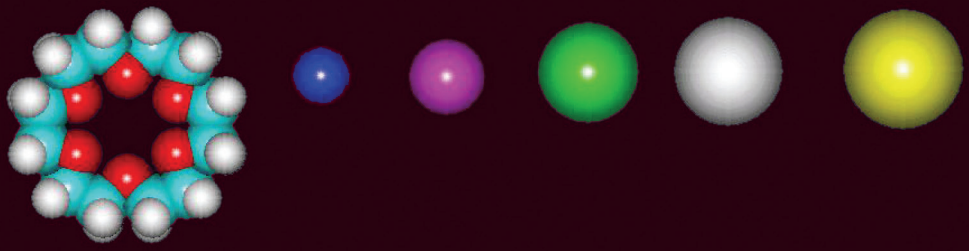

Application of molecular receptors

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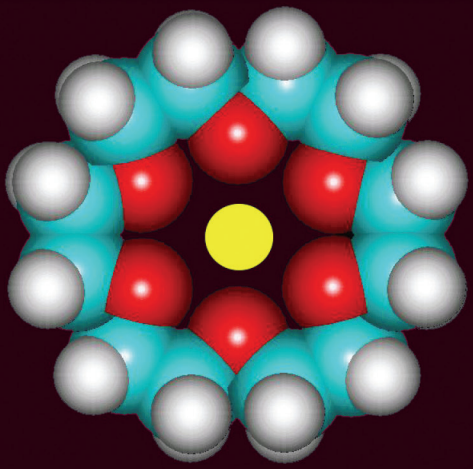

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Contents

List of Contributors

Agnieszka Adamczyk-Woźniak
Warsaw University of Technology
Faculty of Chemistry
Noakowskiego 3
00-664 Warsaw, Poland

Victor Anischenko
L.M. Litvinenko Institute of Physical-Organic and Coal Chemistry
National Academy of Sciences of Ukraine
Department of Spectrochemical Researches
R. Luxemburg 70
81-134 Donetsk, Ukraine

Grażyna Bartkowiak
Adam Mickiewicz University
Faculty of Chemistry
Grunwaldzka 6
60-780 Poznań, Poland

Constantine Chotiy
L.M. Litvinenko Institute of Physica-Organic and Coal Chemistry
National Academy of Sciences of Ukraine
Department of Spectrochemical Researches
R. Luxemburg 70
81-134 Donetsk, Ukraine

Justyna Czupryniak
University of Gdańsk
Department of Chemistry
Sobieskiego 18/19
80-952 Gdańsk, Poland

Aleksey Dykun
L.M. Litvinenko Institute of Physica-Organic and Coal Chemistry
National Academy of Sciences of Ukraine
Department of Spectrochemical Researches
R. Luxemburg 70
81-134 Donetsk, Ukraine

Andrew Redko
L.M. Litvinenko Institute of Physica-Organic and Coal Chemistry
National Academy of Sciences of Ukraine
Department of Spectrochemical Researches
R. Luxemburg 70
81-134 Donetsk, Ukraine

Bogusława Łęska
Adam Mickiewicz University
Faculty of Chemistry
Grunwaldzka 6
60-780 Poznań, Poland

Paweł Niedziałkowski
University of Gdańsk
Department of Chemistry
Sobieskiego 18/19
80-952 Gdańsk, Poland

Izabela Nowak
Adam Mickiewicz University
Faculty of Chemistry
Grunwaldzka 6
60-780 Poznań, Poland

Anna Olejnik
Adam Mickiewicz University
Faculty of Chemistry
Grunwaldzka 6
60-780 Poznań, Poland

Tadeusz Ossowski
University of Gdańsk
Department of Chemistry
Sobieskiego 18/19
80-952 Gdańsk, Poland

Radosław Pankiewicz
Adam Mickiewicz University
Faculty of Chemistry
Grunwaldzka 6
60-780 Poznań, Poland

Andrew Redko
Department of Spectrochemical Researches
L.M. Litvinenko Institute of Physical-Organic and Coal Chemistry
National Academy of Sciences of Ukraine
R. Luxemburg 70
81-134 Donetsk, Ukraine

Volodymyr Rybachenko
L.M. Litvinenko Institute of Physica-Organic and Coal Chemistry
National Academy of Sciences of Ukraine
Department of Spectrochemical Researches
R. Luxemburg 70
81-134 Donetsk, Ukraine

Grzegorz Schroeder
Adam Mickiewicz University
Faculty of Chemistry
Grunwaldzka 6
60-780 Poznań, Poland

Dorota Zarzeczańska
University of Gdańsk
Department of Chemistry
Sobieskiego 18/19
80-952 Gdańsk, Poland

Chapter 1

Phenylboronic compounds as molecular recognition and self-assembling agents

Agnieszka Adamczyk-Woźniak
Department of Physical Chemistry,
Faculty of Chemistry, Warsaw University of Technology

The chemistry of phenylboronic compounds started in 1880 when A. Michaelis and P. Becker described phenylboronic acid (1) for the first time [1]. Most of the properties of boronic acids are derived from the presence of two labile hydroxyl groups. For instance, boronic acids (1) easily undergo spontaneous dehydration that can take place at purifying conditions or under storage, resulting in cyclic boroxines (2). Due to the fact, most of the market-available boronic acids contain various amount of the anhydride (2). As the suppliers claim, the "impurity" does not disqualify the product as the dehydration process is reversible and therefore 1 and 2 can be used interchangeably in most of the cases. As a result of a reversible reaction with other hydroxyl compounds, the corresponding phenylboronic esters (3) are formed. In the case of cis 1,2 and 1,3 diols, the most stable six or five-membered cyclic esters are formed. Distinct classes of boronic compounds are benzoxaboroles (4) –internal hemiesters of 2-(hydroxymethyl)phenylboronic acids, recently rediscovered as biologically active compounds and promising molecular receptors (Fig. 1).

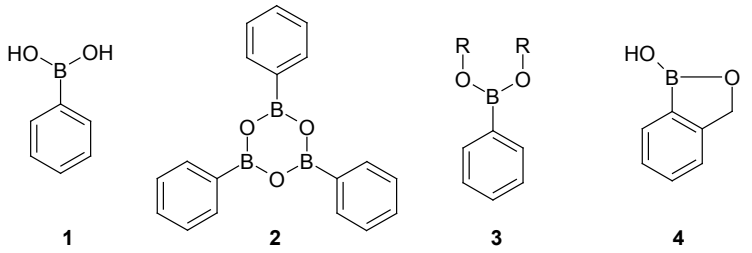


Figure 1. Structures of the considered phenylboronic compounds

Boronic acids (**1**) as well as their esters (**3**) are chemicals of increasing interest due to their wide application in organic as well as in analytical chemistry [2]. Many of them became commercial products due to their wide application as Suzuki coupling agents. Boronic esters (**3**) are in some of the cases even better Suzuki-coupling agents than the corresponding acids, due to their increased stability as well as improved solubility in organic solvents. Benzoxaboroles (**4**) have recently also driven much attention of the researchers, mostly due to their anti-fungal and receptor activity [3].

Boronic compounds, especially *ortho*-substituted boronic acids, display several non-covalent interactions such as hydrogen bonds and donor-acceptor interactions [4] and therefore can be considered as self-assembling agents of huge potential as catalysts, sensors, or new materials [5]. A comprehensive review, covering boronic acids in molecular self-assembly has recently been published by T. D. James and co-workers [6]. The cited review covers such aspects of boronic acids assembly as: molecular imprinting, assembling of monolayers at the air-water interface as well as polymeric systems. Formation of covalent organic frameworks, macrocycles and cages have also been nicely summarized.

1. Boronic acids as molecular recognition agents

1.1. Interactions with hydroxyl compounds

Although the boronic acid-diol interaction is covalent, it is reversible and in rapid equilibrium and thus can be treated analogously to the more classical, non-covalent recognition structures such as hydrogen bonds [7, 8, 9]. The reversibility of the process enables formation of the most stable structures. It also ensures that any errors produced during the assembly process are not permanent [6]. Most of the analytical applications of boronic acids, including sugar sensing, are due to their reversible interaction with hydroxyl compounds with formation of the corresponding boronic esters. The equilibrium in aqueous solution is rather complex, covering both trigonal and tetragonal boronic moiety, which is shown at the probably most frequently cited scheme concerning the subject of boronic acids-diol interaction (Fig. 2) [10].

Several original papers as well as reviews or even books concerning boronic acids as sugar receptors has been recently published [11, 12, 13]. Due to diabetic problems of the humanity, most of the efforts of the boronic acids-scientists concentrate on search for the selective D-glucose receptor. Apart from the most frequently investigated studies in solutions, many researchers built up boronic acids in liquid membranes or as self-assembled monolayers. For example, P. J. Dugan *et al*. [14] have recently reported the usage of lipophilic 2-(aminomethyl)phenylboronic acid (Fig. 3A) for fructose, glucose and

lactose transportation across a thin supported liquid membrane. The resulting membranes are of potential application in food industry. Structures B and C were investigated in analogous research.

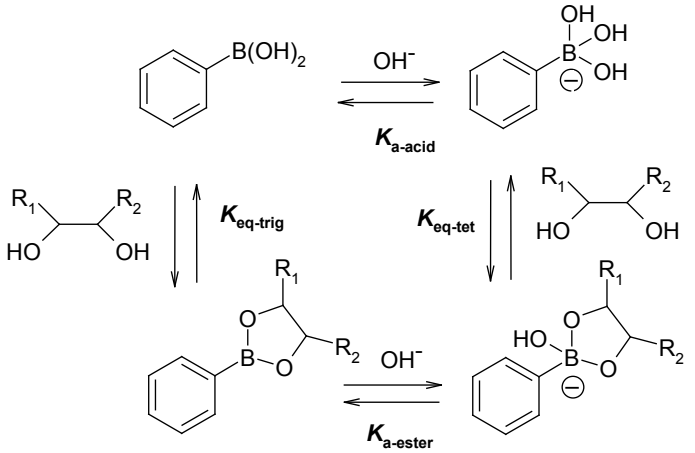


Figure 2. Equilibrium of the boronic acid – diol system in aqueous solution

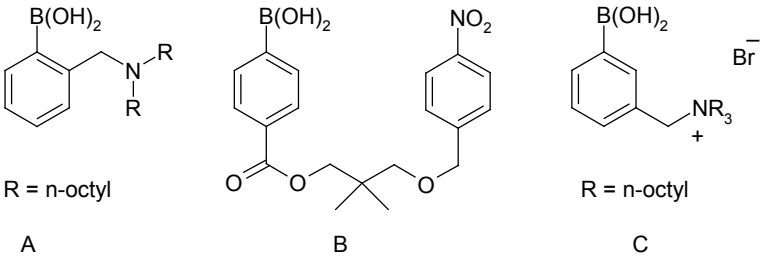
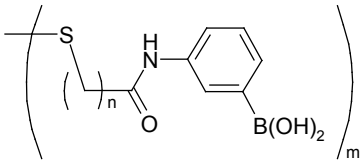


Figure 3. Structures of lipophilic boronic acids used for sugar transportation across liquid membrane [14]

The combination of boronic as well as crown ethers structural motifs were proposed as potential carriers of amino acids across organic membranes. A unique three-component supramolecular complex has been isolated and characterized by X-ray [15].

N. Kanayama and H. Kitano developed a sugar-sensitive self-assembled monolayer at Au surface [16], similar structures have been investigated by H. Chen and co-workers [17]. The saccharide sensing system was sufficiently

useful in detecting monosaccharides (glucose, fructose, galactose, and mannose) even at very low concentrations. Among the three kinds of phenylboronic acid monolayers, monolayer containing structure 3 (Fig. 4) possessing the longest chain displayed the best ordered construction and it showed selectivity for fructose.



m = 2, n = 1, 2 or 3

Figure 4. Structures of phenylboronic acids self-assembled at Au surface [17]

Boronic group forms unique hydrogen bonds with the carboxylic moiety of aminoacids [18] (Fig. 5).

