parts of the molecule is shorter by one atom than in the *S*-alkylated derivatives. Thus, in classes **C** and **D**, the *N*-alkylated benzimidazole fragment can not find the secondary binding pockets due to the steric hindrance (See 3.1. Inhibitor complexes with CA II, Figure 3.1-4). *S*-alkylated phenylalkylthiol derivatives in many cases, stand the best inhibitors of the CA, while the 1,3-thiazoles derivatives in many cases can be attributed to poorly inhibiting compounds.

2.3.2. More detailed analysis of each individual group

N-alkylated benzimidazoles

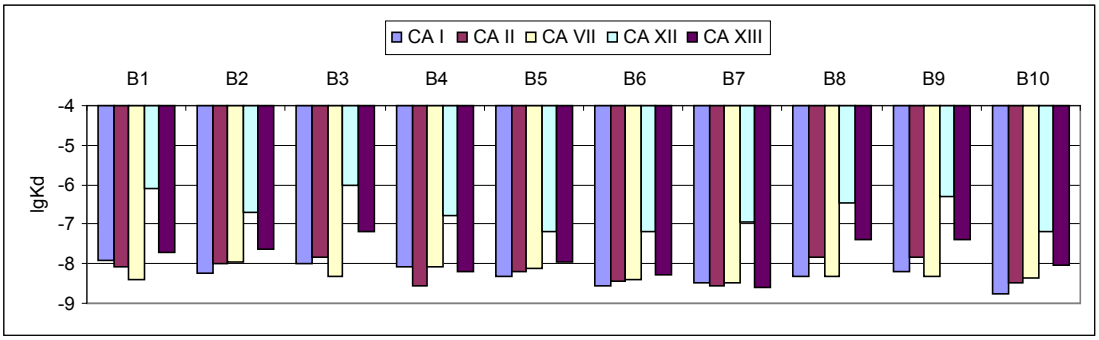


R:	H	Me	CH_2OH	Bn	Et	Pr	Bu	*i*-Pr	CH_2*i*-Pr	SMe

Fig. 2.3.2-1. The averages of Kd logarithms of *N*-alkylated benzimidazoles **A** for each CA.

N-alkylated benzimidazoles **A** with methyl **A2**, hydroxymethyl **A3**, ethyl **A5**, propyl **A6**, butyl **A7** substituents and **A1**, at the 2nd position of benzimidazole has almost identical inhibitory activity to CA (Figure 2.3.2-1). CA II (Kd = 4.00-100 nM) is best inhibited, while CA XII (Kd = 0.480-8.33 μM) - the least. Isopropyl substitution (**A8)** increases inhibitory activity to CA I (Kd = 4.00 nM), and isobutyl substituted **A9** - to CA VII (Kd = 5.56 nM). *S*-methyl substituted **A10** features similar inhibitory activity as the isopropyl substituted derivative.Benzyl substituted **A4** has the weakest CA inhibitory activity, especially with the CA XII (Kd = 8.33 μM) but it is selective to CA VII (Kd = 20 nM).

In case to *N*-alkylated benzimidazoles **B** with methyl **B2**, ethyl **B5**, propyl **B6**, butyl **B7** substituents in the 2nd position of benzimidazole ring, affinity to CA I (Kd = 2.9-5.9 nM), II (Kd = 10.5-2.70 nM), VII (Kd = 11.1-3.33 nM) and XIII (Kd = 23.3-2.50 nM) was slightly increased, while the affinity of CA XII is increased only for compounds **B5** and **B6** (Kd = 66.7 nM, 62.5 nM, respectively) (Fig. 2.3.2-2).

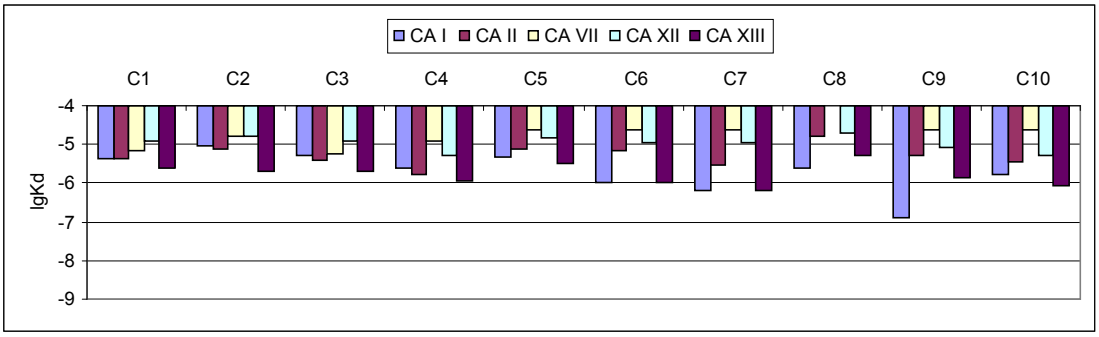


R:	H	Me	CH_2OH	Bn	Et	Pr	Bu	*i*-Pr	CH_2*i*-Pr	SMe

Fig. 2.3.2-2. The averages of Kd logarithms of *N*-alkylated benzimidazoles **B** for each CA.

The compounds **B** are probably the best inhibitors in this group, but have no selectivity. Benzimidazole without substituent **B1** is slightly worse CA I and II inhibitor (Kd = 11.8 nM, 5.88 nM, respectively) and significantly worse CA XII and XIII inhibitor (Kd = 833 nM, 19.2 nM, respectively), compared with alkyl substituted **B(5-7)**. Benzimidazoles **B8** and **B9** containing branched carbon chain have reduced affinity to CA II (Kd = 14.3 nM in both cases), XII (Kd = 333 nM, 500 nM, respectively) and XIII (40.0 nM in both cases), compared with unbranched substituents containing compounds. The introduction of the *S*-methyl substituent **B10**, inhibitory activity remains similar to propyl substituted **B6**, except for enhanced the inhibitory activity to CA I (Kd = 1.67 nM).

N-benzimidazoles **C** are weakest CA inhibitors (Kd = 0.130-100 μM) (Fig. 2.3.2-3).



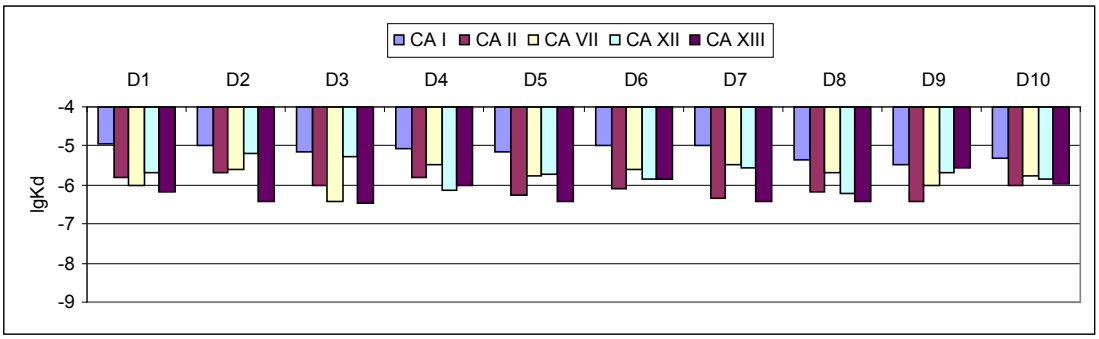
R:	H	Me	CH_2OH	Bn	Et	Pr	Bu	*i*-Pr	CH_2*i*-Pr	SMe

Fig. 2.3.2-3. The averages of Kd logarithms of *N*-alkylated benzimidazoles **C** for each CA.

Many of them have higher affinity for CA XIII (Kd = 0.830-3.33 μM) except **C(6-9)**. Compounds containing propyl **C6**, butyl **C7,** and isobutyl **C9** substituents have the increased

affinity for CA I (Kd = 1.00 μM, 0.667 μM, 0.125 μM) compared with the other compounds in this group.

N-substituted benzimidazoles **D** (Fig. 2.3.2-4) also do not show good CA inhibitory activity (Kd = 0.333-11.1 μM), although it is slightly better than that of class **C** compounds.



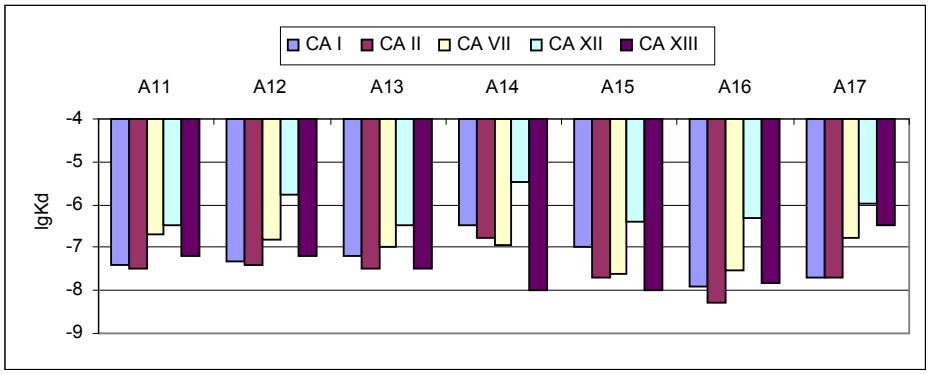
R:	H	Me	CH_2OH	Bn	Et	Pr	Bu	*i*-Pr	CH_2*i*-Pr	SMe

Fig. 2.3.2-4. The averages of Kd logarithms of *N*-alkylated benzimidazoles **D** for each CA.

Half of the compounds best inhibit CA XIII (Kd = 333-667 nM). Compound **D3** with hydroxymethyl substituent from this group distinguishes for its best CA VII inhibition (Kd = 400 nM). The compound **D4** containing benzyl substituent have the highest affinity for CA XII (Kd = 769 nM).

S-alkylated benzimidazoles

Class **A** *S*-alkylated benzimidazoles **A**(**11-13**) act as much more effective inhibitors of the CA I (Kd = 38.5-62.5 nM), II (Kd = 31.3-40.0 nM) and XIII (Kd = 33.3-66.7 nM). CA VII is inhibited weak (Kd = 100-200 nM) and XII - the least (Kd = 0.333-1.67 μM) (Fig. 2.3.2-5).

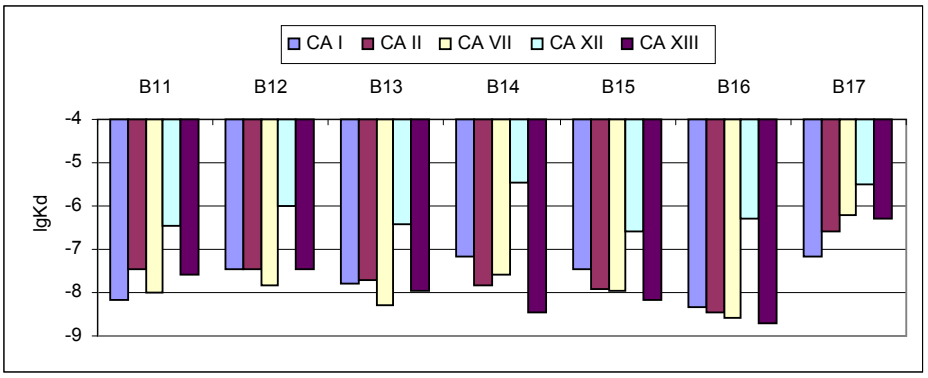


	benzimidazole			imidazole		benzthiazole	benzimidazothiadiazole
R:	H	OCH_2 CH_2O	Br	Ph, Ph	H, Ph		

Fig. 2.3.2-5. The averages of Kd logarithms of *S*-alkylated benzimidazoles **A** for each CA.

Imidazole derivatives **A14** and **A15** are best inhibitors of CA XIII (Kd = 10.0 nM in both cases). In case of imidazole derivative **A15**, with only one phenyl substituent compared to monophenyl derivative **A14**, the inhibition efficiency increases to the rest of CA. Benzothiazole moiety containing compound **A16** is a better CA I, II, VII and XIII inhibitor (Kd = 5.00-12.5 nM) than with benzimidazole moiety **A11**, but less potent inhibitor of CA XII (Kd = 500 nM). Benzimidazothiadiazole derivative **A17** is also a better inhibitor of CA I and II (Kd = 20.0 nM in both cases) then the benzimidazole derivative **A11**, but has the lowest affinity for CA XIII in this group of compounds (Kd = 333 nM).

Although the simple benzimidazole derivative **B11** effectively inhibit CA I and VII (Kd = 6.67 nM and 10.0 nM, respectively), but the compounds with dioxane ring **B12** or bromo substituted **B13** posses selectivity to CA VII (Kd = 14.3 nM, 5.00 nM, respectively) (Fig. 2.3.2-6).

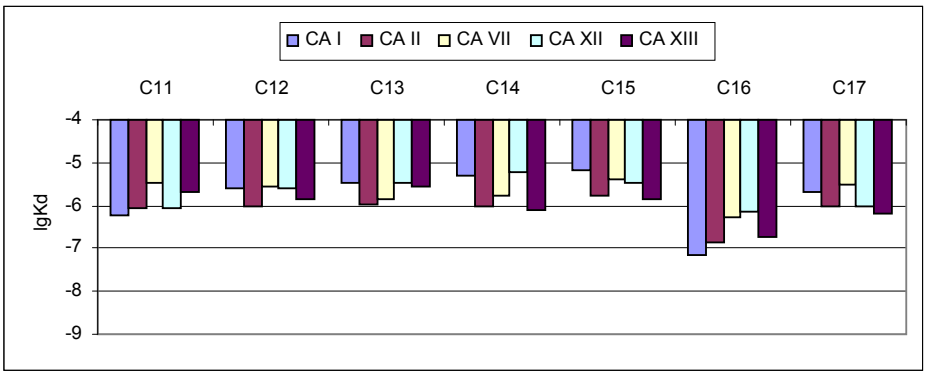


	benzimidazole			imidazole		benzthiazole	benzimidazothiadiazole
R:	H	OCH_2 CH_2O	Br	Ph, Ph	H, Ph		

Fig. 2.3.2-6. The averages of Kd logarithms of *S*-alkylated benzimidazoles **B** for each CA.

Imidazole derivatives **B14** and **B15** are best CA XIII inhibitors (Kd = 3.33 nM, 6.67 nM, respectively). Monophenylimidazole **B15** in this group have the best CA XII inhibition (Kd = 250 nM). Benzothiazole substituted **B16** inhibits CA I, II, VII and XIII, the best in this group (Kd = 4.55 nM, 3.33 nM, 2.50 nM, 2.00 nM, respectively). Benzimidazothiadiazole moiety containing compound **B17** showed reduced these CA inhibitory properties (Kd = 66.7-645 nM).

Again, a simple benzimidazole derivative **C11** has selectivity to CA I (Kd = 588 nM), but the remaining benzimidazole derivatives **C12** and **C13** that CA inhibit weaker and are more selective to CA II (Kd = 1.00 μM, 1.11 μM, respectively) (Fig.2.3.2-7).

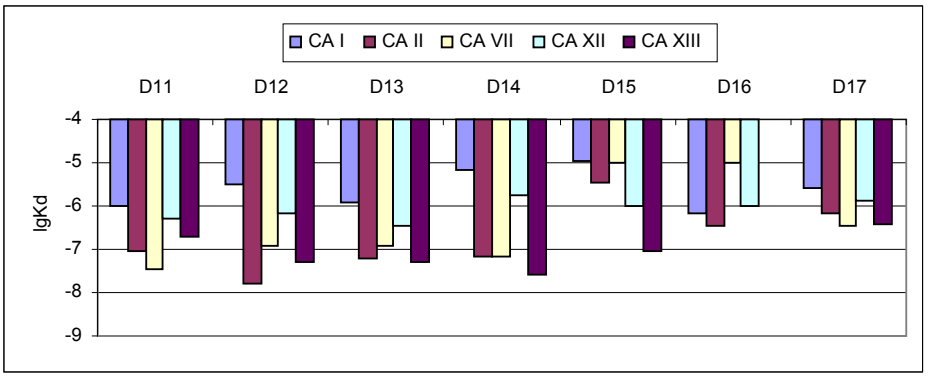


	benzimidazole			imidazole		benzthiazole	benzimidazothiadiazole
R:	H	OCH_2 CH_2O	Br	Ph, Ph	H, Ph		

Fig. 2.3.2-7. The averages of Kd logarithms of *S*-alkylated benzimidazoles **C** for each CA.

Imidazoles **C14** and **C15** inhibit even worse CA I (Kd = 5.00 μM, 6.67 μM, respectively), but it shows better affinity to CA XIII (Kd = 0.769 μM, 1.43 μM, respectively). Benzothiazole derivative **C16** in the group of these compounds has the best CA inhibition (Kd = 71.4-714 nM) and selectivity for CA I (Kd = 71.4 nM).

All compounds of this group weakly inhibit CA I (Kd = 0.714-11.1 μM) (Fig. 2.3.2-8)). Dioxane moiety containing compound **D12** is the best CA II (Kd = 16.7 nM) inhibitor of this group. Imidazoles **D14** and **D15** shows selectivity to CA XIII (Kd = 25.0 nM and 90.9 nM, respectively).



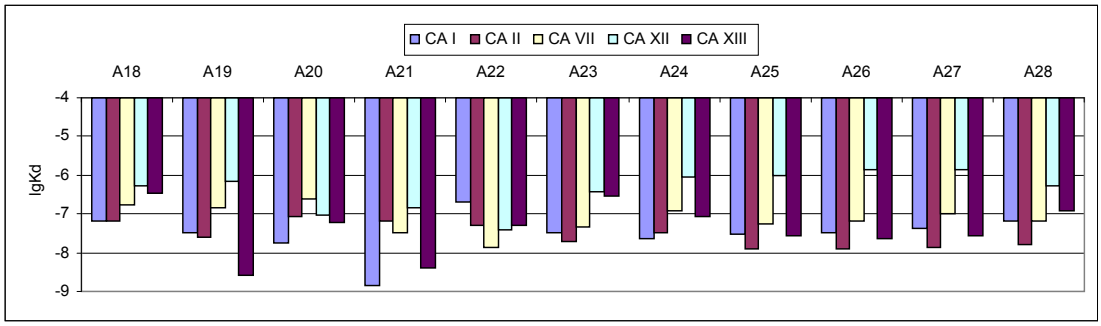
	benzimidazole			imidazole		benzthiazole	benzimidazothiadiazole
R:	H	OCH_2 CH_2O	Br	Ph, Ph	H, Ph		

Fig. 2.3.2-8. The averages of Kd logarithms of *S*-alkylated benzimidazoles **D** for each CA.

Benzothiazole **D16** shows enhanced inhibition activity to CA, especially to CA VII (Kd = 33.3 nM) compared with the benzimidazole **D11**. Thiadiazole moiety containing compound **D17** is selective to CA VII (Kd = 333 nM), another CA inhibition is modest. It is interesting to note that this replacement-containing compounds **(A-D)17** affinity to CA XIII remains more or less constant (Kd = 333-667 nM).

S-alkylated pyrimidines

Introduction of benzyl group to the 5th position in oxopyrimidines (**A19)** significantly increases the affinity to CA XIII (Kd = 2.50 nM) (Figure 2.3.2-9). Ester moiety **A23** gives the reverse effect (Kd = 286 nM, CA XIII). We observe an increase in affinity for CA I and XIII with oxopyrimidines derivative having substituents in the 6th position like methyl **A18**, propyl **A20** or *tert*-butyl **A21** (Kd from 66.7 to 1.43 nM and from 357 to 4.00 nM, respectively), while CA II inhibitory activity remained almost constant (Kd = 66.7-88.3 nM). However, with phenyl substituted **A22** CA I is less inhibited (Kd = 200 nM), inhibition of CA II remains almost unchanged, and inhibition of CA VII and XII increases (Kd = 14.3 nM, 38.5 nM, respectively).

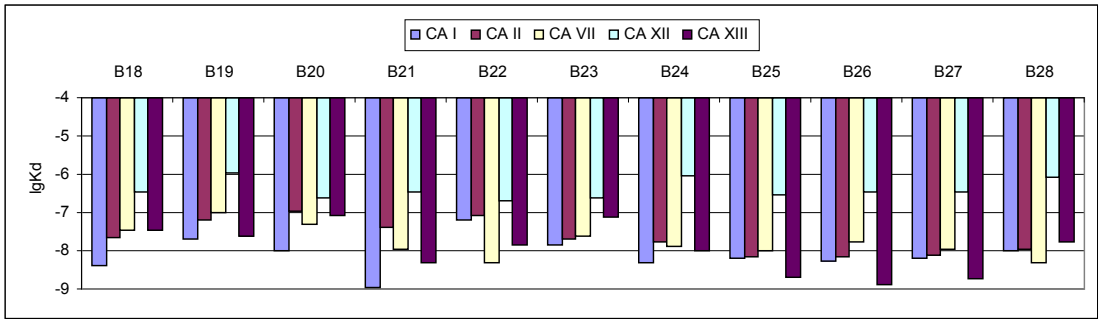


R^1:	OH,	OH,	OH,	OH,	OH,	OH,	Me,	H,	H,	H,	H,
R^2:	H,	Bn,	H,	H,	H,	CO_2Et	H,	Et,	Pr,	Bu,	H,
R^3:	Me	Me	Pr	*t*-Bu	Ph	H	Me	H	H	H	H

Fig. 2.3.2-9. The averages of Kd logarithms of *S*-alkylated pyrimidines **A** for each CA.

Comparing data of 6-methyl-4-oxopyrimidine **A18** with 4,6-dimethylpyrimidine **A24** enhanced affinity for CA I, II, VII and XIII (Kd = 22.2 nM, 33.3 nM, 125 nM, 83.3 nM, respectively) is observed, while the affinity for CA XII is reduced (Kd = 909 nM). Carbon chain elongation in the 5th position of the pyrimidine **A**(**25-27**) does not affect the inhibition activity for CA I, II, and XIII very much (Kd = 30.3-41.7 nM, 12.5-14.3 nM, 22.7-28.6 nM, respectively). Otherwise, CA VII and XII inhibition is even slightly lower (Kd from 55.5 to 100 nM, 1.00 to 1.43 μM, respectively).

Introduction of benzyl group into the 5th position **B19** in oxopyrimidines reduces the affinity to all CA three to five times (Fig. 2.3.2-10.). The introduction of ester moiety **B23** synchronizes inhibitory activity towards CA I, II and VII (Kd = 14.3 nM, 20.0 nM, 25.0 nM, respectively). Oxopyrimidines derivatives containing in the 6th position methyl **B18**, propyl **B20** or *tert*-butyl **B21** substituent, exhibit the best inhibition of CA I (Kd = 4.00 nM, 10.0 nM, 1.11 nM, respectively). Pyrimidine derivative **B21** can be distinguished as the most effective CA I inhibitor.

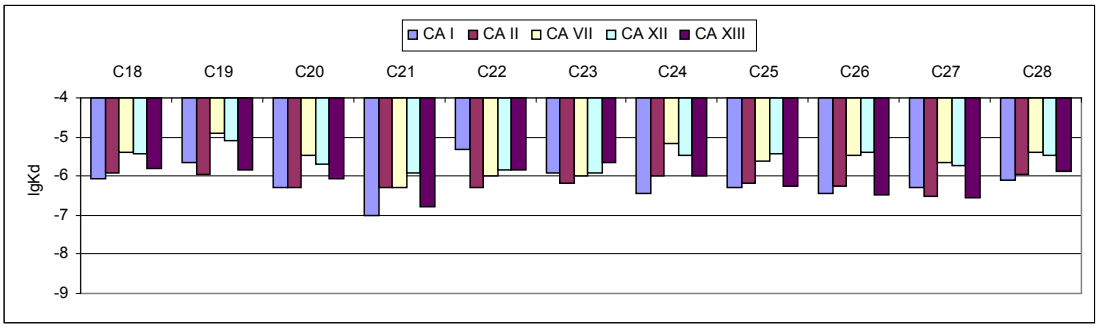


R^1:	OH,	OH,	OH,	OH,	OH,	OH,	Me,	H,	H,	H,	H,
R^2:	H,	Bn,	H,	H,	H,	CO_2Et	H,	Et,	Pr,	Bu,	H,
R^3:	Me	Me	Pr	*t*-Bu	Ph	H	Me	H	H	H	H

Fig. 2.3.2-10. The averages of Kd logarithms of *S*-alkylated pyrimidines **B** for each CA.