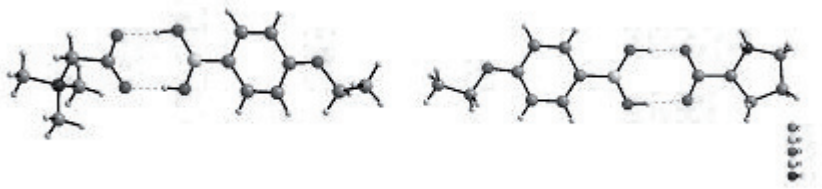
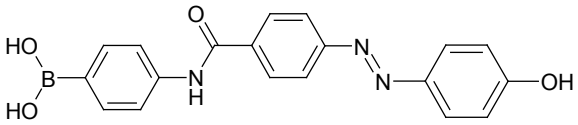


Figure 5. Heterodimeric interactions of phenylboronic acids carboxylic moiety [18]

The feature may result in heterodimers or even polymers [19] in the solid state. Phenylboronic acid moiety itself does not form complexes with metal-ions, however by introducing carboxyl group in the structure, it was possible to obtain metal-organic hybrids with Co(II), Mn(II) and Ni(II) salts [20]. The formation of a 1:2 inclusion complex of γ-cyclodextrin with BA-Azo boronic acid (Fig. 6) turned out to be the basis of glucose selectivity of the resulting supramolecular complex formed in aqueous solution [21].



BA-Azo

Figure 6. Structure of boronic acid that forms complex with γ-cyclodextrin [21]

1.2. Interactions with Lewis bases

Due to electron deficiency of the boron atom, boronic compounds interact with Lewis bases (Fig. 7).

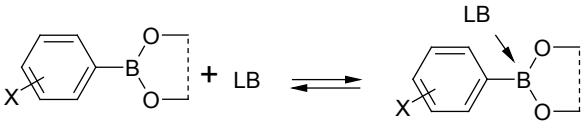


Figure 7. Interaction of boronic compounds with Lewis bases

The useful non-covalent interactions of boronic compounds include binding of anions which is the principle of fluoride anion sensing by boronic acids [22, 23, 24] and esters [25] as well as anion receptor activity of boronic esters [26, 27, 28, 29] and boroxins [30].

2. Common structural motifs in phenylboronic compounds

2.1. Hydrogen bonding

The basic structural motive of phenylboronic acids (1) as well as benzoxaboroles (4) is a dimer with two intermolecular hydrogen bonds [31] (Fig. 8).

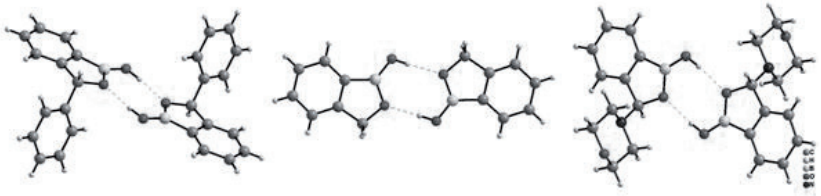


*Figure 8. Dimeric interactions in benzoxaboroles (**4**) [31]*

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In case of benzoxaboroles, only one hydroxy group is present, and hence there is no possibility of lateral hydrogen bond formation to form infinite 2D or 3D networks, as it is observed in the case of phenylboronic acids [32]. The boron center in **4** is always trigonal, but unlike in **1**, the BOO fragment is always coplanar with the phenyl fragment. Substitution at phenyl ring and/or on methylene carbon of oxaborole fragment can influence the molecular interactions both by steric and electronic effects, so more complicated patterns are also observed [3].

2.2. Reversible formation of boroxines

The self-condensation of boronic acid is the ever-concern for the ones

dealing with them, and in many cases it is extremely difficult to obtain spectrum of pure acid or pure boroxin [9, 33]. It is also potential drawback in using boronic acids as supramolecular building blocks [34]. To avoid this, some carry out the crystallization from organic-aqueous solutions [35] or from "wet" solvents [36, 37]. Dehydration of phenyldiboronic acid may however also result in very promising boroxin (**2**) structures, called Covalent Organic Frameworks (COFs) of a surface area and pore volume comparable to those of porous zeolites and carbo-based materials [38]. Similar structures can be obtained by application of the esterification of polyboronic acids with polyhydroxyl compounds [39, 40, 41]. The boroxin ring resulting from 3-pyridineboronic acid was also the basis of a pentameric molecular cage [42].

2.3. N–B dative bond

The Lewis acidity of boronic unit may result in creation of N-B dative bond. Its formation in case of 2-aminomethyl derivatives of phenylboronic acids is said to play a crucial role in action of the compounds as sugar receptors. The N-B dative bond is, however, not so common in crystal structures of the compounds. The nitrogen atom takes part rather in hydrogen bonding with the neighboring oxygen atom [13, 35, 43]. In some cases the formation of N–B dative bond results in self-assembly in the solid state as well as in solution [44]. The N–B dative bond is most commonly present in boroxines, where the formation of hydrogen bonds is impossible. It is quite surprising that despite the presence of three boron atoms in the boroxine ring only one N–B dative bond have been reported in case of intermolecular N-B bond, yet the complexing amine changes its position quickly that all the boron atoms are equal [45, 46]. The reason of that might be the increasing of electron density in boroxin ring and the lowering the Lewis acidity of other boron atoms after coordination of the first boron atom. Surprisingly, in case of boroxines containing *ortho*-aminomethyl substituents, two or even three intramolecular N–B bonds have been observed in crystal structure. The N–B dative bond is said to be favorable in boronic esters [47] and is claimed to be responsible for the enhanced sugar-response in comparison with boronic receptors in which no such an interaction is possible. A four component self-assembly resulted in formation of rotaxanes, that were formed due to the reversibility of the N–B dative bond [48]. Pentameric structures (Fig. 9) have also been obtained [49]. The formation of N–B dative bond resulted also in a deep-purple polymeric material not stable in solution [50].

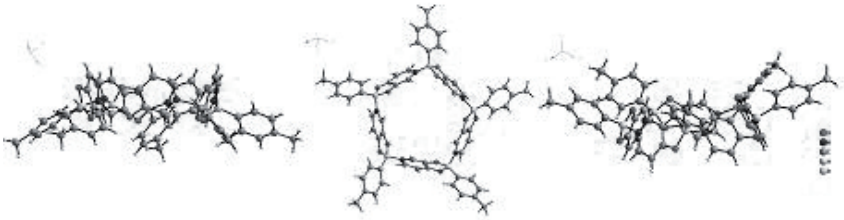

Figure 9. Various views of pentameric rotaxane structure formed due to N-B dative bonds of boronic esters [49]

3. Self-assembly of boronic acids in solution

Boronic acid-containg block-polymers were found to be capable of solution self-assembly into micelles and reverse micelles in response to changes in temperature, pH and sugar concentration [51]. Solution self-assembly of 2-formyl-phenylboronic esters with primary amines was used to enhance boronic Schiff-base formation [52].

4. Boronic acids as building blocks in crystal engineering

Boronic compounds are promissing building-blocks in crystal engineerning [53], mostly due to the formation of hydrogen bonds that seems to be the main force in producing supramolecular species in the solid phase [18]. In case of phenylboronic acids homo- [36, 54, 55] as well as hetero-interactions may take place. *ortho*-Substituted boronic acids seem to be especially prone to creation of supramolecular structures.

Boronic acids are useful building-blocks in the design and synthesis of supramolecular assemblies [56]. Most of the described boronic-assemblies have been created from two or more complementary tectons and belong therefore to the class of multi-component systems. Boron assemblies generated from a single component are very rare so far [42].

Tetraboronic acids for example form open three-dimentional, four-connected networks with significant internal volumes for the inclusion of quests [36]. Investigation of the different structural motifs formed between pyridine and phenyl boronic acids revealed that due to the low H-bonding energy differences, the prediction of the resulting supramolecular structures should be much more difficult than in case of carboxylic acids interactions. Additionally, water molecules play an important role as spacer molecules in the resulting supramolecular structure [57]. Formation of esters and boroxines or/and N–B dative bonds is also useful in crystal engineering. A comprehensive review

covering the subject has been recently published by K. Severin [58]. Careful application of reagents and conditions may result in boronic macrocycles, cages, dendritic structures, rotaxanes or polymers in simple one-pot reactions. For example, condensation of boronic acids with tridendate imine ligands derived from salicylaldehyde and appropriately substituted hydroxylamine resulted in macrocycles with two tetradendate boron centers. The macrocycles are able to act as hosts for primary amines in methanol solutions [59]. By application of amine substituted boronic acid, a calix[3]arene-like shape macrocycles with three boron centers have been obtained [60, 61]. The formation of boronic macrocycles can be based on the formation of covalent [62] as well as coordinating bonds [63, 64, 65]. Condensation of boronic acid with 2,6-dimethanolpyridine resulted in a 20-membered macrocycle [62,66]. Formation of all the macrocycles have been confirmed on the basis of X-ray measurements. Condensation of diboronic acids with tetraols results either in formation of macrocycles or 1-dimentional polymers. Interestingly, the aromatic solvent (benzene) used for crystallization acts as template to control ring size of the formed macrocycle [67]. The condensation of chiral tetrols with diboronic acids resulted in chain polymers of a helical structures [68]. Nanometer-sized macrocycles and cages can be constructed in multicomponent condensation reactions from very simple starting materials such as 2-formyl-phenylboronic acid and 2-aminophenols [69] or differently substituted boronic acids with tetraols [70] (Fig. 10).

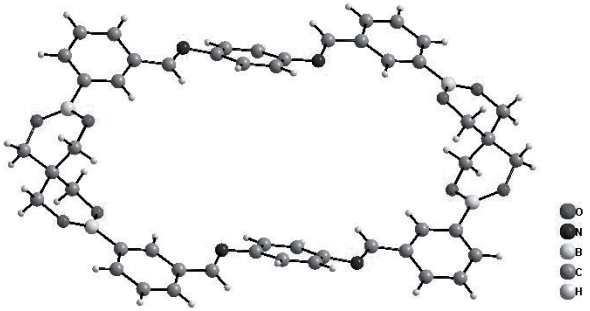

Figure 10. Boronic macrocycle formed by condensation with tetraol [70]

Molecular containers (hemicarcerands) have been obtained in a one-pot reaction through the formation of N–B coordinative bonds. The X-ray structural analysis for one derivative (Fig. 11) showed inclusion of two benzene molecules within the cavity, confirming the capability of the polymacrocyclic compounds to function as molecular receptors [71].

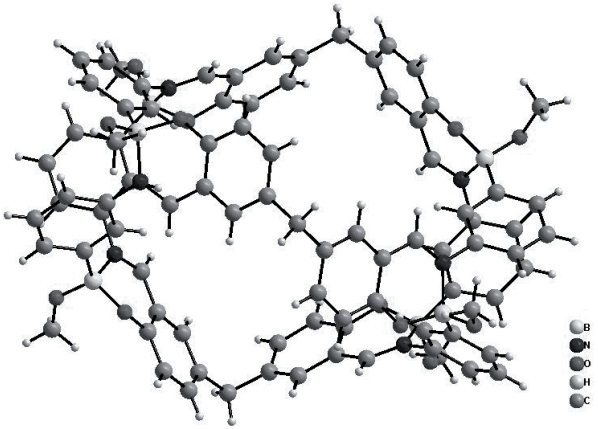

Figure 11. Boronic molecular container formed via N-B coordinative bonds [71]

A two component system of 2-formyl-phenylboronic acid and 1,2-aminoalcohols resulted in formation of dynamic covalent self-assembled macrocycles of containing O-B-O-B-O bridging with a potential binding cavity [8]. The formation of a dynamically controlled capsule via boronate esterification templated by tetrabutylammonium cation [72] has also been reported. Supramolecular triangles [73] and cobaloxime molecular boxes [74] have also been obtained. Reaction is strongly pH dependent, and the formation of the complexes requires a neutral medium. They precipitate at pH = 7 and dissolve after acidification as well as upon alkalization.

Boronic acids have been used as templates in directing the synthesis of multinuclear complexes of cobaloximes [75]. Boronic derivative of pyridine has been used as template in the self-assembly of porphyrin molecules [7].

Due to the large variety of commercially available boronic acids, as well as unique properties of the obtained materials, the application of boronic compounds in supramolecular chemistry is highly likely to receive further increasing interest.

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Chapter 2

The cyclen – amino acid and cyclen – peptide conjugates with methylenecarbonyl linker. Theoretical study of the biological activity

Grażyna Bartkowiak and Grzegorz Schroeder
Adam Mickiewicz University, Faculty of Chemistry, Grunwaldzka 6, 60-780 Poznań, Poland

The biological proprieties of chemicals are determined by structures of compounds and substituents present in molecules.

Polyazamacrocycles (for example cyclam i.e. 1,4,8,11-tetraaza-cyclotetradecane, cyclen i.e.1,4,7,10-tetraazacyclododecane and others aza analogues of the crown ethers) are of interest as starting materials to produce specific chelating agents [1], used in synthesis of MRI contrast factors [2] and in radiopharmaceutical chemistry [3, 4]. Complexes of cyclen derivatives with $^{90}Y^{3+}$ are used in cancer radioimmunotherapy [4]. Generally this class of compounds has found many applications in diagnostic imaging technique and for the medication as therapeutic agents and auxiliary pharmaceutics.

One of the strategies in drug research and development is to find new uses for the old drugs or to link chemical compounds with the known pharmaceutical activity in order to obtain new biologically active substances. Several computer programs are used to predict the possible activity of new compounds designed like this.

Polyazamacrocycles as well as amino acids exhibit various kinds of interesting activities which are already proven. Lipophilic derivatives of cyclam, in contrast to non-lipophilic analogues, were found to be inhibitors of tumor cell (mouse leukemia L1210) growth *in vitro* [5]. Cyclen analogues exhibit antiparasitic [6, 7], antihelmintic and antiarthritic activity and it seems highly probable that they can find many more applications as pharmacologically active compounds or as new drugs precursors.

Biological activity of many amino acids is also known, especially for these ones which contain additional amino functionalities, like lysine and arginine. Both of these compounds are antiviral agents and arginine has additionally antitoxic effect. However, many types of bioactivity are possible for these

compounds but they are not revealed yet or there was no research performed in such area. It seems also interesting how the activity of these compounds would change when they were used as building blocks to form larger, covalently bound units like peptides or peptide-cyclen conjugates. The activity of so built molecules can be prognosed by means of computer-assisted methods [8].

The computer-aided prediction of possible activities of resulting compounds has recently attracted a lot of attention [9] as a method, which enables to save a lot of time and financial expenses (unavoidable during biological tests *in vivo* and *in vitro*) and makes possible to eliminate potentially toxic, harmful or pharmacologically non-safe compounds.

Among the computer programs used for estimating the biological activity spectrum of substances the special role plays PASS (Prediction of Activity Spectra for Substances). A basic principle of this program is that the activity of the chemical compound is a function of its structure. This will imply the conclusion that by comparing the structure of a new substance with the structures of compounds with known activity it is possible to predict if a new compound may be useful for the treatment of particular disease.

The computer system PASS Inet encloses about 1000 possible activities, i.e. pharmacological effects, mechanisms of action and adverse side effects (like toxicity, mutagenicity) and predicts biological activity spectrum of compound studied on the basis of its structural formula. The leave-one-out (LOO) cross-validation has been used to validate this prediction method. The prediction is based on the training set containing over 50 000 compounds of known biological activity. The mean accuracy in LOO cross-validation is about 87% and the activity is described qualitatively (active or inactive). The results of prediction are presented as the lists of activities with appropriate Pa (probable activity) and Pi (probable inactivity) in descending sequence of the difference (Pa-Pi) > 0.

If Pa for the particular activity type is over 0.7 the compound is very likely to exhibit this kind of activity in tests but it is also highly probable that similar pharmaceutical agent are already known. If $0.5 < Pa < 0.7$ the compound is likely to reveal this type of activity, but with lower probability, and the compound is supposedly not very similar to the known pharmaceutical agent. If $Pa < 0.5$, the compound is unlikely to reveal this kind of activity, but if this activity would be confirmed in experiment the compound might be a precursor of the new class of pharmacologically valuable compounds.

In order to design and synthesize novel biologically active compounds, the building blocks of established biological activity have been used, i.e. simple amino acids (lysine and arginine), peptides consisting of 2-4 identical amino acid units and 1,4,7,10-tetraazacyclododecane (cyclen). It seemed interesting

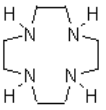
what kind of effects will have the introduction of short peptidic side chain into azamacrocycle like cyclen on the selected types of bioactivity. It is strongly suggested the elongation of peptide chain would change smoothly the lipophilicity and bioavailability of compounds yielded.

Several kinds of bioactivity for cyclen, lysine and arginine are detected in biological tests. Much more types of activity are expected and prognosed on the basis of their structural characteristics using PASS program. The list of possible activities, postulated for above-mentioned building blocks is very long, for example:

compound	number of predicted types of activity	
	Pa>0.3	Pa>0.7
cyclen	231	29
lysine	759	108
arginine	566	55

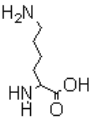
It means that, according to PASS, cyclen can exhibit 231 types of activity with Pa>0.3 and 29 with Pa> 0.7, while lysine 759 and 108 and arginine 566 and 55, respectively.

The synthetic di-, tri- and tetrapeptides, cyclen – amino acid and cyclen – peptide conjugates with methylenecarbonyl linker of structures shown below were obtained:



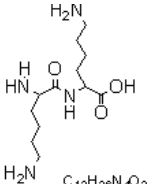
$C_8H_{20}N_4$
Exact Mass: 172,17
Mol. Wt.: 172,27
C 55,78; H 11,70; N 32,52

1 cyclen



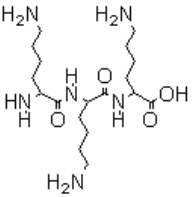
$C_6H_{14}N_2O_2$
Exact Mass: 146.11
Mol. Wt.: 146.19
C, 49.30; H, 9.65; N, 19.16; O, 21.89

2 Liz



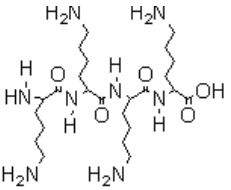
$C_{12}H_{26}N_4O_3$
Exact Mass: 274.20
Mol. Wt.: 274.36
C, 52.53; H, 9.55; N, 20.42; O, 17.49

3 Liz2



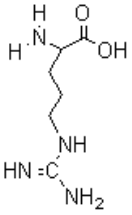
$C_{18}H_{38}N_6O_4$
Exact Mass: 402.30
Mol. Wt.: 402.53
C, 53.71; H, 9.52; N, 20.88; O, 15.90

4 Liz3



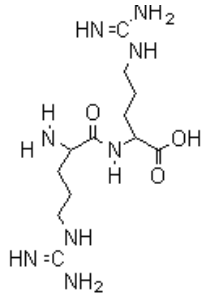
$C_{24}H_{50}N_8O_5$
Exact Mass: 530.39
Mol. Wt.: 530.70
C, 54.32; H, 9.50; N, 21.11; O, 15.07

5 Liz4



$C_6H_{14}N_4O_2$
Exact Mass: 174,11
Mol. Wt.: 174,20
C 41,37; H 8,10; N 32,16; O 18,37

6 Arg1



$C_{12}H_{26}N_8O_3$
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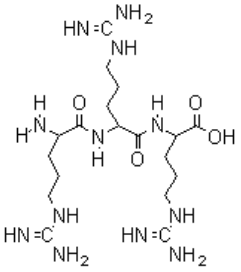
Mol. Wt.: 330,39
C 43,62; H 7,93; N 33,92; O 14,53

7 Arg2



$C_{18}H_{38}N_{12}O_4$
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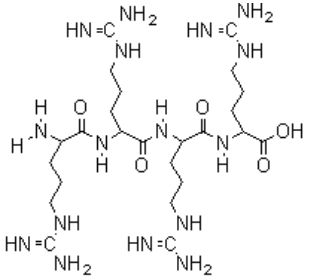
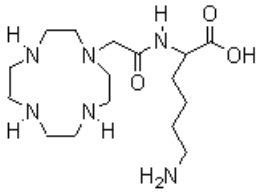
Mol. Wt.: 486,57
C 44,43; H 7,87; N 34,54; O 13,15

8 Arg3



$C_{24}H_{50}N_{16}O_5$
Exact Mass: 642,42
Mol. Wt.: 642,76
C 44,85; H 7,84; N 34,87; O 12,45

9 Arg4



$C_{16}H_{34}N_6O_3$
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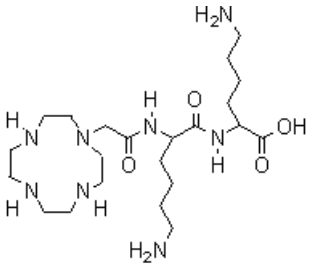
Mol. Wt.: 358.48
C, 53.61; H, 9.56; N, 23.44; O, 13.39

10 cyclen/Liz1



$C_{22}H_{46}N_8O_4$
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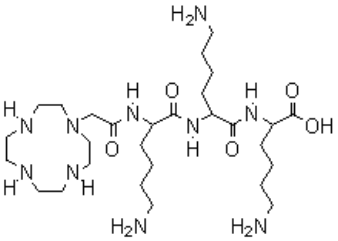
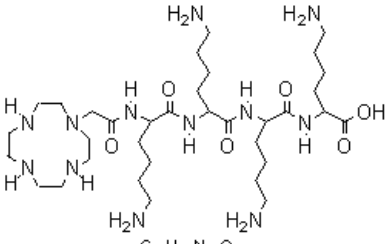
Mol. Wt.: 486.65
C, 54.30; H, 9.53; N, 23.03; O, 13.15

11 cyclen/Liz2



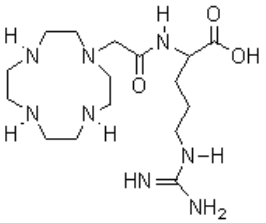
$C_{28}H_{58}N_{10}O_5$
Exact Mass: 614.46
Mol. Wt.: 614.82
C, 54.70; H, 9.51; N, 22.78; O, 13.01

12 cyclen/Liz3



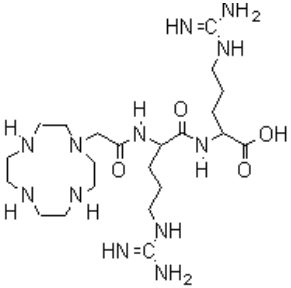
$C_{34}H_{70}N_{12}O_6$
Exact Mass: 742.55
Mol. Wt.: 743.00
C, 54.96; H, 9.50; N, 22.62; O, 12.92

13 cyclen/Liz4



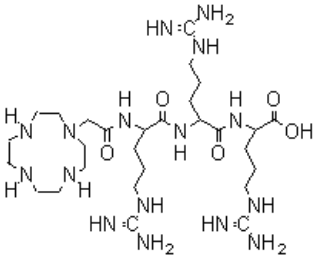
$C_{16}H_{34}N_8O_3$
Exact Mass: 386.28
Mol. Wt.: 386.49
C, 49.72; H, 8.87; N, 28.99; O, 12.42

14 cyclen/Arg1



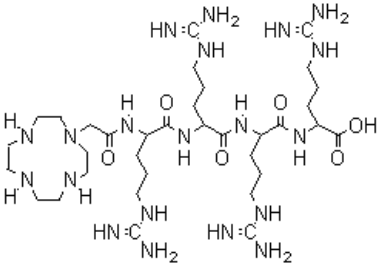
$C_{22}H_{46}N_{12}O_4$
Exact Mass: 542.38
Mol. Wt.: 542.68
C, 48.69; H, 8.54; N, 30.97; O, 11.79

15 cyclen/Arg2



$C_{28}H_{58}N_{16}O_5$
Exact Mass: 698.48
Mol. Wt.: 698.86
C, 48.12; H, 8.37; N, 32.07; O, 11.45

16 cyclen/Arg3



$C_{34}H_{70}N_{20}O_6$
Exact Mass: 854.58
Mol. Wt.: 855.05
C, 47.76; H, 8.25; N, 32.76; O, 11.23

17 cyclen/Arg4

From among the theoretically possible types of activity the ones of high probability have been chosen, i.e. antineoplastic, anticoagulative, several kinds of psychotropic, antiinflammatory, antiviral (different types of viruses), antiasthmatic, autoimmune disorder and sickle-cell anemia treatment as well as toxicity. The possible activity of substrates and compounds obtained was predicted *in silico* [10] and subsequently the comparison was performed how the predicted activity will change for cyclen, lysine (Lys1), peptides containing 2 (Lys2), 3 (Lys3) or 4 (Lys4) lysine molecules, arginine (Arg1), peptides containing 2 (Arg2), 3 (Arg3) or 4 (Arg 4) molecules of arginine, cyclen-lysine conjugates (cyclen/Lys1, cyclen/Lys2, cyclen/Lys3, cyclen/Lys4) and cyclen-arginine conjugates (cyclen/Arg1, cyclen/Arg2, cyclen/Arg3 and cyclen/Arg4) where the binding group between nitrogen atom of cyclen and NH group of amino acids or peptide is CH_2CO.