Comparing data of 6-methyl-4-oxopyrimidine **B18** derivative with a 4,6-dimethylpyrimidine derivative **B24**, the increase of affinity for CA II, VII and XIII of the latter case (Kd = 16.7 nM, 12.5 nM, 10.0 nM, respectively) and decrease of the affinity for CA XII (Kd = 909 nM) can be observed. Carbon chain elongation in the 5th position **B**(**25-27**) CA inhibitory activity did not significantly affect, but the fact of introduction of carbon chain significantly increases the affinity for CA XIII (Kd = 1.25-2.08 nM) compared with a unsubstituted pyrimidine **B28** (Kd = 16.7 nM). Pyrimidine **B28** becomes more potential inhibitor for CA II and VII (Kd = 11.1 nM, 5.00 nM, respectively), while the affinity of CA I falls down (Kd = 10.0 nM).

Introduction of benzyl group into the 5th position **C19** in oxopyrimidines reduces affinity for CA I, VII and XII about three times, as compared to the 6-methyl derivative **C18** (Fig. 2.3.2-11). The introduction of an ester group (compound **C23)** increases the inhibitory activity of CA II, VII and XII about two - four times when compared with **C18**.



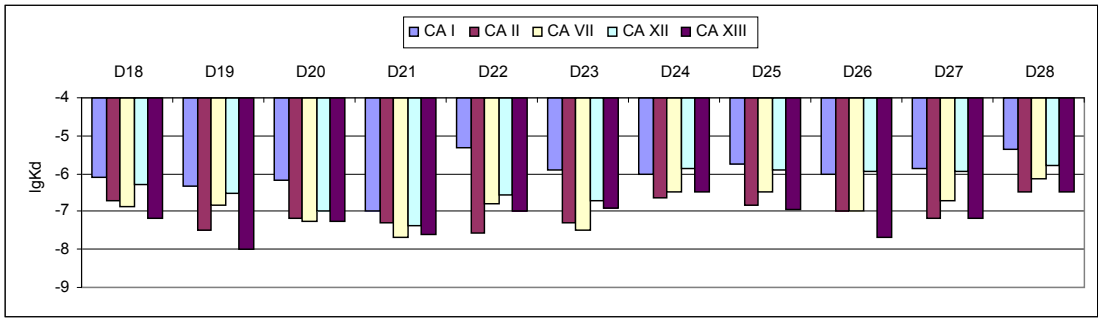
R^1:	OH,	OH,	OH,	OH,	OH,	OH,	Me,	H,	H,	H,	H,
R^2:	H,	Bn,	H,	H,	H,	CO_2Et	H,	Et,	Pr,	Bu,	H,
R^3:	Me	Me	Pr	*t*-Bu	Ph	H	Me	H	H	H	H

Fig. 2.3.2-11. The averages of Kd logarithms of *S*-alkylated pyrimidines **C** for each CA.

We observed an increase in affinity for all CA with oxopyrimidines derivative bearing substituents in the 6th position like methyl **C18**, propyl **C20** or *tert*-butyl **C21.** *tert*-Butyl substituted **C21** of all relevant compounds in the group has the highest CA I and XIII inhibitory activity (Kd = 100 nM, 167 nM, respectively). Phenyl substituted **C22** within the whole group of compounds exhibited a worst CA I inhibition (Kd = 5.00 μM). 4,6-dimethyl derivative **C24** has a similar inhibitory activity as pyrimidines **C28** and **C18.** The introduction of the carbon

chain in the 5th position (**C(25-27)**) improves binding to CA I, II and XIII, but inhibitory activity of these compounds is almost constant (Kd = 370-500 nM (CA I), 313- 667 nM (CA II), 278-556 nM (CA XIII)).

Compound **D28** with a simple pyrimidine substituent has a worst inhibition activity in this group (Kd = 0.333 to 4.55 μM) (Fig. 2.3.2-12). 4,6-dimethylpyrimidine derivative **D24** has a better inhibition of CA I (Kd = 1.00 μM). 4-Oxo-6-methyl derivative **D18**, compared with them, shows the better inhibition of all CA (Kd = 66.7 to 833 nM). Oxopyrimidine with 5-benzyl substituent **D19** becomes better CA inhibitors as compared to **D18**, is particularly enhanced CA II and XIII-inhibitory activity (Kd = 33.3 nM, 10.0 nM respectively). An introduction of ester substituent **D23** increases the affinity for CA VII (Kd = 33.3 nM).



R^1:	OH,	OH,	OH,	OH,	OH,	OH,	Me,	H,	H,	H,	H,
R^2:	H,	Bn,	H,	H,	H,	CO_2Et	H,	Et,	Pr,	Bu,	H,
R^3:	Me	Me	Pr	*t*-Bu	Ph	H	Me	H	H	H	H

Fig. 2.3.2-12. The averages of Kd logarithms of *S*-alkylated pyrimidines **D** for each CA.

We observe increase in affinity for all CA with oxopyrimidines derivative having substitutes in the 6th position lined methyl-**D18**, propyl **D20** or *tert*-butyl **D21** but the best from this trio CA inhibitor compound **D21** case falls selectivity (Kd = 20.0-100 nM). Phenyl substituted **D22** has worst inhibition activity for CA I (Kd = 5.00 μM) in the *S*-alkylated pyrimidine **D** group, but has the best inhibition activity towards CA II (Kd = 26.3 nM). An introduction of a carbon chain (**D(25-27)**) in 5th position of the pyrimidine increases the affinity of CA II, VII and XIII, as compared with the simple pyrimidine **D28**. Of these, the best CA XIII inhibition shows 5-propyl substituted **D26** (Kd = 20.0 nM).

S-alkylated phenylalkylthiols

These compounds with the exception of compounds **D** show better inhibitory properties to CA I (Kd = 1.00-263 nM), where CA I placed among the least inhibited (Kd = 400-833 nM) (Fig. 2.3.2-13). Carbon chain elongation between the sulfur atom and the phenyl ring provides consistent patterns. In all classes of compounds (except **C**) is decreased affinity for CA XII (Kd from 71.4-250 nM to 0.200-1.25 μM) while the inhibitory activity of CA XIII is increased (Kd = from 66.7 to 286 nM to 10.0 -172 nM) (except for **B**). In classes **A** and **D** increase in affinity for CA I is observed (Kd from 6.67-833 nM to 2.33-400 nM, respectively), while the compounds **B** and **C** show the decreased affinity to that CA (Kd of 1.00-100 nM to 1.82-233 nM).

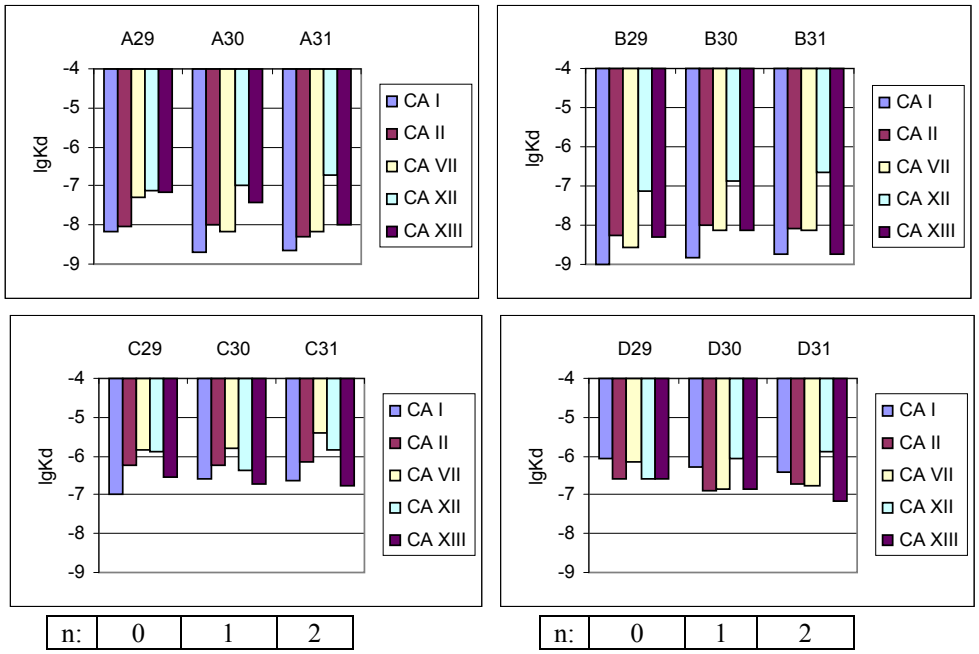


Fig. 2.3.2-13. The averages of Kd logarithms of *S*-alkylated phenylalkylthiols for each CA.

The more expressed inhibitory activity for CA VII is observed only in compounds **A** (Kd from 50.0 to 6.67 nM). The lengthening of the chain on the binding effect to CA II is not obvious.

1,3-thiazole derivatives

Compounds **A** and **B** inhibit best CA I (Kd = 6.67-125 nM), but the affinity of compounds **C** and **D** are significantly reduced (Kd = 1.00-20.0 μM) (Fig. 2.3.2-14). Compounds containing phenylamino substituent **(A-D)34** are weakest CA XII inhibitors(Kd = 8.33-100 μM) among all compounds of this group.

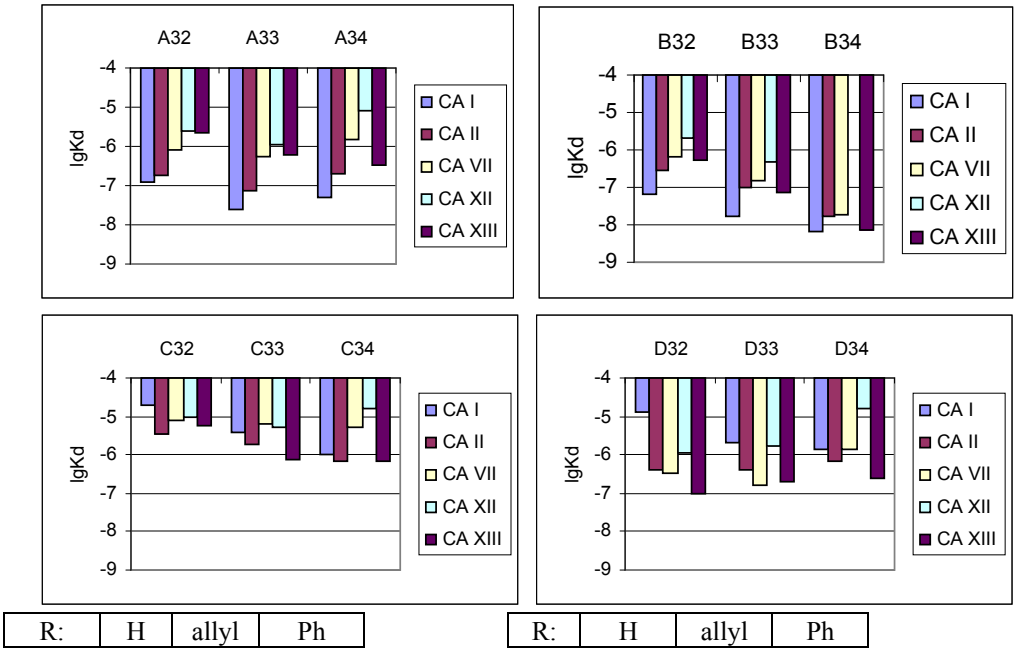


Fig. 2.3.2-14. The averages of Kd logarithms of 1,3-thiazole derivatives for each CA.

Compounds can be sorted by affinity for CA I in ascending order: **32**<**33**<**34**, apparently in all classes (except **A**). In classes **B** and **C,** an increase in affinity for CA II is observed (Kd from 0.286-3.33 μM and from 16.7-667 nM, respectively), while the compounds **D** show

the decreased affinity to CAII (Kd from 400 to 667 nM). In compounds **B,** there is an increased affinity for CA VII (Kd from 667 to 18.2 nM). In classes **A**, **B** and **C,** an increased affinity is observed for CA XIII (Kd from 0.526-5.56 μM and 7.69-667 nM) while in class **D** it is decreased (Kd from 100 to 250 nM).

2.4. Analysis of selectivity

Compounds were selected for each CA that inhibit it more than twice stronger in comparison with the others. The margin of two-fold has been chosen due to the standard deviation of up to 50 percent.

Compounds selective to CA I

The selectivity ratios of the tested CAIs against CA I over the other CAs are bigest with CA XII, while smallest - with CA XIII (Table 2.4-1).

Table 2.4-1. The selectivity ratios of the CA I-selective compounds against CA I over to other CA are given.

	II/I (Kd)	VII/I (Kd)	XII/I (Kd)	XIII/I (Kd)	CA I (Kd, nM)	CA II (Kd, nM)	CA VII (Kd, nM)	CA XII (Kd, nM)	CA XIII (Kd, nM)
B21	36,0	10,0	300	4,50	1,11	40,0	11,1	333	5,00
C9	40,0	200	66,7	11,4	125	5000	25000	8330	1430
A34	4,00	28,6	167	6,67	50,0	200	1430	8330	333
A21	46,7	23,3	100	2,80	1,43	66,7	33,3	143	4,00
B30	7,00	5,38	93,3	5,38	1,43	10,0	7,69	133	7,69
B18	5,32	8,93	83,3	8,33	4,00	21,3	35,7	333	33,3
A31	2,15	2,87	86,0	4,30	2,33	5,00	6,67	200	10,0
A33	2,86	22,2	44,4	25,0	25,0	71,4	556	1110	625
B29	5,56	2,86	71,4	5,00	1,00	5,56	2,86	71,4	5,00
A30	5,00	3,57	50,0	19,2	2,00	10,0	7,14	100	38,5
B17	3,75	9,68	45,5	7,50	66,7	250	645	3030	500
B32	4,29	10,0	30,0	7,89	66,7	286	667	2000	526
B33	6,00	9,23	30,0	4,62	16,7	100	154	500	76,9
B20	11,1	5,00	25,0	8,33	10,0	111	50,0	250	83,3
C29	5,56	14,3	12,5	2,86	100	556	1430	1250	286
C24	2,70	18,0	9,00	2,70	370	1000	6670	3330	1000
A20	4,83	14,5	5,27	3,63	17,2	83,3	250	90,9	62,5

We can exclude 2-isobutyl-benzimidazole **C9** which is the weakest inhibitor of the maximum number of CA (II, VII, and XII, from 40 to 200 times), only CA XIII inhibition of 11.4 times less potent among the CA I selective compounds. 4-*tert*-butyl-6-oxo-pyrimidine **A21** also has a higher selectivity - CA II, VII and XII are inhibited from 23.3 to 100 times less, only CA XIII is inhibited just 2.8 times less potent.

1,3-thiazole **A33** is one of the least inhibited CA XIII in this group (25 times), CA VII and XII are inhibited respectively by 22.2 and 44.4 times less potent, and CA II is inhibited only about three times weaker. 4-*tert*-butyl-6-oxo-pyrimidine **B21** has the biggest Kd ratio against CA I over to CA XII (300 times), it is also 36 times less potent inhibitor of CA II, but CA VII and XIII are inhibited only 10 and 4.5 times less potent.

Compounds selective to CA II

N-alkylated benzimidazole **A1** being a good CA II inhibitor (Kd = 4.00 nM), inhibits other CAs from 5 to 227-fold less potent and is a much better candidate for the CA II selective inhibitor than *N*-alkylated 2-benzyl-benzimidazole **B4** (Kd = 2.86 nM), as that other CAs are inhibited only 2.33 to 58.3 times less potent (Table 2.4-2). *S*-alkylated benzimidazole **D12** and pyrimidine **D22** inhibit CA I 188 and 190-fold less. Compound **D12** inhibit remaining CAs from 3 to 40 times less, and **D22** - from 3.8 to 10.9 times less potent.

Table 2.4-2. The selectivity ratios of the CA II-selective compounds against CA II over to other CA are given.

	I/II (Kd)	VII/II (Kd)	XII/II (Kd)	XIII/II (Kd)	CA I (Kd, nM)	CA II (Kd, nM)	CA VII (Kd, nM)	CA XII (Kd, nM)	CA XIII (Kd, nM)
D16	2,14	30,0	3,00	600	714	333	10000	1000	200000
A1	8,33	5,00	227	20,8	33,3	4,00	20,0	909	83,3
A7	2,67	4,80	240	12,0	11,1	4,17	20,0	1000	50,0
D12	188	7,50	40,0	3,00	3130	16,7	125	667	50,0
D22	190	6,33	10,9	3,80	5000	26,3	167	286	100
A16	2,50	5,71	100	3,08	12,5	5,00	28,6	500	15,4
A25	2,42	4,44	80,0	2,29	30,3	12,5	55,6	1000	28,6
B4	2,92	2,92	58,3	2,33	8,33	2,86	8,33	167	6,67
A28	4,00	4,00	33,3	7,50	66,7	16,7	66,7	556	125
D9	8,67	2,60	5,20	7,43	3330	385	1000	2000	2860

Compounds selective to CA VII

The largest selectivity ratio for CA VII versus other CAs has been mainly observed for CA XII (Table 2.4-3).

We can exclude 4-phenyl-6-oxo-pyrimidine **B22** (Kd = 5.00 nM) which is the weakest inhibitor of the maximum number of CAs (I, II, and XII, from 13.3 to 40 times), only CA XIII inhibition is of about 3-fold lower.

Table 2.4-3. The selectivity ratios of the CA VII-selective compounds against CA VII over to other CA are given.

	I/VII (Kd)	II/VII (Kd)	XII/VII (Kd)	XIII/VII (Kd)	CA I (Kd, nM)	CA II (Kd, nM)	CA VII (Kd, nM)	CA XII (Kd, nM)	CA XIII (Kd, nM)
A4	7,14	5,00	417	14,3	143	100	20,0	8330	286
B1	2,94	2,08	208	4,81	11,8	8,33	4,00	833	19,2
B13	3,33	4,00	76,9	2,22	16,7	20,0	5,00	385	11,1
B12	2,33	2,33	70,0	2,33	33,3	33,3	14,3	1000	33,3
B22	13,3	16,7	40,0	2,86	66,7	83,3	5,00	200	14,3
D11	30,0	2,73	15,0	6,00	1000	90,9	33,3	500	200
A22	14,0	3,50	2,69	3,50	200	50,0	14,3	38,5	50,0

Compounds selective to CA XII

Only one compound - *N*-alkylated 2-benzylbenzimidazole **D4** - inhibits CA XII at least 1.3 times more than any of the CAs (Table 2.4-4). This compound is not very strong CA XII inhibitor (Kd = 769 nM), has similar inhibition for CA XIII. CA II and VII is inhibited 2-4 times less potent, but CA I is inhibited 10.8 times less potent.

Table 2.4-4. The selectivity ratios of the CA-XII selective compound against CA XII over to other CA are given.

	I/XII (Kd)	II/XII (Kd)	VII/XII (Kd)	XIII/XII (Kd)	CA I (Kd, nM)	CA II (Kd, nM)	CA VII (Kd, nM)	CA XII (Kd, nM)	CA XIII (Kd, nM)
D4	10,8	2,03	4,33	1,30	8330	1560	3330	769	1000

Compounds selective to CA XIII

The biggest selectivity ratio for CA XIII versus other CAs is mainly observed for CA I and XII, and the lowest - mostly for CA II and VII (Table 2.4-5).

We can exclude 4-phenylimidazole **D15** (Kd = 90.9 nM) which is the weakest inhibitor of the maximum number of CA (I, II, and VII, from 36.7 to 122 times), only CA XIII inhibition of 11 times less potent among the CA XIII selective compounds. Several of the very best CA XIII inhibiting compounds - 5-propylpyrimidine **B26**, 5-butylpyrimidine **B27** and 5-ethylpyrimidine **B25** (Kd = 1.25 to 2.08 nM) inhibits CA I, II and VII only 3.20 to 13.3 times less potent, CA XII is inhibited from 137 to 276-fold less potent.

Table 2.4-5. The selectivity ratios of the CA XIII selectives compounds against CA XIII over to other CA are given.

	I/XIII (Kd)	II/XIII (Kd)	VII/XIII (Kd)	XII/XIII (Kd)	CA I (Kd, nM)	CA II (Kd, nM)	CA VII (Kd, nM)	CA XII (Kd, nM)	CA XIII (Kd, nM)
B14	20,0	4,29	7,50	1000	66,7	14,3	25,0	3330	3,33
A14	33,3	16,7	11,8	333	333	167	118	3330	10,0
A19	13,3	10,0	57,1	286	33,3	25,0	143	714	2,50
D14	267	2,67	2,67	72,7	6670	66,7	66,7	1820	25,0
B26	4,21	5,71	13,3	276	5,26	7,14	16,7	345	1,25
D15	122	36,7	110	11,0	11100	3330	10000	1000	90,9
B27	3,44	4,23	6,11	196	6,25	7,69	11,1	357	1,82
B25	3,20	3,43	4,80	137	6,67	7,14	10,0	286	2,08
D32	125	4,00	3,33	11,1	12500	400	333	1110	100
D26	50,0	5,00	5,00	55,6	1000	100	100	1110	20,0
D19	45,5	3,33	14,3	29,4	455	33,3	143	294	10,0
D34	5,71	2,67	5,71	66,7	1430	667	1430	16700	250
D2	25,0	5,00	6,25	15,6	10000	2000	2500	6250	400
D31	5,60	2,55	2,33	17,5	400	182	167	1250	71,4
C2	4,55	3,85	8,33	8,33	9090	7690	16700	16700	2000
C33	5,20	2,36	8,13	6,50	4000	1820	6250	5000	769

4,5-diphenylimidazole **A14** (Kd = 10.0 nM) inhibits CA I, II and VII from 11.8 to 33.3 times less, and CA XII - 333 times less potent. The introduction of chlorine substituent into benzenesulfonamide moiety (compound **B14**), improves an affinity not only towards CA XIII

(Kd = 3.33 nM), but towards CA I, II and VII - from 4.72 to 11.7 times - so selectivity properties decreases. Sulfonamide group counterchange with chlorine substituent (compound **D14**), causes decrease of affinity to CA XIII about 8-fold (Kd = 25.0 nM). Also **D14** exhibited the reduced inhibitory activity to CA I (100-fold) and II, VII (3-fold), although the affinity to CA XII slightly increased several times compared with the **B14**. Compound **D14**, compared with **A14** and **B14**, are weaker and less selective CA XIII inhibitor.

Similar trends can be found in 5-benzyl-4-methyl-6-oxopyrimidines **A19** and **D19** pair case.In summary, most of the compounds are possessing selectivity to CA I and CA XIII, while selectivity to CA XII almost is not observed. Although CA II is the most inhibited, but according to the number of CA II selective compounds is in the middle.

The major number of compounds which shows selectivity towards any CA is from class **B**, and **A**. Class **D** compounds does not show selectivity to CA I and II, class **C** does not shows selectivity VII and XII, and class **A** - to CA XII.

Compounds with 1,3-thiazole and phenylalkylthiol moieties exibit selectivity towards most CAs, while *N*-alkylated benzimidazoles appear to be the least selective.

2.5. Comparison of CA inhibitory activity of the synthesized compounds with pharmaceutical drugs.

Binding data of some drugs (Figure 2.5-1) to CA I, II, VII, XII and XIII are given in Table 2.5-1 (VU, Institute of Biotechnology, TSA method).