The relations obtained for different types of activity are visualised in Figures 1 – 10:

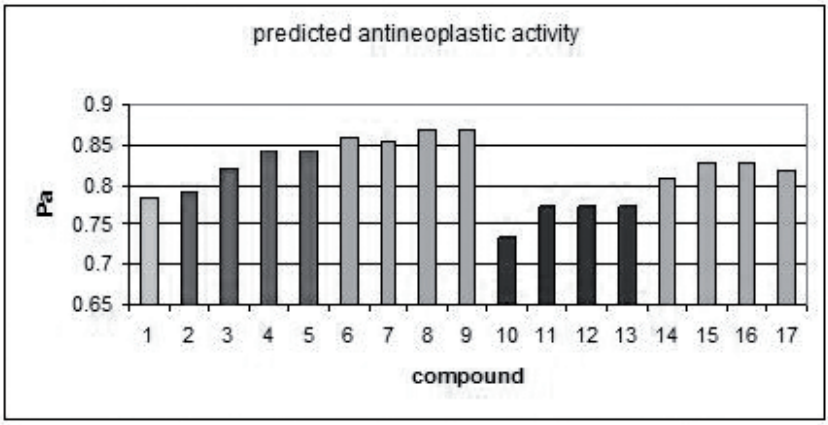


*Figure1. Prediction of antineoplastic activity for compounds studied (***1** – **17***)*

There is a high probability that cyclen will have antineoplastic activity (Pa 0.78). For lysine is this probability slightly higher and it increases with the next two molecules of lysine added into peptide chain. The predicted antineoplastic activity of arginine is higher as for cyclen and lysine and the highest for 3-4 arginine-containing peptides. The cyclen-amino acid or cyclen-peptide conjugates show lower predicted activity than starting compounds.

The similar relations, with small differences, can be observed for the prognostic antiinflammatory (Figure 4), antiasthmatic (Figure 6) and autoimmune

disorder treatment (Figure7) activity. A bit surprising general trend is that the cyclen conjugates are supposed to be less active than a free cyclen and less than the building amino acids or peptides.

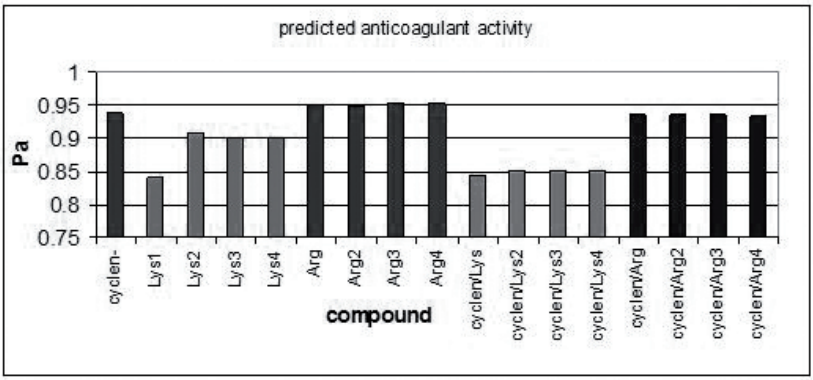


Figure 2. Predicted anticoagulative activity for the compounds studied

The presumption can be made that all compounds of the group studied are able to be good anticoagulants ($P_A > 0.8$) but the most prospective seem to be arginine derivatives (Figure 2) and the arginine itself.

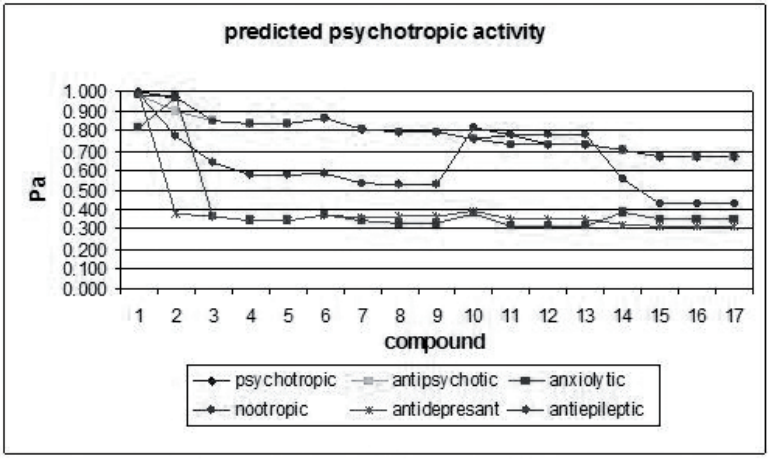


Figure 3. Probability of different types of psychotropic activity

Figure 3 presents the results of psychotropic activity prediction in general and for specific types of such an activity, i.e. antipsychotic, anxiolytic, nootropic, antidepresant and antiepileptic. Cyclen itself exhibits high Pa as antipsychotic and antidepressant factor, but these Pa values are lower for lysine, arginine and respective peptides. The probable anxiolytic activity is high (>0,8) only for cyclen and lysine and for all remaining compounds is much lower, so these compounds do not promise themselves too well as anxiolytics. The cyclen-lysine conjugates seem to be prospective antiepileptic agents.

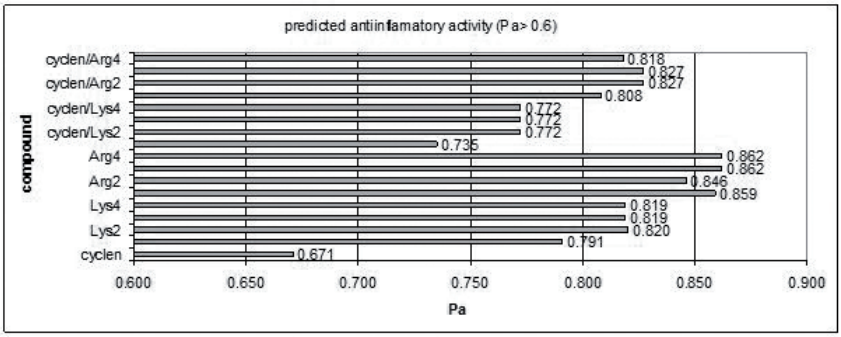


Figure 4. Predicted antiinflammatory activity of compounds **1** – **17**

All the compounds of interest may be likely efficient antiinflamatory agents with Pa > 0.67 (Figure 4). The highest probability of such working (Pa 0.846 – 0.862) is prognosed for arginine-containing peptides (Arg1 – Arg4).

Figure 5 shows general antiviral activity prediction results and a comparison of the activities against selected types of viruses (influenza, hepatitis, herpes and HIV). It is worth to notice that all lysine- or arginine-containing compounds are likely to act as anti-hepatitis drugs.

The anti-HIV activity is postulated mainly for simple peptides studied, and not for the ones bound with the azamacrocyclic compound.

Prognosed antiasthmatic activity of lysine- and arginine-containing peptides is high (Pa over 0.78, Figure 6). However, for cyclen-peptide conjugates the probability of being active against asthma is lower than for each peptide alone.

Figure 7 presents possibility of usage of the compounds studied in autoimmune diseases therapy i.e. in the treatment of diseases connected with overactive immune response of the body against its own tissues. The most prospective agents against autoimmune diseases seem to be lysine, arginine and their synthetic peptides. The functionalization of these compounds with

polyazamacrocyclic ring lowers the probability of being active towards immune system.

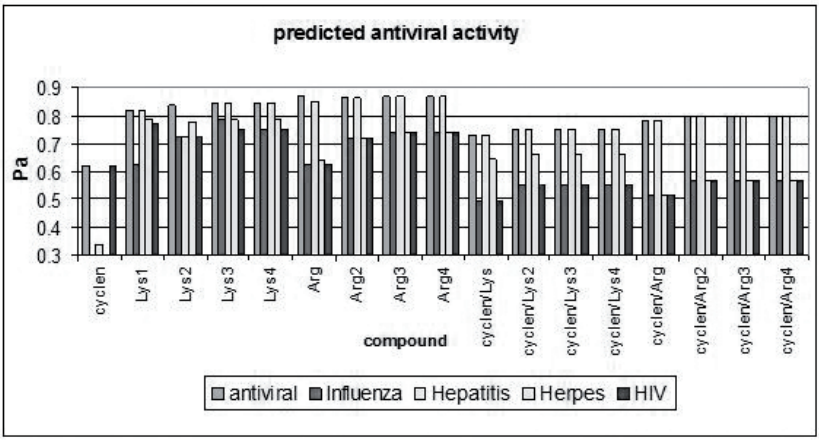


Figure 5. Results of antiviral activity prediction against various types of viruses for compounds **1** – **17** *(common antiviral activity and prognesed activity agains influenza, hepatitis, herpes and HIV viruses)*

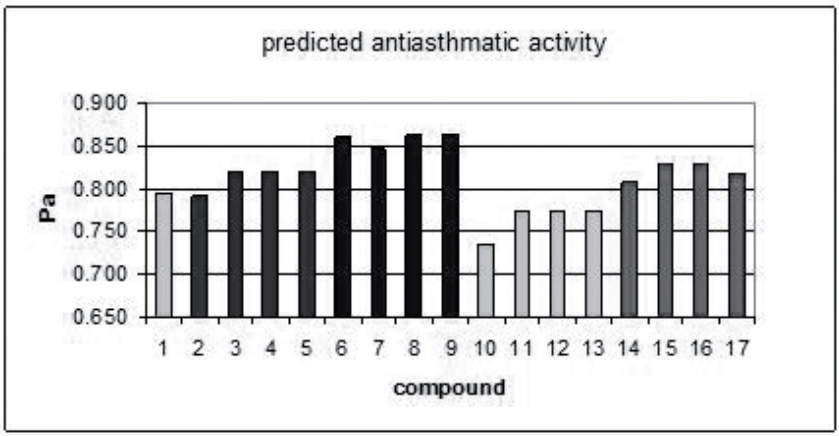


Figure 6. Predicted antiasthmatic activity for compounds **1** – **17**

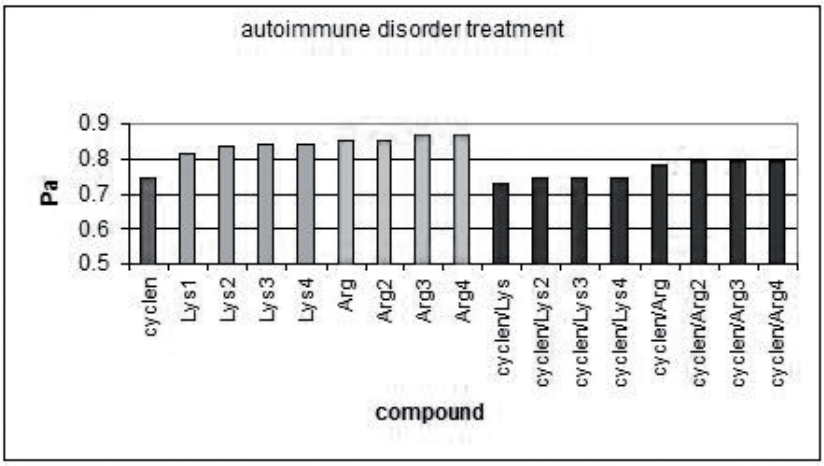


Figure 7. Results of prediction of activity **1** – **17** *in autoimmune diseases therapy*

All lysine-containing compounds studied (2-5, 10-13) exhibit high probability of having anti-sickle-cell anemia activity (Figure 8).

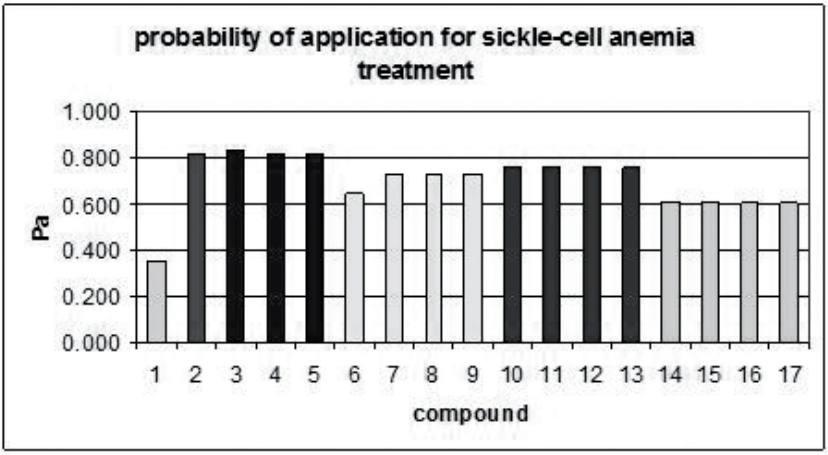


Figure 8. Results of prediction of activity **1** – **17** *as a sickle-cell anemia treatment*

Expected antiosteoporotic activity is highest for arginine-containing peptides **6-9** (Figure 9).