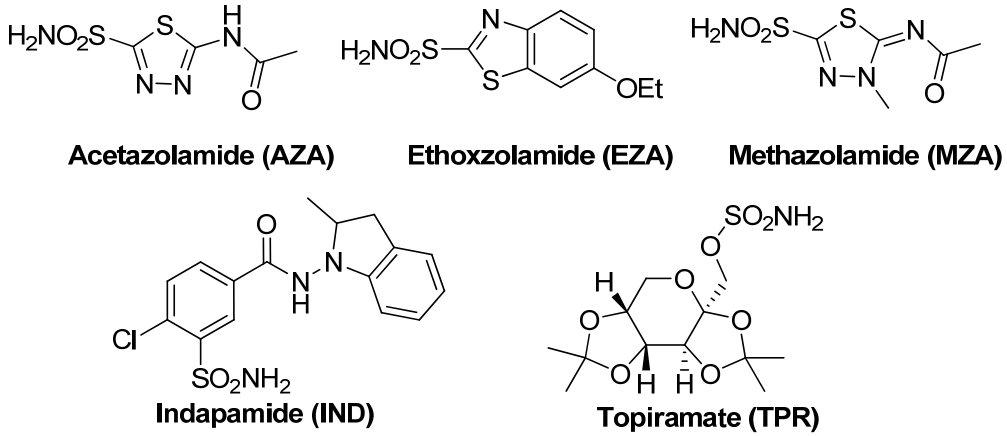


Fig. 2.5-1. Structures of pharmaceutical drugs.

Acetazolamide, methazolamide and ethoxzolamide are used to treat glaucoma via CA II inhibition. 48 compounds synthesized in this work have similar CA II inhibition properties (Kd = 2.70 to 26.3 nM). Eight CA II inhibitors (**A1**, **A7**, **A16**, **A25**, **A28**, **B4**, **D12**, and **D22**) (Table 2.4-2) have a similar or better selectivity towards CA II against CA I, XII, and XIII in comparison with the above mentioned drugs. It is important to note that CA VII is less inhibited (from 2.92 to 7.50 times), while the drugs do not show selectivity to CA II against CA VII. Compound **D12** (Kd = 16.7 nM (CA II)) exhibits higher selectivity than acetazolamide, compound **A1** (Kd = 4.00 nM) and compounds ((**A7**, **A16**, **A28**) (Kd = 4.17 nM, 5.00 nM, 16.7 nM, respectively)) are more selective than ethoxzolamide and methazolamide, correspondingly.

Anticonvulsant drug topiramate is rather selective towards CA VII (Kd = 25.0 nM). 42 compounds synthesized in this work have similar or better CA VII (Kd = 2.50 to 25.0 nM) inhibitory properties than topiramate. CA VII selective compounds (Table 2.4-3) are less selective against CA XIII, and especially against CA I as compared with topiramate. However, they are more selective against CA XII in comparison with this drug. For example, compounds **A4**, **B1** inhibit CA XII 417 and 208 times less potently.

Table 2.5-1. Kd (nM) of drugs binding to CAs and the selectivity ratios are given.

	CA I	CA II	CA VII	CA XII	CA XIII				
AZA	CA II and VII are best inhibited					I/II (I/VII)	XII/II (XII/VII)	XIII/II (XIII/VII)	
	1400	**17,0**	**16,7**	100	50,0	82,4 (83,8)	5,88 (5,99)	2,94 (2,99)	
EZA	CA II and VII are best inhibited					I/II(VII)	XII/II(VII)	XIII/II(VII)	
	7,41	**1,00**	**1,00**	43,5	8,45	7,41	43,5	8,45	
MZA	CA II is best inhibited					I/II	VII/II	XII/II	XIII/II
	58,0	**27,0**	50,0	400	81,6	2,15	1,85	14,8	3,02
TPR	CA VII is best inhibited					I/VII	II/VII	XII/VII	XIII/VII
	58800	227	**25,0**	133	714	2352	9,08	5,32	28,6
IND	CA XIII is best inhibited					I/XIII	II/XIII	VII/XIII	XII/XIII
	10000	300	300	1430	**100**	100	3	3	14.3

The obtained indapamide CA VII and XIII inhibitory activity data shows discrepancies to comparing with the published data[14]. This discrepancy may be due to the different nature of the techniques employed. Indapamide inhibits CA XIII (Kd = 100 nM) better than the other CAs (our data). 71 compounds have a similar and better inhibitory activity towards CA XIII (Kd = 1.25 to 100 nM). Among them, the majority of CA XIII selective compounds (**A14**, **A19**, **B**(**25-27**), **D14**, **D15**, **D19**, **D26**, and **D32**) (Table 2.4-5) exhibits better selectivity ratio against CA II and VII than indapamide.

[14] C. Temperini, A. Cecchi, A. Scozzafava, C. T. Supuran. *J. Med. Chem.* 52 (2), **2009**, 322-328.

3. X-Ray analysis of CA-inhibitor complexes

Crystal structures of compounds **D12**, **D13**, **D24**, **D25**, **A18**, **A27**, **A24**, **A28** and **D1** bound to CA II (PDB codes for the 3M67, 3M96, 3S9T, 3SAX, 3S8X, 3SAP, 3SBH, 3SBI, 3M98), CA II (**A29**, **D19**, **D14**, **B18**, **B24**, **B28** and **C18**), CA XII (**C28**) and CA XIII (**B18**, **B24** and **B28**) (not yet published) were determined by X-Ray crystallography at the Institute of Biotechnology (VU).

3.1. Inhibitor complexes with CA II

There are 16 solved structures of inhibitors bound to CAs in this work. The sulfonamide group of all inhibitors in all crystal structures is bound to the catalytic Zn ion. The position of the benzenesulfonamide moiety is different for each class of compounds **A**-**D**. All pictures in this section were generated using Discovery Studio Visualizer 1.3.

The position of the ligands of the "head" in all five **A**-class structures is almost the same (Fig. 3.1-1). This position is probably defined by hydrophobic contacts with protein side chains. Leu198 supports the benzene ring, while Thr200 and Val121 restrain its mobility from both sides. There are DMSO molecules trapped between the pyrimidine moiety of **A24** or **A28** and the protein, which was carried over from the solution of inhibitors used for soaking CA II crystals. The linker is positioned in such a way that carbonyl is too far away to make hydrogen bonds with polar amino acids of the substrate binding cavity of CA II. The interaction of this group of ligands observed in crystals is mainly hydrophobic.

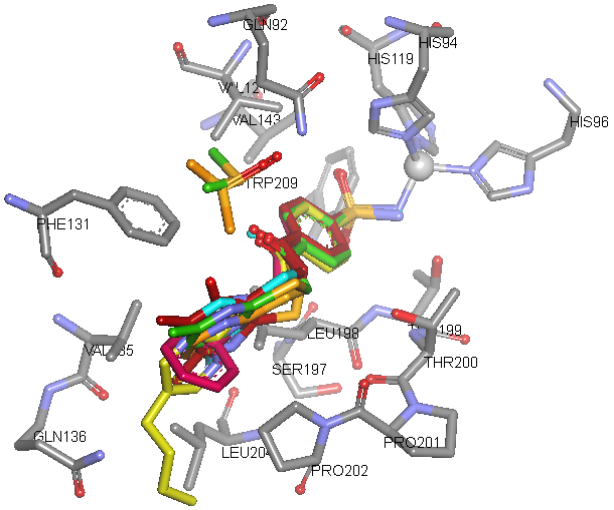


Fig. 3.1-1. The view of compounds **A18** (red, both conformations, the heteroatoms of the second conformation is colored blue), **A24** (green, with DMSO molecule), **A27** (yellow), **A28** (orange with DMSO molecule) and **A29** (rose-coloured) position in the active site of CA II. Protein amino acids are shown in grey. The zinc ion is shown as a grey sphere.

The second part of the ligand **A18** exists in two alternative conformations. The linker on the sulfur atom "splits" into two positions, and pyrimidine rings are positioned relatively to each other in the same plane. In the compound **A29** case is not clear the exact position of the phenyl substitent, figure is showing the most likely position. This phenomenon is observed for the mobile, not fixed part of the molecule, which exists in various positions in the crystal structure.

The benzenesulfonamide moieties of compounds **B** are overlaped in the same position (Fig. 3.1-2). The first ring of compound **B18** is located in two alternative conformations; due to the complexity of the electron-density interpretation for the second part of the molecule the two most likely alternative conformations are shown. The position of the chlorine atom is the same in the ligands **B24** and **B28** case, also in one of the conformation of **B18**. The chlorine atom is found in a hydrophobic pocket formed by residues Leu141, Val143, Val207, Val121 and Leu198.

The benzene ring in another **B18** conformation is turned at an angle of 180°, the chlorine atom is located on the opposite side, between the amino acids Thr200 and His94 side chains.

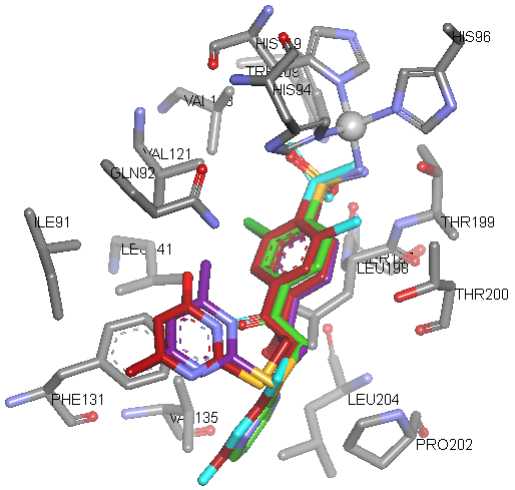


Fig. 3.1-2. The view of compounds **B18** (red, both conformations, the heteroatoms of the second conformation colored blue), **B24** (violet) ir **B28** (green) position in the active site of CA II. Protein amino acids are shown in grey. The zinc ion is shown as a grey sphere.

Comparing compounds **B24** and **B18** (one conformation) with a pair of **B28** and **B18** (the other conformation) compounds is observed the rotation of the carbonyl groups in the linker against each other approximately 90°. The carbonyl oxygen atom in both cases is too far away to make hydrogen bonds with polar amino acids of the substrate binding cavity of CA II.

The pyrimidine rings of the first pair of compounds (**B24** and **B18** (one conformation)) are involved in the interaction with the Phe131 benzene ring and Ile91 hydrophobic chain as well as pyrimidine oxo group makes strong hydrogen bonds (2.4 Å) with a polar amino acid side chain of Gln92. The second pair (**B28** and **B18** (second conformation)) pyrimidine ring falls into the hydrophobic pocket composed of Phe131, Val135, Leu198 and Pr202. The pyrimidine ring is oriented parallel to Phe131 benzene ring in the first pair case, and perpendicular to the Phe131 benzene ring in the second pair case.

There is a single crystal structure of the compounds **C** with CA II. The benzenesulfonamide moiety is observed in two alternative conformations (Fig. 3.1-3).

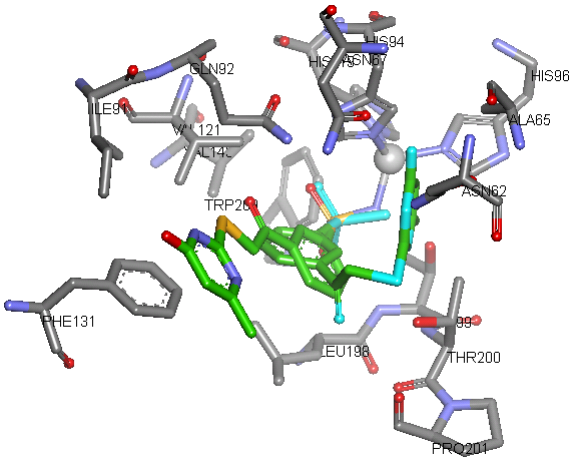


Fig. 3.1-3. The view of compound **C18** (green, both conformations, the heteroatoms of the second conformation is colored blue) position in the active site of CA II. Protein amino acids are shown in grey. The zinc ion is shown as a grey sphere.

The second part of the molecule is visible satisfactorily in one of them, while in other conformation case with a worse electron density, the most likely position (blue heteroatoms) is shown. The benzene rings in the first and second conformations are turned approximately 30°

angle to each other.The benzene ring plane in a better electron density conformation is located between His94 and Leu198, and surrounded by Val121, Leu198 and Thr200 hydrophobic amino acid residues. The carbonyl oxygen of the linker is turned toward Gln92 amino group and forms hydrogen bonds (3.35 Å). The pyrimidine ring is oriented parallel to the Phe131 benzene ring and is situated in the Ile91 hydrophobic chain.