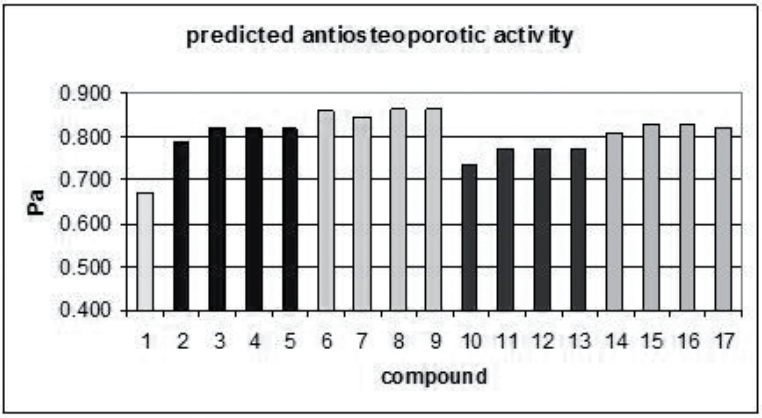


Figure 9. Predicted antiosteoporotic activity of compounds **1** – **17**

Analysis of data collected in Figures 6 – 9 implies a conclusion that cyclen-peptide conjugates are supposed to be less active against many diseases as the peptides themselves or initial amino acids (i.e. lysine or arginine). However, it should be noticed that the probable toxicity is also higher for cyclen and lysine as for their more complex conjugates and that the predicted toxicity of resulting compound decreases with the consecutive molecule of amino acid added to the peptide side chain (Figure 10). The arginine analogues seem likely to be generally less toxic than lysine derivatives. It is strongly suggested that for cyclen-arginine conjugates probability of toxic effect would be very low.

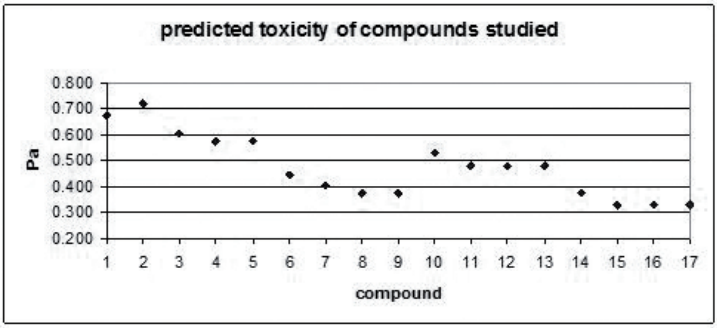


Figure 10. Results of toxicity prediction for compounds **1** – **17**

The representative numerical data – Pa for several types of predicted activity, obtained for compounds **1** -**17**, are collected in Table 1.

Table 1 .Results of prediction of biological activity for compounds **1** – **17**. *Values of Pa (probable activity) for various possible types of activity, estimated by PASS*

type of activity	Pa																
compound	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
antineoplastic	0.784	0.818	0.820	0.843	0.843	0.859	0.855	0.868	0.868	0.735	0.772	0.772	0.772	0.808	0.827	0.827	0.818
anticoagulant	0.937	0.853	0.908	0.901	0.901	0.953	0.948	0.952	0.952	0.843	0.864	0.852	0.852	0.941	0.940	0.940	0.940
psychotropic	0.999	0.969	0.850	0.839	0.839	0.866	0.808	0.796	0.797	0.759	0.779	0.732	0.732	0.703	0.672	0.672	0.672
antipsychotic	0.985	0.904	0.850	0.840	0.840	0.866	0.808	0.796	0.797	0.759	0.733	0.732	0.732	0.703	0.672	0.672	0.672
anxiolytic	0.817	0.969	0.364	0.348	0.348	0.370	0.346	0.332	0.332	0.377	0.316	0.316	0.316	0.384	0.354	0.354	0.354
nootropic	0.984	0.969	0.850	0.840	0.840	0.866	0.808	0.796	0.797	0.759	0.733	0.732	0.732	0.703	0.672	0.672	0.672
antidepresant	0.999	0.378	0.364	0.348	0.348	0.370	0.358	0.369	0.369	0.391	0.349	0.349	0.349	0.321	0.319	0.319	0.319
antiepileptic	0.993	0.774	0.640	0.579	0.579	0.581	0.536	0.526	0.526	0.815	0.779	0.779	0.779	0.554	0.431	0.431	0.431
neuroprotector	0.985	0.969	0.846	0.840	0.840	0.866	0.808	0.796	0.797	0.759	0.733	0.733	0.732	0.703	0.672	0.672	0.672
antiinflamatory	0.671	0.791	0.820	0.819	0.819	0.859	0.846	0.862	0.862	0.735	0.772	0.772	0.772	0.808	0.827	0.827	0.818
gynecological disorders treat.	0.511	0.531	0.608	0.669	0.669	0.808	0.798	0.820	0.820	0.551	0.620	0.620	0.620	0.762	0.782	0.783	0.773
antiviral	0.619	0.818	0.835	0.843	0.843	0.866	0.864	0.868	0.868	0.730	0.750	0.750	0.750	0.783	0.795	0.795	0.795
antiviral (Influenza)	0.000	0.627	0.722	0.787	0.749	0.626	0.716	0.739	0.739	0.492	0.552	0.552	0.552	0.517	0.567	0.567	0.567
antiviral (Hepatitis)	0.338	0.818	0.722	0.843	0.843	0.851	0.864	0.868	0.868	0.730	0.750	0.750	0.750	0.783	0.795	0.795	0.795
antiviral (Herpes)	0.000	0.786	0.778	0.787	0.787	0.641	0.716	0.739	0.739	0.643	0.662	0.662	0.662	0.517	0.567	0.567	0.567
antiviral (HIV)	0.619	0.773	0.722	0.749	0.749	0.626	0.716	0.739	0.739	0.492	0.552	0.552	0.552	0.517	0.567	0.567	0.567
antiasthmatic	0.794	0.791	0.820	0.819	0.819	0.859	0.846	0.862	0.862	0.735	0.772	0.772	0.772	0.808	0.827	0.827	0.818
inflammatory Bowel disease treatment.	0.671	0.818	0.820	0.843	0.843	0.859	0.855	0.868	0.868	0.735	0.772	0.772	0.772	0.808	0.827	0.827	0.818
antiosteoporotic	0.671	0.791	0.820	0.819	0.819	0.859	0.846	0.862	0.862	0.735	0.772	0.772	0.772	0.808	0.827	0.827	0.818
Gaucher disease treatment	0.435	0.832	0.868	0.860	0.860	0.737	0.794	0.794	0.794	0.846	0.830	0.830	0.830	0.786	0.764	0.764	0.753
autoimmune disorder treat.	0.749	0.818	0.835	0.843	0.843	0.851	0.855	0.868	0.868	0.730	0.750	0.750	0.750	0.783	0.795	0.795	0.795
multiple sclerosis treat.	0.619	0.818	0.835	0.843	0.843	0.851	0.855	0.868	0.868	0.730	0.750	0.750	0.750	0.783	0.795	0.795	0.795
sickle-cell anemia treatment	0.355	0.817	0.830	0.822	0.822	0.645	0.726	0.726	0.726	0.765	0.759	0.759	0.759	0.609	0.602	0.602	0.602
toxic	0.675	0.719	0.607	0.573	0.573	0.443	0.405	0.377	0.377	0.530	0.480	0.480	0.480	0.376	0.330	0.330	0.330

Conclusions

The wide variety of the biological effects of amino acids, peptides and polyazamacrocycles is the reason for the search for new promising drug structures among them. The probable activity of the obtained cyclen-amino acid and cyclen-peptide conjugates can be efficiently evaluated *in silico* using the PASS computer system (Prediction of Activity Spectra for Substances). This computer prediction can be applied to the evaluation of mechanism of action and effects of the compounds studied with high accuracy (70-80%) and to obtain early indications if the new compounds might be useful.

The evaluation performed in the present work shows that linking amino acids and peptides with cyclen leads to the promising biologically active compounds which toxicities would be significantly lower than the ones of the starting building blocks, so these newly constucted compounds **1-17** would be valuable drug candidates in many therapeutic areas.

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Chapter 3

Distributive equilibrium in chemistry and partition coefficient

Volodymyr Rybachenko, Constantine Chotiy,
Andrew Redko, Aleksey Dykun and Grzegorz Schroeder*

L.M. Litvinenko Institute of Physica-Organic and Coal Chemistry, National Academy of Sciences of Ukraine, Department of Spectrochemical Researches, R. Luxemburg 70; 81-134 Donetsk, Ukraine
**Faculty of Chemistry, Adam Mickiewicz University, Grunwaldzka 6, 60-780 Poznań, Poland*

The partition coefficient of matter between water and organic phase is a key value in different areas of chemical industry. It also plays an important role in prognosis of the state of environment. This value regulates many processes of distributing, for example absorption in soil and deposits, bioconcentration in organisms [1].

Application in pharmacokinetics

In the context of pharmacokinetics (what the body does to a drug), the distribution coefficient has a strong influence on ADME properties (**A**bsorption, **D**istribution, **M**etabolism and **E**xcretion) of the drug. Hence the hydrophobicity of a compound (as measured by its distribution coefficient) is a major determinant of how drug-like it is. More specifically, in order for a drug to be orally absorbed, it normally must first pass through lipid bilayers in the intestinal epithelium (a process known as transcellular transport). For efficient transport, the drug must be hydrophobic enough to partition into the lipid bilayer, but not so hydrophobic, that once it is in the bilayer, it will not partition out again [2]. Likewise, the hydrophobicity plays a major role in determining where drugs are distributed within the body after adsorption and as a consequence in how rapidly they are metabolized and excreted [3, 4].

Application in pharmacodynamics

In the context of pharmacodynamics (what a drug does to the body), the hydrophobic effect is the major driving force for the binding of drugs to their receptor targets [5, 6]. On the other hand, hydrophobic drugs tend to be more toxic because they in general are retained longer, have a wider distribution within the body (e.g., intracellular), are somewhat less selective in their binding to proteins, and finally are often extensively metabolized. In some cases the metabolites may be chemically reactive [7]. Hence it is advisable to make the drug as hydrophilic as possible while it still retains adequate binding affinity to the therapeutic protein target. Therefore the ideal distribution coefficient for a drug is usually intermediate (not too hydrophobic nor too hydrophilic).

Many other industries take into account distribution coefficients for example in the formulation of make-up, topical ointments, dyes, hair colors and many other consumer products.

Application in agrochemicals

Hydrophobic insecticides and herbicides tend to be more active. On the other hand, hydrophobic agrochemicals in general have longer half lives and therefore display increased risk of adverse environmental impact [7].

Application in metallurgy

In metallurgy, the partition coefficient is an important factor in determining how different impurities are distributed between molten and solidified metal. It is a critical parameter for purification using zone melting, and determines how effective an impurity can be removed using directional solidification, described by the Scheil equation.

Application in environmental protection

The hydrophobicity of a compound can give scientists an indication of how easily a compound might be taken up in groundwater to pollute waterways, and its toxicity to animals and aquatic life [8]. Distribution coefficients may be measured or predicted for compounds currently causing problems or with foresight to gauge the structural modifications necessary to make a compound environmentally more friendly in the research phase.

In the field of hydrogeology, the octanol water partition coefficient, or K_{ow}, is used to predict and model the migration of dissolved hydrophobic organic compounds in soil and groundwater.

Usage of partition coefficient in extraction

Besides that the sizes of partition coefficients are widely used in chemistry for example in the processes of extraction. In spite of the fact that extraction as a method of division is used in analytical chemistry and chemical technology a long time, theoretical bases of this method long time remained not enough studied. In particular, a long time there were not enough studied basic quantitative descriptions of extraction processes, which was a certain obstacle for wide introduction of extraction in practice. For the calculation of amount of matter which is extracted organic solvents, it is necessary to know a partition and distribution coefficients [9].

M. Bertlo and U. Yungfleysh were the first researchers, who in 1872 on the basis of experimental information showed that a relation of equilibrium concentrations of matter, distributed between two liquid phases, was constant. This relation was shown out with thermodynamic way by V. Nernst and in 1891 was formulated distributing law.

According to the distributing law, a matter dissolved in two immiscible or limitedly mixed up liquids is distributed between them in a permanent relation [10]. This relation for the ideal systems depends only from temperature, nature of matter and does not depend from concentration. It is consequent from this law that at simultaneous dissolution of a few matters, each of them is distributed between both liquid phases thus, as though in the system there are no other matters subjects distributing. A distributing law is fair only in case when the distributed matter in both phases is in the same form.

It is necessary to make important reservation, speaking about the distribution coefficient (D) as about constant, characterizing properties of the extraction system. The point is that a distribution coefficient is constant only in case when the concentration of extraction agent essentially surpasses the concentration of the extracted matter, because the distribution coefficient is the special case of well-known thermodynamics constant of equilibrium. We will consider a general case, when the testing matter (A) form connection with a few molecules of extraction agent (S) in an organic phase:

$$A + mS \leftrightarrow AS_m. \quad (1)$$

The constant of equilibrium of connection will have a next kind:

$$K = [A][S]^m/[AS_m]. \quad (2)$$

If to use the concept of distribution coefficient, that

$$K = [S]^m/D,$$
because
$$D = [AS_m]/[A]. \quad (3)$$

From these equations evidently, that a distribution coefficient (D) will be permanent only in case if the spending of extraction agent on formation of connection $[AS_m]$ will be vanishing small.

On the basis of information about the distribution coefficient it is possible to get data about co-ordination of the extracted matter with the molecules of extraction agent. For this purpose distribution coefficients at the different concentrations of extraction agent are determining. Then a chart of experimental results in the co-ordinates of lgD from lg[S] will be direct line, inclination of which is equal to the number of molecules of extraction agent to one molecule of the extracted matter. It appears from equation (3), because $lgD = m\ lg[S] + lg(1/k)$.

The equation is of the used to describe process of extraction the concept of partition coefficient (P) of matter is used. It is a permanent size, expressing the relation of concentrations of the distributed matter, being in both phases (after the offensive of equilibrium) in the same form. The size of partition coefficient depends on nature of the distributed matter, composition and properties of applied extraction agent, temperature which extraction is made at. This constant does not depend on the equilibrium concentrations of the extracted matter and volumes of water and organic phases. At the calculations of partition coefficient of matter it is necessary to carry conviction that the distributed matter in both phases is in an identical form (in the identical molecular state). However the condition indicated higher is not observed in many extraction systems. In one of liquid phases can take place dissociation, association, hydrolysis, formation of complexes of the distributed matter et cetera. For the calculation of extraction equilibrium in such systems does not take into account the form of existence of matter in each phase, but take into account only the relation of total (analytical) concentrations of the distributed matter in both phases [11].

On the basis of determination of total concentrations it is possible to calculate not only partition coefficient, but the distribution coefficient of this matter in the applied system of solvents (water — organic solvent). The distribution coefficient — it is attitude of total analytical concentration of matter in the phase of organic solvent toward the total analytical concentration of this matter in a water phase (without an account that, in what form a matter is in every phase) [3, 11].

For un-ionizable compounds, log P = log D at any pH.

Using in chemical synthesis

The partition coefficient is also a size which characterizes catalytic properties of matters, used as compound which transfer reagent from one phase to another at the synthesis of some organic compounds. Such examples are known in the reactions of inverse phase transfer catalysis (IPTC). The reactions of IPTC are very perspective direction in modern chemistry. Therefore the correct selection of catalysts is very important (using for that the sizes of partition coefficients) [12].

The determination of such physical-chemical descriptions as partition coefficients is a very important and actual task as for the receive of more detailed information about the state of environment, level of contamination and prognostication of operating under prevention and removal of problems of ecology, so for researches in area of synthesis of new compounds and study of their properties. There's more comfortable to use in practice lgP instead of value P.

Table 1. Some octanol-1 – water partition coefficient data [13]

Component	lgP_{OW}	T (°C)	Literature
Acetamide	-1.16	25	[14]
Methanol	-0.82	19	[15]
Formic acid	-0.41	25	[16]
Diethyl ether	0.83	20	[15]
p-Dichlorobenzene	3.37	25	[17]
Hexamethylbenzene	4.61	25	[17]
2,2',4,4',5-Pentachlorobiphenyl	6.41	Ambient	[18]

From the matters resulted in Table 1 acetamide is most hydrophilic and 2,2',4,4',5-Pentachlorobiphenyl is most lipophilic.

Partition coefficients determination

So determination of partition coefficient of octanol-1/water (P_{ow}) is required for drafting of base set of data, presented during registration into ES of new matters and existent matters fight against contamination of which requires primary measures. As P_{ow} not always can be define experimentally, for example

in the case of well water-soluble matters and very hydrophobic matters, it is possible to use a value P_{ow}, certain on the basis of Quantitative Structure-Property Relationship (QSPR) [19]. However it is necessary to show an extreme carefulness at the use of QSPR in the case of matters for which it is impossible to define a partition coefficient experimentally (for example surfactant).

Experimental determination of P_{ow}

For experimental determination of values of partition coefficient described shake flask (or tube) method and HPLC in standard leading principles, for example OECD 107 (1995); OECD 117 (1983); EEC A.8 (1992); EPA-OTS (1982); EPA-FIFRA (1982); ASTM (1993). Information which is got by the shake flask method and by the method of HPLC in accordance with standard leading principles is not the uniquely recommended information [19]. For the highly hydrophobic matters which slowly dissolve in water, more reliable is information which is got by the method of slow interfusion. The method of slow interfusion passes presently control tests with the purpose of development of final guidance.

Shake flask method

Basic principle of this method is a measuring of dissolution of matters in two different phases – in water and octanol-1. To define a partition coefficient, equilibrium must be attained between all interactive components of the system and then the concentration of matter dissolved in two phases will be determined. Shake flask method is used when values of lgP_{ow} are in a range from -2 to 4. Shake flask method is used only to the practically clean matters which is water-soluble and soluble in octanol-1, and must be executed at a stationary temperature (±1°C) from 20°C to 25°C [4, 19, 20].

Advantages:

- Most accurate method.
- Accurate for broadest range of solutes (neutral and charged compounds applicable).
- Chemical structure does not have to be known beforehand.

Defects:

- Time consuming (>30 minutes per sample).
- Octanol-1 and water must be premixed and equilibrated (takes at least 24 hours to equilibrate).
- Complete solubility must be attained, and it can be difficult to detect small amounts of undissolved material.
- The concentration vs. UV-Vis response must be linear over the solute's

concentration range (Beer-Lambert law).

- If the compound is extremely lipophilic or hydrophilic, the concentration in one of the phases will be exceedingly small, and thus difficult to quantify.
- Relative to chromatographic methods, large amounts of material are required.

The process by which a solute is transferred from one phase to a new phase.

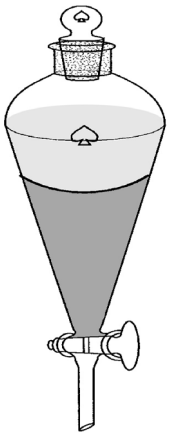

Figure 1. Separator funnel for use in a liquid–liquid extraction

HPLC method

HPLC method is executed on analytical columns, filled with hard phase with a long hydrocarbon chain ($C_8 - C_{18}$) chemically bonded with silica gel. Chemical matters, placed in such column, move with different speeds, is stipulated by different degrees of distributing between a mobile water phase and immobile hydrocarbon phase [21]. HPLC method is not used to strong acids and bases, complexes of metals, surfactants or matters which have chemical reaction with a solvent. HPLC method is used when a value of lgP_{ow} is in a range from 0 to 6 (OECD 117, 1989). HPLC method is less sensible to the admixtures in examinee connection as compared to the shake flask method [21, 22].

Advantages:

- Fast method of determination (5-20 minutes per sample).

Defects:

- The solute's chemical structure must be known beforehand.
- Since the value of log P is determined by linear regression, several

compounds with similar structures must have known log P values.
- Different chemical classes will have different correlation coefficients; between-class comparisons are not significant.

Slow interfusion method

Slow interfusion method allows exactly to define P_{ow}, lpP_{ow} of which reaches 8,2 (De Bruijn et al., 1989). In the case of high lipophilic compounds, shake flask method has a tendency to cause formation of micro drops, and the HPLC method requires extrapolation outside the range of values which are used for verification at calculation of P_{ow}.

To define a partition coefficient at the system of water – octanol-1 examinee compounds become at equilibrium with each other, and then the concentration of the probed connection is determined in both phases. Experimental difficulties, related to formation of micro drops during shaking in a funnel is possible to overcome by the method of slow interfusion in which water, octanol-1 and probed connection is become at equilibrium in a reactionary vehicle, in which they are slowly mixed [23]. Interfusion creates a laminar flow between octanol-1 and water, and also improves an exchange between phases without formation of micro drops.

Laboratory column method

Another one flexible method of measuring of lgP_{ow} is a method of laboratory column. In this method a column is used for distributing of examinee matter between phases of oktanol-1 and water. In a column entered solid transmitter which is saturated with the set concentration of examinee matter in oktanole-1. An examinee matter is extracted from the impregnated with oktanol-1 column by water. Going out from a column water solution has an equilibrium concentration of examinee matter which was distributed between the phases of oktanol-1 and water. The main advantage of laboratory column method as compared to the shake flask method is that, it allows fully avoid formation of micro emulsion [24, 25]. Therefore this method is especially effective at measuring of P_{ow} for matters, lgP_{ow} of which more than 4,5 (Doucette and Andren, 1987 and 1988; Shiu et al., 1988). One of the lacks of laboratory column method is that, it requires complicated equipment. Detailed description of laboratory column method is contained in a law «About control of toxic matters: leading principles» (USEPA 1985).

Usage of QSPR for determination of lgP_{ow}

For calculation of P_{ow}, have developed and continue to be developed

numerous QSPR (Quantitative Structure-Property Relationship). The widely used methods are based on fragmentary approaches. The usual adding up of lipophilicy of separate fragments for the molecule lies in basis of fragmentary approaches, because of absence of the set of experimental data. The program CLOGP (Daylight Chemical Information Systems, 1995) was originally developed for the use in area of creation of new medicinal preparations. This model is based on the method of calculation, created by Hansh and Leo. The program calculates lgP_{ow} for organic compounds, which are containing C, H, N, O, Hal, P, S. It is impossible to calculate lgP_{ow} for salts and matters, which are containing some admixture (except for nitrogen compounds and oxides of nitrogen). Results of calculations lgP_{ow} for the ionized compounds, such as phenols, amines and organic acids, reflect neutral or un-ionized form and depend on the hydrogen index (pH). In most cases this program allows to do accuracy calculations in the range of lgP_{ow} from 0 to 5 (European Commission, 1996, part 3). In the case of chelate compounds and biologically active matters, the program CLOGP provides only limited authenticity of information [26].

Structural fragments and correlation coefficients are used in the program KOWWIN (LOGKOW). By this program can be calculated lgP_{ow} for organic compounds, containing atoms C, H, N, O, halogens, Si, P, Se, Li, Na, K, Hg. It also allows calculating lgP_{ow} for compounds containing such admixtures, as nitrogen oxides and other nitrogen compounds. Calculation of lgP_{ow} for the ionized matters, such as phenols, amines and organic acids, reflects neutral or un-ionized form, and its values will depend on the hydrogen index (pH). KOWWIN can give prognoses for some surfactants (for some dye-stuffs). This program allows to get accuracy data in the range of values lgP_{ow} from 0 to 9 [27].

The program AUTOLOGP was created on the basis of set of information about 800 organic matters which were collected from scientific articles. This program allows to calculate the values of lgP_{ow} for organic compounds, which contain C, H, N, Hal, P, S. It can not make calculations for salts. It can be calculated values for the ionized chemical compounds, such as phenols, amines and organic acids, but these values depend on pH [28].

There is also a range of more new programs for the calculation of lgP_{ow}. The programs which it is possible to use on-line are created nowadays. Some of them are given below: COSMOFrag, ABLogP, PreADMET, XLOGP3, Molecular Property Explorer, Acd/LogP DB [29, 30, 31].

Limitations

The value of LgP is not an accurate determinant of lipophilicity for ionizable compounds because it only correctly describes the partition coefficient of neutral

(uncharged) molecules. Taking the example of drug discovery we see how the limitations of lgP can affect research. Since the majority of drugs (approximately 80%) are ionizable, lgP is not an appropriate predictor of a compound's behavior in the changing pH environments of the body. The distribution coefficient (lgD) is the correct descriptor for ionizable systems. Alternatively, use may be made of the apparent partition coefficient which is defined as follows: (true partition coefficient) * (fraction of the drug which is un-ionized). Clearly, if the drug is 100% un-ionized then $P_{apparent} = P_{true}$ [4, 5, 6].

Conclusions

In conclusion it should be noted that partition coefficient has a great importance both in chemical industry and in chemistry as science. It is widely used for researches of distributing of matters in an environment.