The benzenesulfonamide moiety position in the crystal structures with the inhibitors of class **D** overlaps well (Fig. 3.1-4). The chlorine atom is found in a hydrophobic pocket formed by residues Leu141, Val143, Val207, Val121 and Leu198. The electron density of compound **D14** is observed only in the first ring, while the position of the second part of the molecule is not clear. The linker position is fixed by hydrogen bonds ocurred between carbonyl oxygen atoms and amino acids Gln92 (3.23 Å) and Asn67 (3.52 Å) residues, but the position of the carbonyl group in **D1** is tilted in the opposite direction. In all class **D** structures, with the exception of compound **D1**, near to the linker is monitored DMSO molecule derived from the solvent.

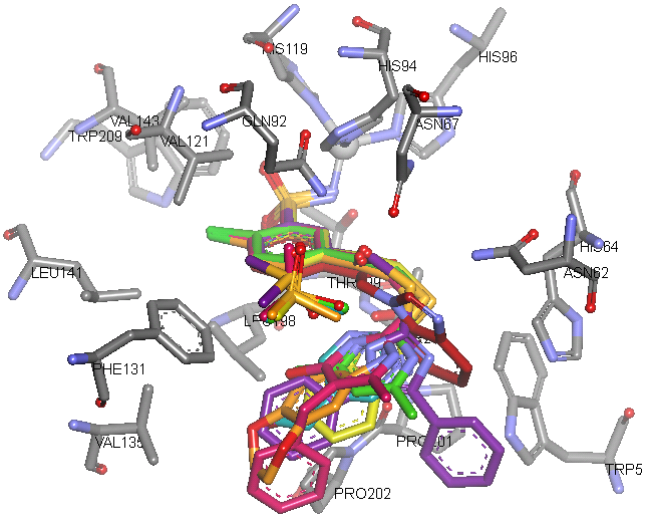
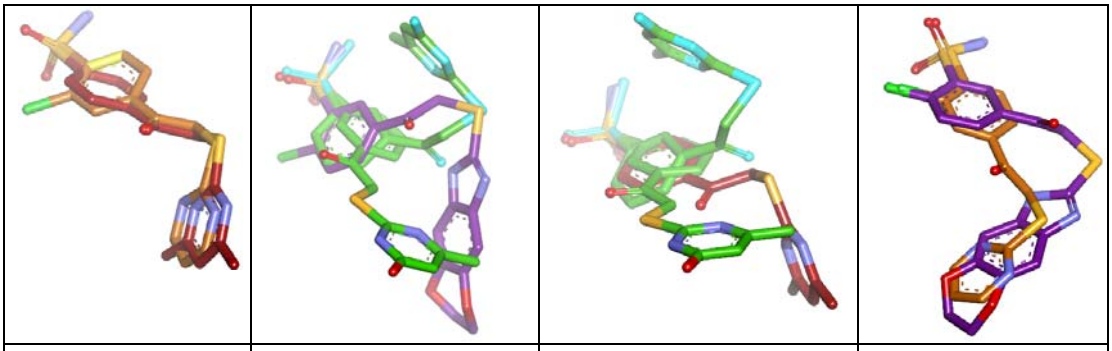


Fig. 3.1-4. The view of compounds **D1** (red), **D12** (orange, with DMSO molecule), **D13** (yellow, with DMSO molecule), **D14** (violet), **D19** (rose-coloured, with DMSO molecule), **D24** (green, with DMSO molecule) ir **D25** (blue, with DMSO molecule) position in the active center of CA II. Protein amino acids are shown in grey. The zinc ion is shown as grey sphere.

The position of the second part of the molecule in all ligands, except **D1** is very similar. This fragment is involved in van der Waals'o interaction with the protein amino acids residues that forms the hydrophobic cavity (Phe131, Val135, Pro202, Leu198 and Thr200). The benzimidazole ring of compound **D1** is surrounded by Asn67, Asn62, His64, Pro201 and Trp5 amino acid residues. The compound **D1** is not effective CA II inhibitor (Kd = 1.56 μM) while other compounds **D12-14**, **D19**, **D24** and **D25** have better CA II inhibitory activity (Kd = 16.7 to 222 nM).

Comparing the **A** and **B** structures (Fig. 3.1-5), the rotation of the first ring relative to one another at an angle of 30° is observed, but the carbonyl positions coincide, and pyrimidine rings are situated in one plane, almost with one another shifted (other class **B** compounds **B18** and **B24** the pyrimidine ring is situated in another position (Fig. 3.1-2).

In **C** and **D** structures the discrepancy in the positions of the first ring is also observed. Its position is overlaped only in class **B** and **D** structures. The chlorine atom being in the *ortho*-position with regard to the sulfonamide group interacts with the amino acids residues in a hydrophobic cavity formed in the active center near the zinc ion.



a: A24 ir **B28**	**b: C18** ir **D12**	**c: A24** ir **C18**	**d: B28** ir **D12**

Fig. 3.1-5. The view of compounds **A24** (red), **B28** (orange), **C18** (green, both conformations, the heteroatoms of second conformation colored blue) ir **D12** (violet) position in the active site of CA II.

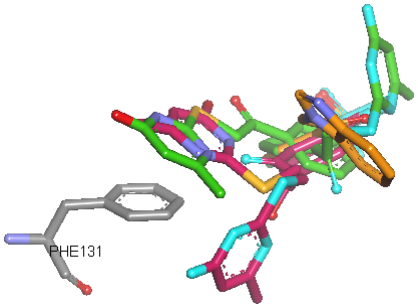


Fig. 3.1-6. The view of compounds **B18** (rose-coloured, both conformations, the heteroatoms of the second conformation is colored blue), **C18** (green, both conformations, the heteroatoms of second conformation colored blue) ir **D1** (orange) position in the active site of CA II. Phe131 is shown in grey.

In most cases, the second part of the molecule is observed in the same proteine site, except for **B24**, **B18** (one alternative conformation) and **C18**, where the second ring lies parallel to the Phe131 benzene ring, and **D1** (shorter linker does not allow a pocket) (Fig. 3.1-6).

3.2. The complex of compound C28 with CA XII

The first ring is located among Leu198, Val121, and Thr200 (Fig. 3.2-1). The carbonyl oxygen atom of the linker make weak hydrogen bonds with Gln92 (3.54 Å) residue. The pyrimidine ring is surrounded with Pro202, Leu198 and Ser135 and fixed by the hydrogen bonds of nitrogen with Ser135 side chains (2.61 Å).

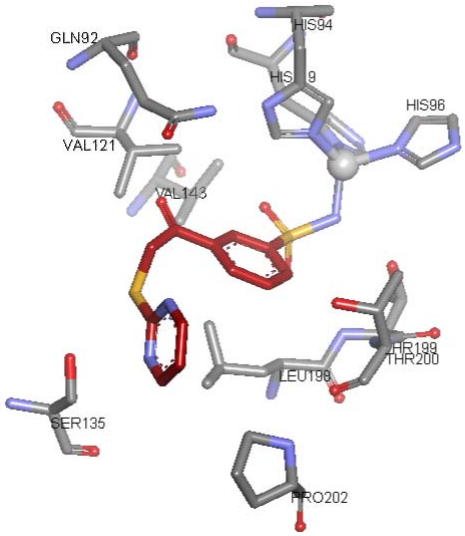


Fig. 3.2-1. The view of compound **C28** (red) position in the active site of CA XII. Protein amino acids are shown in grey. The zinc ion is shown as grey sphere.

Comparing CA II and XII active centers are visible six different amino acid pairs (Fig. 3.2-2). They are located away from the zinc ion. In CA XII active center non-polar Ile91 is replaced with polar Thr91, Phe131 is replaced with smaller side chain Ala13. Instead of non-polar Gly132 and Leu204 the polar Ser132 and Asn204 are located in CA XII. On the opposite side of the active site cavity Asn67 is replaced with basic Lys67, and non-polar Leu60 is replaced with polar Thr60.

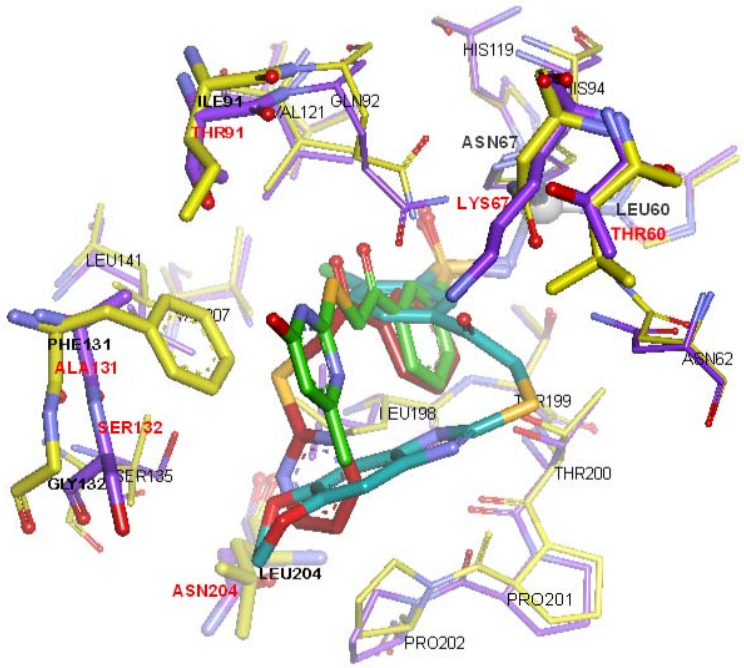


Fig. 3.2-2. The superposition of the active site regions in the CA II (yellow)-**C18** (green, conformer with better electron density) and **D12** (blue) complexes with the CA XII (violet)-**C28** (red) complex. An amino acids pairs with discrepancy are thickened. CA II amino acid names are written in black and CA XII-red colors. The zinc ion is shown as a grey sphere.

The benzene ring is overlapping and the orientation of carbonyl group of linker to Gln92 is similar as determined by the superposition of CA II-**C18** with CA XII-**C28** complexes (Fig. 3.2-2). In CA II-**C18** complex pyrimidine ring interacts with Phe131, while in CA XII-**C28** complex, instead of Phe131 at Ala131, a pyrimidine ring rotates at Ser135, pyrimidine nitrogen atom participates in hydrogen bonding (2.61 Å).

Based on the fact that the first ring surrounding amino acids in both CA isoforms are not different at a given **C28** (CA XII) and **C18** (better electron density conformer) (CA II), the position of the ring of benzenesulfonamides in both ligands overlaps, it can be assumed that the first position of the ring in compounds of other classes in complexes with CA XII also meet in CA II complexes observed position.

Unfortunately, with only one X-ray crystal structure of CA XII with the inhibitor, it is difficult to explain the poor affinity of compound to CA XII in this work study. Maybe the more presence of hydrophilic amino acids Ala131, Ser132 and Asn204 instead of Phe131, Gly132, and Leu204 reduces the hydrophobicity of the protein and it becomes less attractive location for binding of the second molecule fragment of what is observed in almost all well binding compounds to the CA II in their complexes with CA II.

3.3. Inhibitors B18, B24 and B28 complexes with CA XIII

In X-ray crystal structure of CA XIII with the **B18**, a good electron density is observed only in the first ring, while the situation of the second part of the molecule is not clear (Fig. 3.3-1).

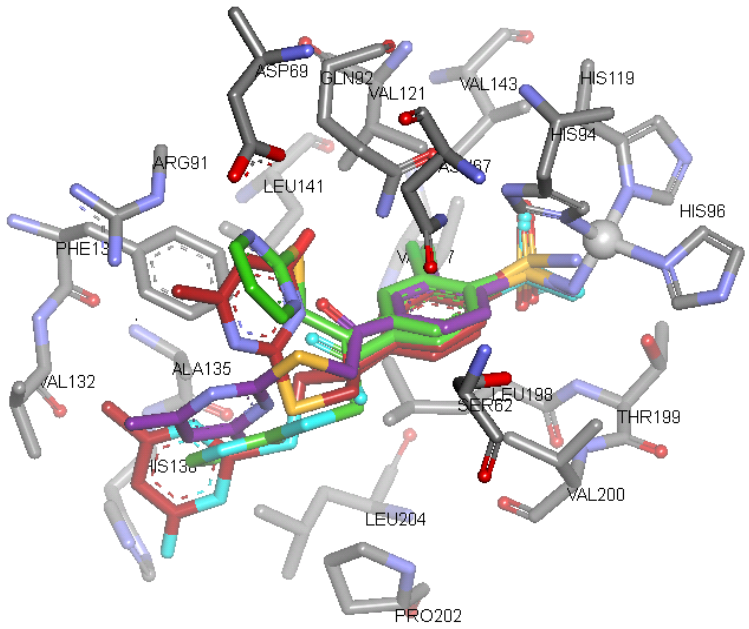


Fig. 3.3-1. The view of compounds **B18** (red, both conformations, the heteroatoms of second conformation is colored blue), **B24** (violet) ir **B28** (green, both conformations, the heteroatoms of second conformation colored blue) position in the active site of CA II. Protein amino acids are shown in grey. The zinc ion is shown as a grey sphere.