In most cases at research of distributing of matter between two immiscible phases, one of them it is water. Water is universal and ecologically safe solvent. The second phase is organic solvent. There are a row of requirements to the organic solvents, which are used for partition coefficient determination (and in extraction): the organic solvent must extract the probed matter from a water phase, and must extract from solutions only one matter or group of identical compounds, and must have insignificant solubility in water, and water must not notedly dissolve in this solvent. At the use of organic solvents, which are dissolved in water or are dissolved water, the final volumes of phases after shaking will not be equal to the initial volumes of these phases. It can be the source of errors at the calculations of partition coefficient and distribution coefficient, or also at the calculation of degree of extraction. To eliminate possible errors at calculations, an organic solvent is satiated by the water, and water is satiated by the organic solvent. The organic solvent must have not low boiling temperature (higher then 50°C). Therefore at extraction their volumes are being decreased, and the concentration of the extracted matters in these solvents is being increased. It can be one of sources of errors at the calculations of partition coefficient or distribution coefficient of the extracted matter. However a low temperature of boiling of organic solvents is a positive factor for their regeneration (similarly it can be comfortably at the synthesis of some organic compounds).

1. The density of organic solvents at possibility must differ from the density of water and water solutions. At the large difference of densities of these liquids, the separation of phases takes place quickly.
2. Solvents must not be inflammable or poisonous [9].

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Chapter 4

Inverse phase transfer catalysis

Constantine Chotiy, Andrew Redko,
Victor Anischenko and Grzegorz Schroeder*
L.M. Litvinenko Institute of Physical-Organic and Coal Chemistry, National Academy of Sciences of Ukraine, Department of Spectrochemical Researches, R. Luxemburg 70; 81-134 Donetsk, Ukraine
**Faculty of Chemistry, Adam Mickiewicz University, Grunwaldzka 6, 60-780 Poznań, Poland*

Phase transfer catalysis (PTC) is a well-known technique in chemistry and has been widely used for organic synthesis, particularly for nucleophilic substitution [1]. This technique to allow react the reagents that present in different phases. It is possible because of catalyst, which transfers anions, in the form of an ion pair, from the aqueous phase into the organic phase. The reaction with water-insoluble hydrophobic substances takes place in organic phase. Crown ethers, quaternary ammonium salts, poly(ethyleneglycol)s, and cryptates have been used as phase transfer catalysts.

Main advantage of organic synthesis by PTC is absence of necessity of heating reaction mixture to get a large reaction rate. The PTC techniques have been successfully applied to synthesis of various organic substances.

In 1986 Mathias and Vaidya presented a new PTC methodology, namely inverse phase transfer catalysis (IPTC) [2]. In contrast to the classic PTC in case IPTC system, the organic reagent reacts with the catalyst in organic phase to form a water soluble intermediate, which is active and reacts rapidly with the aqueous reactant to produce the desired product. The catalyst is regenerated in the aqueous-phase reaction.

The problem of environmental protection is very significant, because many reactions still involve the use of toxic organic solvents, which are partly discharged into the atmosphere with a host of negative environmental effects. Replacement of these solvents with environmental benign reaction media such as water brings about potential advantages, such as the opportunity for environmentally

benign processing, lower costs, and easy separation of the catalysts from the reaction medium. And IPTC method allows decreasing quantity of toxic organic solvents thanks to using water as a main solvent.

In inverse phase transfer catalysts has sewed application the following catalysts pyridine derivatives, cyclodextrin derivatives, transition metal complexes, tetramethylammonium salts and calix[n]arens. Further, the different IPTC catalysts and their applications will be considered.

Transition metal complexes

Cuprous chloride, water-soluble rhodium- and palladium-based catalysts are used as an IPTC catalyst. Ability of cuprous chloride to form water-soluble complexes with the lowest olefins has been used for catalyze by IPTC method in the two-phase hydrolysis and the Prins reactions [3, 4]. Analogously, water-soluble rhodium-based catalysts were used as IPTC catalysts for the hydroformylation of olefins to produce aldehydes for the fine chemicals market [5]. Various catalysts on the basis of palladium complexes with organic ligands have been used for dehalogenation reactions of allyl and benzyl halides, and as for alkylation of allylic substrates and nucleophiles such as ethyl acetoacetate [6]. As a result of alkylation have been received regio- and stereospecific products in quantitative yields. Ito et al. used a self-national assembled nanocage, based on chelate palladium complex, as an IPTC catalyst for Walker oxidation of styrene [7].

Tetramethylammonium salts

Due to its low organophilicity low molecular weight quaternary salts, such as tetramethyl or tetraethylammonium are normally a poor PTC catalysts for transferring reactant anions into the organic phase.

However, these salts could act as the IPTC catalyst, e.g., tetramethylammonium bromide have been employed as IPTC catalysts to carry out highly selective carbohydrate reactions in the aqueous phase. Trimethylammonium groups attached to ion-exchange resins also act as IPT catalysts in the oxidation of benzyl alcohol by NaOCl to yield benzaldehyde, the fluorination of chlorobenzaldehydes [8], the acetalization of sorbitol with benzaldehyde to produce dibenzalsorbitol [9]. Commonly, there are other methods for obtaining these substances, but all of they have disadvantages. The most popular disadvantage is formation of by-product resulting from hydrolysis one of reactant.

In case of synthesis of 2,4-dichlorophenoxyacetic acid in presents tetramethylammonium bromide, authors have analyzed several effects [10]. The dependents of conversations from speed of agitation have a maximum point.

The effect of phase volume ratio was studied for 1:1, 1:2 and 2:1 ratios of organic phase to aqueous phase volumes under otherwise similar experimental conditions. When the phase ratio of organic to aqueous phase is 1:2, the best conversion values are obtained. Suggested, that the organic phase as a dispersed phase leads to higher interfacial values that in turn enhance the mass transfer rates.

Thus, for certain systems where the reaction in the organic phase was not possible, the quaternary salts could react as IPTC catalysts.

Surfactants

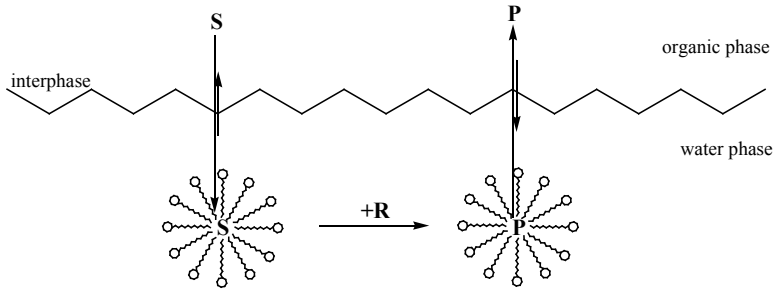
Boyer and co-workers explored a general method dealing with the IPTC [11-14]. It is based upon the fact that any lipophilic substrate could be transferred into the aqueous phase by means of hydrosoluble surfactants. The fact, that micelles formed in the aqueous phase, above the CMC, are able to solubilize a fraction of the hydrophobic substrate in equilibrium with the organic phase, allow to investigated the following IPTC reactions using surfactants as the IPTC catalysts: the epoxidation of α,β-unsaturated ketones by H_2O_2 in heptane – 0.5 M NaOH(aq) system [11-13] and the reduction of ketones by sodium borohydride [14]. Dodecenyltrimethylammonium bromide was used as a catalyst.

According to the mechanism of IPTC, reaction takes place at the surface of the micelles in the water phase. The reaction product, normally lipophilic, is transferred into the organic phase (Scheme 1).

However, in presents of surfactant micelles two catalytic processes are involved: an inverse phase transfer – the surfactant allows the transfer of the lipophilic substrate into the water phase and the micellar catalysis – the charged transition state formed at the micelle surface can be stabilized by the surfactant ionic head groups only in the situation where the charges are opposite.

The results indicated that the reaction was catalyzed by water-soluble micellar aggregates of the surfactant and the catalytic effects depended strongly on the hydrophobicity of the substrate. Interesting results were observed in the study of the effect of surfactant concentration on the epoxidation of chalcone by H_2O_2. It appeared that under slow agitation (100 rpm), the reaction occurred mainly via IPTC, while under vigorous agitation (1200 rpm) it took place mainly at the interface due to the formation of an emulsion.

This type of IPTC is of interest especially in the reduction of highly hydrophobic ketones by sodium borohydride and in the epoxidation of α,β-unsaturated ketones by hydrogen peroxide.



Scheme 1. IPTC principle involving a surfactant as catalyst

Cyclodextrines

Trifonov and Nikiforov reported that in the presence of β-cyclodextrin, the rate of the nucleophilic displacement of neat octyl bromide with aqueous cyanide, iodide, and thiocyanate anions was considerably increased [15]. Thus, unmodified α- and β-cyclodextrins were investigated as carriers of organic molecules into aqua phase before the concept of IPTC was rationalized.

Cyclodextrins are cyclic glucose oligomers, and the 6-, 7-, and 8-units terms are commercially available under the names of α-, β-, and γ-cyclodextrin. These compounds are characterized by a typical shape that can be described as barrel-like (Figure 1).

The inner cavity is essentially hydrophobic and can host organic guests, whereas hydrophilic-OH groups span from the upper and the lower rim, ensuring water solubility to the molecule. The free hydroxyl groups can be selectively functionalized, thus making possible the fine-tuning of the inclusion and solubility properties of these compounds. After the IPTC concept was established, cyclodextrins found applications as IPTC catalysts in several important organic reactions, which heretofore catalyzed by transition metals substances, e.g. the aerobic oxidation of olefins to ketones in water [16], isomerization of 4-allylanisole [17], the hydrogenation of conjugated dienes to monoolefins [18].

In 1994 Mortreux and co-workers reexamined the application of IPTC to the Wacker process. They used amphiphilic β-cyclodextrin, in which about 60% of the free-OH groups were methylated, as IPTC catalysts for transporting the organic substrate into the aqueous phase and obtained excellent yield 98% [19]. Other cyclodextrins (native α-, β-, γ-, fully acetylated β-cyclodextrin or 2-hydroxypropyl-β-cyclodextrins with different degrees of substitution) were tested with limited success. The same modified β-cyclodextrin was successfully used as IPTC catalysts for the hydroformylation of water-insoluble olefins in an

aqueous two-phase system free of organic solvent.

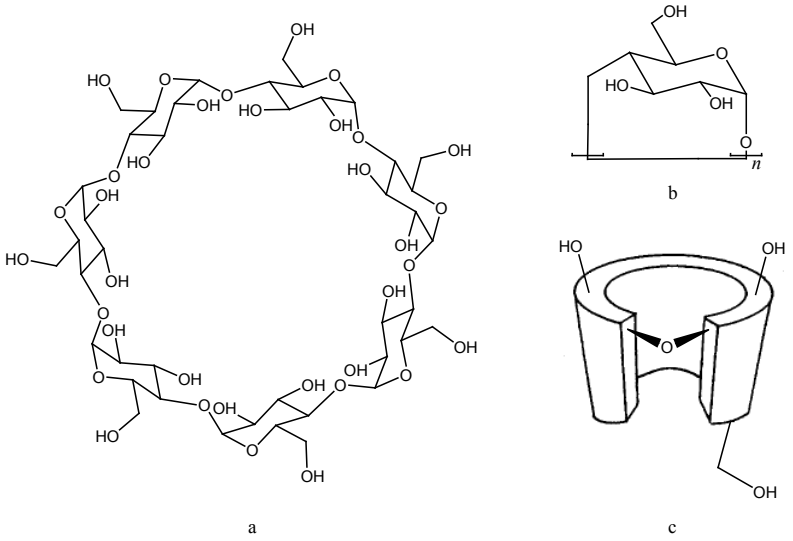


Figure 1. Cyclodextrins: a – chemical structure; b – elementary unit; c – space form

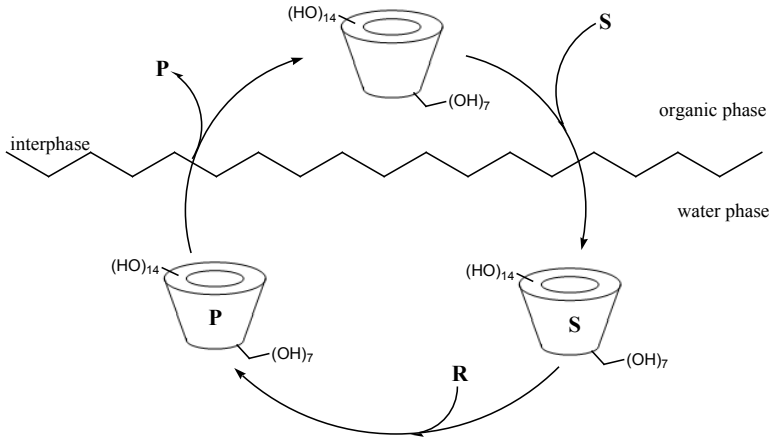
Two factors have an importance for the activity of cyclodextrins as IPTC catalysts: main is the correct balance between lipophilic and hydrophilic character and the possible role of molecular recognition based on reversible interactions between the cyclodextrins host cavity and substrate.