It may be distinguished two alternative positions. Figure 3.3-1 shows the most likely positions of **B18**. Two alternative conformations with good electron density are observed in compound **B28** case. In all three compounds structures of class **B** first ring positions are almost overlaped. The chlorine atom is surrounded by hydrophobic Leu198, Leu141, Val143, Val121, and Val207 amino acid residues. The positions of linkers are more distinct making distance from the benzenesufonamide moiety, oxygen atoms of carbonyl groups are directed to Phe131 side, and make a slight hydrogen bonds with Gln92 residue (3.54 Å), or in the opposite direction, and is too far away from the polar amino acids in order to form the hydrogen bonding. The pyrimidine rings are dispersed in space to Asp69, Arg91, Phe131, Ala135, Leu204, Leu198 and Pro202 amino acid side chains.

Comparing CA II and XIII active sites are visible seven different amino acid pairs (Fig. 3.3-2). They are located away from the zinc ion. In CA XIII in place Glu69 is shorter chain Asp69, the non-polar Ile91 is replaced by the strong basic Arg91, non-polar Gly132 and Val135 is replaced by similar non-polar Val132 and Ala135, and polar neutral Gln136 is replaced by the weak basic His136. In the reverse side of the active site polar Asn62 is replaced by a similar Ser62, and polar Thr200 replaced by non-polar Val200.

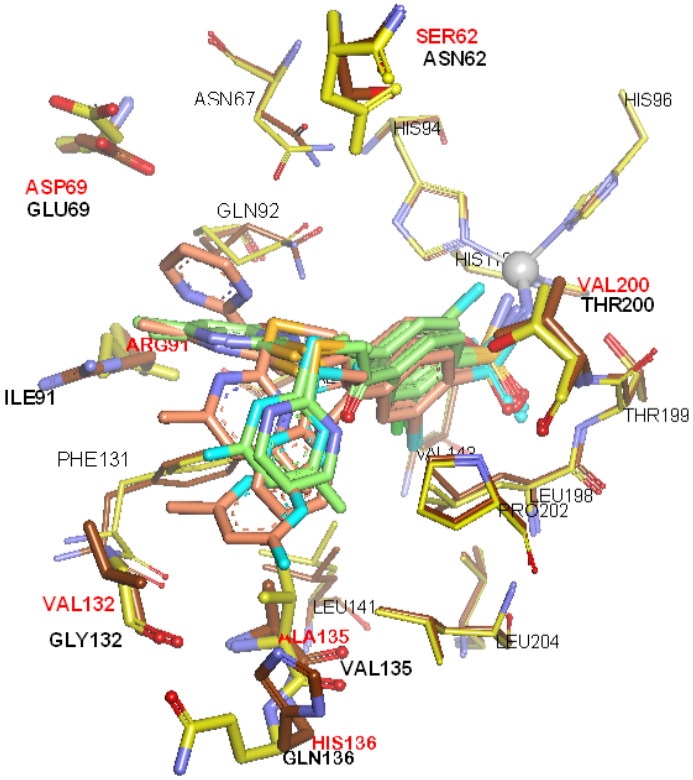


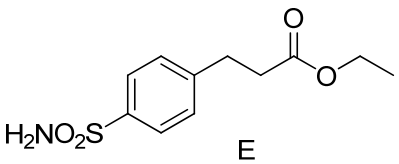
Fig. 3.3-2. The superposition of the active site regions of the CA II (yellow)-**B18, B24**, **B28** (pale green) complexes with CA XIII (brown)-**B18, B24**, **B28** (light brown) complexes. The heteroatoms of the second conformations are colored blue An amino acids pairs with discrepancy are thickened. CA II amino acid names are written in black and CA XIII-red colors. The zinc ion is shown as grey sphere.

In compounds **B18**, **B24** and **B28** complexes with CA II and XIII, the first ring positions are very similar (except for one alternative conformation **B18** in complex with CA II, which benzene ring is turned through 180° against other rings) (Fig. 3.3-2). The presence of chlorine substituent determines the position of the first ring. Since CA II and XIII does not have the differences between the amino acids surrounding the first ring (as well as CA XII), can surmise that the first position of the ring in these three CA could be unique to each class.

3.4. Comparison of CA I and II active site

Without any X-ray crystal structure of CA I-inhibitor in this work, CA II-**A24** complex was compared with CA I-ethyl-3-(4-sulfamoilfenil) propanoate (**E**) (Fig. 3.4-1) complex (Fig. 3.4-2) (Protein Data Bank: www.rcsb.org, PDB id.: 2NN7).

Comparing CA II and I active centers nine different amino acid pairs are visible. Closer to the zinc ion, in the space around the first part of the molecule, Val121 is replaced by a shorter side chain Ala121, polar neutral Asn67 - a weak basic His67, and instead of the polar neutral Thr200 is His200. Away from the active site, the non-polar neutral Ile91, Phe 131, Gly132, Val135, and Leu204 are replaced as well as non-polar neutral Phe91, Leu131, Ala132, Ala135, and Tyr204. Thus, there are different hydrophobic surface slopes in these CA. As far away from the hydrophobic pocket the polar acidic Glu69 is replaced by a neutral polar Asn69.



E

Fig. 3.4-1. The structure of ethyl-3-(4-sulfamoylphenyl)propanoate (**E**).

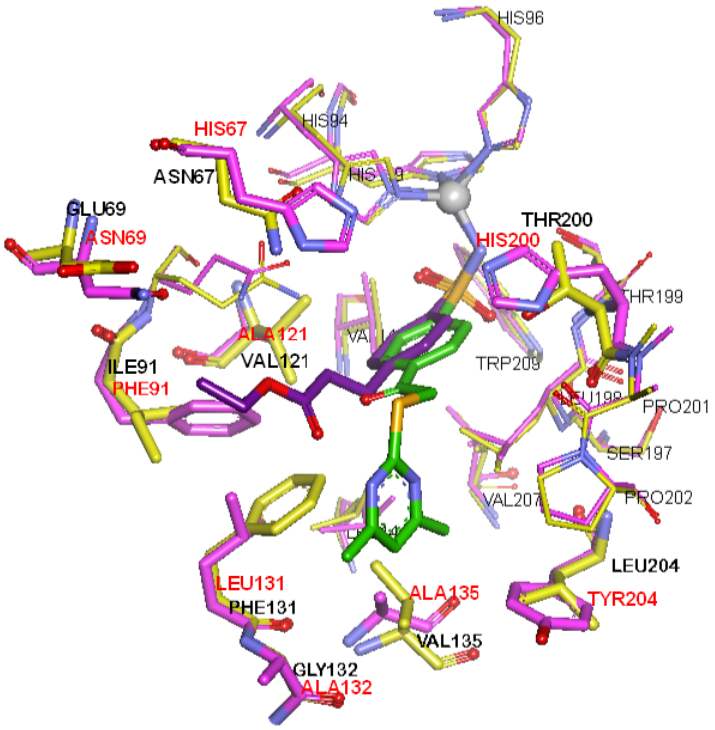


Fig. 3.4-2. The superposition of the active site regions of the CA II (yellow)-**A24** (green) complex with the CA I (pale purple color)-**E** (violet) (2NN7) complex. An amino acids pairs with discrepancy are thickened. CA II amino acid names ae written in black and CA I-red colors. Thes zinc ion is shown as a grey sphere.

The benzenesulfonamide moieties of compound **A24** and **E** are located in a similar plane, but are slightly shifted relative to each other. The pocket that accept chlorine atom remains almost unchanged, but a cause of the other two amino acids (His67 and His200) differences in the first ring environment is difficult to predict the position of the first part of the molecule in **B** and **D** classes' compounds into the active site of CA.

The second part of the compound **E** is more shifted to the Phe91 in CA I active site and the second part of the compound **A24**-located near Phe131 in CA II active site. Looking at the location of the second part of compound **E** to Phe91 could be predicted in this work described the second ring of inhibitors interaction with Phe91.

In X-ray complexes of compounds of class **D** with CA II, the linker of carbonyl group participates in hydrogen bonding with Gln92 and Asn67 residues, but in the CA I active site Asn67 is replaced by more space occupant His67. Also Thr200 (near to linker of class **D** compounds in CA II active site) is replaced by more bulky His200. It can be assumed that the class **D** compounds are undergoing greater disturbance to the active cite of CA but not in CA II. With this it may be explained the reduced affinity of compounds of class **D** to CA I. The most compounds of class **C** have the worst affinity for all the CA, and **D**-much better, but in CA I case is observed the inversion (it is not characteristic only to compounds **D32-34**, with a thiazole fragment, probably due to the absence of the linker).

Conclusions

1. An efficient method for *N*- and *S*-alkylation of benzimidazole derivatives, *S*-alkylation of benzimidazothiadiazole, imidazole, benzothiazole, pyrimidine, phenylalkylthiol derivatives with 3- and 4-(bromoacetyl)-benzenesulfonamides and 4- and 5-(bromoacetyl)-2-chlorobenzenesulfonamides has been developed. It was found that in the *N*-alkylation reaction using an equivalent amounts of benzimidazole and bromoacetophenone along with the main *N*-alkylated product formation of *N*, *N*′-dialkylderivative occurs. Using an excess of benzimidazole formation of byproduct was avoided.
2. 2-[(6-oksopyrimidine-2-yl)sulfanyl]acetylbenzenesulfonamides in dimethyl-sulfoxide solution were found to exists in two forms - open chain and cyclic.
3. The synthesized 136 new compounds were subjected to the studies of their inhibitory activity towards CA I, II, VII, XII and XIII. According to the analysis of binding of the synthesized compounds to CA, CA can be sorted in an ascending order - CA XII <VII <I <XIII ≤ II by binding activity.
4. 4-(Hetarylmethylcarbonyl)benzenesulfonamides exhibit better inhibitory activity towards CA than the 3-(hetarylmethylcarbony)benzenesulfonamides.
5. Sulfonamide group position on the benzene ring is more important for CA binding than the acidic properties of sulfonamide group.
6. Among all compound studied *S*-alkylated phenylalkylthiol derivatives exhibited the best CA inhibitory properties, while 1,3-thiazole derivatives - the lowest. The length of linker between the two molecular fragments is an important for heterocyclic/aromatic moiety interaction with amino acids of protein active site.
7. The inhibitors having the selectivity of any one CA were selected from synthesized compounds. Most of the compounds possess selectivity to CA I and XIII, while selectivity to CA XII almost is not observed. The major number of compounds which shows selectivity towards CA I, II, VII, and XIII is from class **B** and **A**. Compounds **D** do not show selectivity for CA I and II, compounds **C** do not show selectivity for CA VII and XII, and compounds **A** - for CA XII.
8. One third of the synthesized compounds has a similar inhibition to CA II, as currently used drugs acetazolamide, ethoxzolamide, methazolamide, a third - similar inhibition to CA VII as topiramate, and a half of compounds exhibits a similar inhibition to CA XIII as indapamide. Some of the compounds have better selectivity for some CA than currently used drugs.
9. X-ray analysis of some compounds cocrystals with CA II, XII and XIII showed that the binding is determined by interaction of sulfonamide group with the zinc ion and stacking of the second part of molecules with phenyl group of amino acid Phe131 (CA II and XIII). The presence of the flexible linker between the "head" and the "tail" enhances the interaction with the secondary binding site.
10. The benzenesulfonamide moiety position in CA II active site is unique to each class of compounds as shown in the superposition of X-ray cocrystal structures
11. The comparison of CA II active site with CA I, XII and XIII active sites in complexes of CA II, XII, XIII with the synthesized compounds was performed. Decreased 5-substituted-2-chlorobenzenesulfonamides binding affinity to CA I is caused by the arising steric hindrances in the shifted secondary binding site (Phe131 (CA II) is shifted to Phe91 (CA I) comparing with CA II). Decreased affinity of compounds to CA XII, compared with CA II can be explained by environmental change in secondary binding site from hydrophobic to a more hydrophilic and substitution of amino acid Phe131 by Ala131.

List of publications

Articles

1. E. Čapkauskaitė, L. Baranauskienė, D. Golovenko, E. Manakova, S. Gražulis, S. Tumkevičius, D. Matulis. Indapamide-like benzenesulfonamides as inhibitors of carbonic anhydrases I, II, VII, and XIII. *Bioorganic & Medicinal Chemistry*, **2010**, *18*, 7357-7364
2. E. Čapkauskaitė, L. Baranauskienė, A. Zubrienė, G. Tamulaitienė, E. Manakova, V. Kairys, S. Gražulis, S. Tumkevičius, D. Matulis. Design of [(2-pyrimidinylthio)acetyl]benzenesulfonamides as inhibitors of human carbonic anhydrases. *European Journal of Medicinal Chemistry*, **2012**, *51*, 259-270.

International conference abstracts

1. E. Čapkauskaitė, L. Baranauskienė, D. Golovenko, E. Manakova, S. Gražulis, S. Tumkevičius, D. Matulis. Synthesis of Benzimidazole Derivatives as Inhibitors of Carbonic Anhydrases. BOS 2010 International Conference on Organic Synthesis, June 27-30, 2010, Riga, Latvia. Program and abstracts, p. 67
2. A. Zubrienė, L. Baranauskienė, E. Kazlauskas, Z. Toleikis, R. Chaleckis, V., Petrikaitė V. Michailovienė, E. Čapkauskaitė, V. Dudutienė, J. Matulienė, D. Matulis. Drug Binding Energetics by Titration Calorimetry, Thermal and Pressure Shift Assay. COST Action TD0905 Epigenetics - Bench to Bedside, November 22-25, 2010, Brno, Czechia.
3. A. Zubrienė, L. Baranauskienė, E. Kazlauskas, E. Čapkauskaitė, V. Dudutienė, Z. Toleikis, V. Jogaitė, R. Chaleckis, V. Michailovienė, J. Šližytė, J. Torresan, V. Petrikaitė, V.Petrauskas, J. Matulienė, D. Matulis. Thermodynamic-structure correlations of drug lead binding to target proteins. The 66th Calorimetry Conference, June 12 – 17, 2011, Oahu, Hawaii, USA, p. 53
4. V. Jogaitė, A. Zubrienė, L. Baranauskienė, V. Dudutienė, E. Čapkauskaitė, V. Michailovienė, J. Gylytė, H. Šebėka, D. Matulis. Inhibitor binding thermodynamics and stability characterization of human carbonic anhydrases. FEBS satellite CA meeting, June 22-24, 2011, Montecatini, Italia.
5. E. Čapkauskaitė, L. Baranauskienė, A. Zubrienė, G. Tamulaitienė, E. Manakova, S. Gražulis, S. Tumkevičius, D. Matulis. Synthesis of Pyrimidine Derivatives as Inhibitors of Carbonic Anhydrases. 23rd International Congress of Heterocyclic Chemistry, 31 July - 4 August, 2011, Glasgow, UK. Program and abstracts, p. 221
6. E. Čapkauskaitė. Synthesis of benzenesulfonamides containing azaheterocyclic moieties. Paul Walden 7th Symposium in Organic Chemistry, September 12-13, 2011, Riga, Latvia. Conference publication - abstract, Latvian Journal of Chemistry, 2012, vol. 51, Nr.1, p. 54
7. E. Čapkauskaite, J. Gylytė, A. Zubrienė, L. Baranauskienė, A. Smirnov, D. Golovenko, E. Manakova, S. Gražulis, S. Tumkevičius, D. Matulis. Synthesis of *S*-alkylated Benzimidazole and Imidazole Derivatives as Inhibitors of Carbonic Anhydrases. 13th Tetrahedron Symposium, June 26-29, 2012, Amsterdam, The Netherlands, Program and abstracts, P1.78.

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1. E. Čapkauskaitė, S. Tumkevičius, D. Matulis. 2-chlor-5-[(2-pakeistų 1-benzimidazolil)-acetil]benzensulfonamidų sintezė. Scientific conference "Organinė chemija". Book of abstracts. - Kaunas: Technologija, 2009, p. 46-47
2. E. Čapkauskaitė, V. Dudutienė, L. Baranauskienė, D. Matulis. Chinolino darinių, slopinančių karboanhidrazes, sintezė. Scientific conference "Organinė chemija". Book of abstracts. - Kaunas: Technologija, 2009, p. 48-50
3. E. Čapkauskaitė, L. Baranauskienė, S. Tumkevičius, D.Matulis. *N*- ir *S*-[(3-aminosulfonil)-4-chlorbenzoil]metilbenzimidazolai: sintezė ir karboanhidrazes slopinantis aktyvumas. Scientific conference "Organinė chemija". Book of abstracts. - Kaunas: Technologija, 2010, p. 67-68
4. E. Čapkauskaitė, L. Baranauskienė, A. Zubrienė, S. Tumkevičius, D. Matulis. Pirimidino darinių, slopinančių karboanhidrazes, sintezė. Scientific conference "Organinė chemija". Book of abstracts. - Kaunas: Technologija, 2011, p. 15.

Reziume

Karboanhidrazės (CA) (EC 4.2.1.1) yra gyvoje gamtoje paplitę cinko metalofermentai, katalizuojantys grįžtamą anglies dioksido hidratacijos reakciją. Žmogaus organizme nustatyta 15 α-CA izofermentų, įvairiai pasiskirsčiusių organizme. CA veiklos nukrypimai, pvz., padidėjęs aktyvumas, sukelia įvairias ligas. Dėl CA II hiperfunkcijos išsivysto glaukoma, CA IX ir XII yra siejamos su vėžio vystymusi, nerviniuose audiniuose esančių karboanhidrazių - CA II, IV, V, VII ir XIV - veiklos sutrikimai sukelia epilepsiją, migreną. Kaulų audiniuose esančių CA II, IV ir XII aktyvumo padidėjimas sukelia osteoporozę. Šiuo metu medicinoje naudojami CA slopikliai turi trūkumų. Neatrankiai slopindami ne tik su liga susijusią CA izoformą, bet ir daugelį kitų CA izoformų, sukelia įvairius šalutinius poveikius. Todėl skirtingoms izoformoms specifinių ir atrankių slopiklių sukūrimas išlieka labai aktualia ir svarbia užduotimi.

Atsižvelgiant į tai, buvo suformuluotas šio darbo tikslas – potencialių žmogaus karboanhidrazių slopiklių sintezė ir jų struktūros – aktyvumo tyrimas.

CA slopinimo tyrimams buvo susintetinti 136 nauji įvairiais heterociklais pakeisti benzensulfonamidai ir išmatuotas jų CA I, II, VI, XII ir XIII slopinimo aktyvumas bendradarbiaujant su VU BTI mokslininkais, kurie atliko CA slopinimo tyrimus. Paruoštos efektyvios benzimidazolų *N*- ir *S*-alkilinimo, imidazolų, benztiazolo, benzimidazotiadiazolo, pirimidinų, fenilalkiltiolių *S*-alkilinimo 3- ir 4-(bromacetil)benzensulfonamidais bei 4- ir 5-(bromacetil)-2-chlorbenzensulfonamidais metodikos. Nustatyta, kad [(6-oksopirimidin-2-il)sulfanil]acetilbenzensulfonamidai tirpaluose egzistuoja atviroje ir ciklinėje formose. Išnagrinėjus benzensulfonamidinio ir heterociklinio/aromatinio fragmentų struktūros įtaka CA I, II, VII, XII ir XIII slopinančioms savybėms nustatyta, kad benzensulfonamidinės dalies įtaka CA slopinančiam aktyvumui yra didesnė nei heterociklinės/aromatinės dalies. Sulfonamidinės grupės padėties benzeno žiede įtaka jungimuisi prie CA yra reikšmingesnė nei sulfonamidinės grupės rūgštingumo įtaka. Iš visų šiame darbe tirtų junginių *S*-alkilinti fenilalkiltiolio dariniai pasižymi geriausiomis CA slopiklio savybėmis, o 1,3-tiazolo dariniai – prasčiausiomis. Jungtuko ilgis tarp dviejų molekulės fragmentų yra svarbus heterociklinės molekulės dalies sąveikai su baltymo aktyvaus centro aminorūgštimis.

Iš susintetintų buvo junginių atrinkti slopikliai, pasižymintys atrankumu kuriai nors vienai CA ir nustatyta, kad iš visų junginių daugiausiai atrankių yra CA I ir CA XIII, o CA XII atrankių junginių beveik nėra. Daugiausiai kuriai nors CA atrankių junginių yra tarp 4-(hetarilmetilkarbonil)benzensulfonamidų, o analizuojant junginius pagal antrąjį molekulės fragmentą tarp atrankiausių patenka turintys 1,3-tiazolo ir fenilalkiltiolio fragmentą. Nustatyta, kad dalis susintetintų junginių pasižymi geresnėmis CA slopinančiomis savybėmis bei atrankumu kai kurioms CA izoformoms nei šiuo metu naudojami vaistiniai preparatai - acetazolamidas, etoksazolamidas, metazolamidas, topiramatas, indapamidas, arba prilygsta jiems.

Remiantis rentgenostruktūrine CA II, XII ir XIII kompleksų su kai kuriais slopikliais analize, kurią atliko VU BTI mokslininkai, nustatyta, kad sąveiką tarp slopiklių ir CA apsprendžia sulfonamido grupės sąveika su katalitiniu cinko jonu ir antrojo molekulės fragmento (heterociklas/fenilas) sanglauda su antrine atpažinimo vieta, paaiškintas sumažėjęs junginių gimiringumas CA XII, lyginant su CA II, bei sumažėjęs 5-pakeistų-2-chlorbenzensulfonamidų gimiringumas CA I. Parodyta, kad kristaliniuose junginių kompleksuose su CA II benzensulfonamidinio fragmento padėtis aktyviajame centre yra savita kiekvienai junginių klasei.

Curriculum Vitae

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Acknowledgment

This project would not have been possible without the support of many people.

First and foremost I offer my sincere gratitude to the supervisor Sigitas Tumkevičius and consultant Daumantas Matulis for their support, advice and guidance.

In my daily work, I had a great opportunity to work with different groups of scientists and colleagues, whom I would like to thank – Marijona Krenevičienė for NMR spectral measurements and advice, Audronė Karosienė for IR spectral measurements, Asta Zubrienė, Lina Baranauskienė, Joana Gylytė, David Timm, and Miglė Kišonaitė (Department of Biothermodynamics and Drug Design (BDD), Institute of Biotechnology (IBT), VU) for the compound inhibitory activity measurements, Dmitrij Golovenko, Giedrė Tamulaitienė, Alexey Smirnov, Elena Manakova, and Saulius Gražulis (Department of Protein - DNA Interaction, IBT, VU) for crystallographic analysis, Zita Liutkevičiūtė (Department of Biological DNA Modification, IBT, VU) for her help during the HRMS measurements, colleagues from Department of BDD for recombinant CA production and other help, and the people of Liquid Crystals Laboratory (LCL), Institute of Applied Research (IAR), VU, Virginija Dudutienė (BDD, IBT, VU), Algirdas Brukštus, Virginija Jakubkienė, and Milda Malvina Burbulienė for some the initial reagents.

I would like to thank Audrius Zakšauskas (LCL, IAR, VU) and Virginija Dudutienė (BDD, IBT, VU) for valuable advice, optimistic attitude and sharing their experiences at work.

I would also like to thank all of the Faculty of Chemistry colleagues for their help and advice.

Finally, thanks to my family for their patience, understanding and support.