A common picture emerged from these studies: the amphiphilic cyclodextrin acts as a host, wrapping the hydrophobic substrate and transferring it into the water phase, where the reaction occurs. The stability constant of the new host-guest complex is lower, and the product is then released into the organic phase (Fig. 3).

The validity of these working hypotheses was tested in other catalytic reactions, e.g. the biphasic hydrogenation of water-insoluble aldehydes to alcohols [20], the cleavage of allylic substrates [21, 22] and the hydrocarboxylation of olefins to give carboxylic acids [23].

In the result of two independent studies, in which the influence of partly methylated β-cyclodextrins on the other component of the catalytic system was been evidenced in the case of the hydroformylation of alkenes, was concluded that chemically modified cyclodextrins influence the biphasic reaction not only by acting as inverse phase-transfer agents but also by modifying the equilibrium

between the components of the catalytic system. Kalck and co-workers proposed that a gradual supramolecular organization in the interphase occurs for the catalytically active organometalic complex, which should involve at least two cyclodextrin molecules [24]. Monflier and co-workers refined their previous model, taking into account the formation of inclusion complexes between the cyclodextrin and some components of the catalytic system [25].



Scheme 2. Proposed role of cyclodextrins as carriers in aqueous-phase organometallic catalysis

A more sophisticated β-cyclodextrin-based catalytic system combining different functions in the same molecule was also conceived. Rhodium complexes of multi-component ligands featuring a chelating diphosphine covalently linked to the upper rim of β-cyclodextrin. These supramolecular catalysts showed higher substrate selectivity and activity than simple methylated β-cyclodextrin. Such improvements were explained with the formation of an inclusion complex at the phase boundary, with the cyclodextrin host fixing the substrate in the proximity of the catalytically active metal center [26].

Calix[n]arenes

Shimizu and co-workers proposed water-soluble calix[n]arenes (n – 4, 6, 8) as IPTC catalysts [27]. They used the well known calix[n]arenes bearing trimethylammoniomethyl groups (Figure 2) in the nucleophilic displacement of alkyl and arylalkyl halides in water. Regardless of the fact that the monomer unit of the calix[n]arene catalysts did not show any activity, the catalytic activity of these compounds exceeded that of β-cyclodextrin. It was investigated that the

efficiency of the calix[n]arenes varied depending on the sizes and shapes of the substrates. The following assumption has been made that they behave similarly to cyclodextrins, forming inclusion compounds with the substrate that is thus transferred into the aqueous phase.

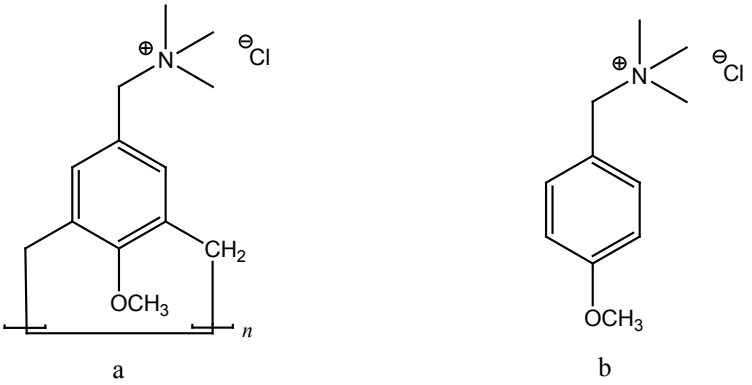


Figure 2. Calix[n]arene; a – water-soluble calix[n]arenes; b – monomeric unit

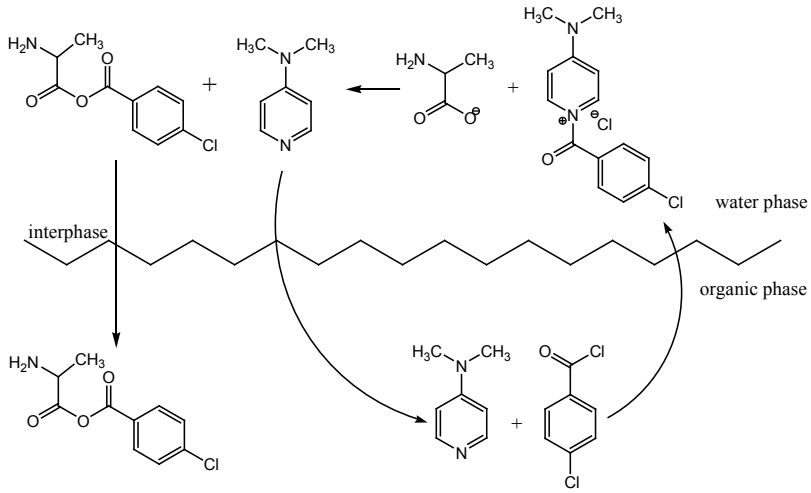
Calix[n]arenes were used as IPTC catalysts in the alkylation of active methylene compounds with alkyl halides in aqueous NaOH solutions [28], in aldol-type condensation and Michael addition reactions [29]. In these reactions calix[n]arenes enhanced the main reaction versus secondary reactions, with respect to those observed under classical PTC reactions in the presence of tetrabutylammonium bromide or hexadecyltributylammonium bromide [13]. Moreover, the aqueous catalyst solution was easily separated from the organic phase containing the products, and no organic solvent was required.

Generally association constants of aromatic compounds with calix[n]arenes are close to those observe with β-cyclodextrin, although there is the exception of iodobenzene, which showed a remarkable higher affinity to β-cyclodextrin than to the calixarenes.

Pyridine derivatives

Pyridine derivatives are of interest as IPTC catalysts, because of their low cost and availabilities in comparison with cyclodextrins and calix[n]arenes. Pyridines relate to an important class of IPTC catalyst, which can react with hydrophobic substrate in organic phase with formation of intermediate. These compounds have an ionic structure that's why it passes into aqua phase and here take place main reaction between ionic intermediate and hydrophilic substrate. In result of that reaction form products and regenerate catalyst. This class of IPTC

catalysts includes 4-(dimethylamino)pyridine (DMAP), 4-pyrrolidinopyridine, pyridine-1-oxide (PNO), 4-methoxypyridine-1-oxide, 4-methylpyridine-1-oxide, etc.



Scheme 3. Scheme of reaction between 4-chlorobenzoyl chloride and alanine catalyzed by DMAP in H_2O – CH_2Cl_2 system

Mathias and Vaidya were first who studied the acylation reaction of alanine with decanoyl- or 4-chlorobenzoyl chloride catalyzed by DMAP in H_2O – CH_2Cl_2 system (Scheme 3) [2]. DMAP was also used as IPTC catalyst to improve the tosylation of alcohols and amines with tosyl chloride [30].

The IPTC reaction of acid chloride with carboxylate ions [31] and with phenols [32] catalyzed by PNO in H_2O – CH_2Cl_2 system to produce accordingly acid anhydride and aromatic ester were reported. In the latter case was observed that the IPTC reaction was more efficient than the normal PTC reaction catalyzed by quaternary ammonium salts.

Further, DMAP- and PNO-catalyzed IPTC reactions will be considered more detail.

DMAP as a catalyst in IPTC system

Two independent investigations of the DMAP-catalyzed IPTC reaction of benzoyl chloride with glycine were made. The main different was in the pH of water phase.

Wang and co-workers investigated this reaction in H_2O – CH_2Cl_2 system and pH of water phase was between 7 and 11 [33]. It was observed that the rates of

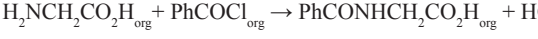
both the uncatalyzed and DMAP-catalyzed reactions were fast and the yields of hippuric acid were very high (up to 100%). Also was founded that the reaction rate depended on the shape of the reaction vessel and on agitation rate below 1200 rpm.

The mechanism of the DMAP-catalyzed reaction can be described as follows:

1

2

3



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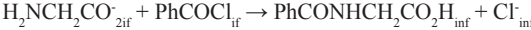

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The $DMAPCOPh^+Cl^-$ is the active ionic intermediate, formed by the reaction of benzoyl chloride and DMAP in the organic phase. The reactions 1-4 described the uncatalyzed reaction between benzoyl chloride and glycine in $H_2O - CH_2Cl_2$ system. The hippuric acid can be generated via reaction 3 in the organic phase (org) and reaction 4 in the interfacial region (if). In case of DMAP-catalyzed reaction the hippuric acid mainly generated via reactions 9-10 in water phase (aq).

The reaction has taken place in the kinetic region. It was confirmed by parallel experiments in which DMAP was presented initially in organic and in the aqueous phase, respectively. In all cases the reaction rates were similar, which implied that the mass transfer of DMAP between the two phases was extremely rapid. Both the uncatalyzed and DMAP-catalyzed reactions followed pseudo-first-order kinetics in the initial presence of excess amount of the sodium salt of glycine.

The effective pseudo-first-order rate constants increased with the initial

concentrations of sodium salt of glycine and DMAP in the aqueous phase in cases of uncatalyzed and DMAP-catalyzed reaction, respectively. These facts confirm that in the uncatalyzed reaction, the reaction rate determines by reactions 3 and 4 and in the DMAP-catalyzed reaction it control by reactions 6, 8 and 10.

The nucleophilicity of RNH_2 is considerably higher than that of the $RCOO^-$ ion, because the pK_a values relative to water are $pK_a(RNH^+_3)$ = 10-11, $pK_a(RCOOH)$ = 4-5, and $pK_a(H_2O)$ = -1,74. Therefore, the reaction of PhCOCl with $H_2NCH_2CO^-_2$ to yield $PhCOOCOCH_2NH_2$ is negligible, as observed [34]. The hydrolysis of PhCOCl was also negligible, because no benzoic acid was detected.

Also, analogous experiments with sodium salts of other α-amino acids were realized and similar results were obtained. These reactions proceeded rapidly to produce PhCONRCHR'COOH with high yields (85 - 100%). In the follow row the reactivity's of amino acids increased 2-methylalanine < DL-alanine « glycine « N-methylglycine ≈ L-prolinene [35]. There are three facts, which determined reactivity's of these amino acids: nucleopholicities, organophilicities (solubility's in CH_2Cl_2) and the steric hindrance, e.g., the low reactivity of 2-methylalanine was due to both the low solubility in CH_2Cl_2 and the steric hindrance of the methyl group.

In contrast Asai and co-workers studied that reaction in H_2O – CH_2Cl_2 system in the absence of alkali [36]. They obtained high yields (up to 94%) of hippuric acid. It was observed that the overall reaction rates were proportional to the interfacial concentration of ionic intermediate in the aqueous phase. In the absence of DMAP, the reaction was about three to four orders slower than that of the DMAP-catalyzed reaction. The yield of hippuric acid decreased with increasing amounts of NaOH added, due to the hydrolysis of benzoyl chloride. In that case the mechanism of the DMAP-catalyzed reaction can be described by reactions 2, 3 and 5-9.

An attractive application of the IPTC technique was demonstrated in the protection of the amino group of DL-serine with carbobenzoxy chloride (benzyl chloroformate) in H_2O – dichloroethane system catalyzed by DMAP [37]. This method is useful for preparing the precursor for synthesizing the peptide containing the serine moiety, since the protection of amino acids by the carbobenzoxy group is generally made in the alkaline solution, which is not applicable to DL-serine due to its decomposition in the alkaline solution to produce byproducts such as glycine.

PNO as a catalyst in IPTC system

Acid anhydrides are very important precursors for the synthesis of esters,

amides, and peptides and they being less reactive than acyl chlorides. An attractive application of the IPTC technique was demonstrated in produce of acid anhydride from acid chlorides and carboxylate ions by Fife and co-workers [31, 38]. They have proposed the next scheme of catalytic process (Scheme 4):

Jwo and co-workers [39-49] have investigated the kinetics and mechanism of the reactions of benzoyl chlorides and carboxylate ions using PNO as the IPTC catalyst in H_2O – CH_2Cl_2 system. Based on the kinetic results, a detailed mechanism was proposed for the PNO-catalyzed substitution reaction of benzoyl chloride and benzoate ion in this system [39]. The main elementary acts are shown as follows:

$$PNO_{org} \rightleftharpoons PNO_{aq} \quad 11$$

$$PhCOCl_{org} + PNO_{org} \longrightarrow PhCOONP^{+}Cl^{-}_{org} \quad 12$$

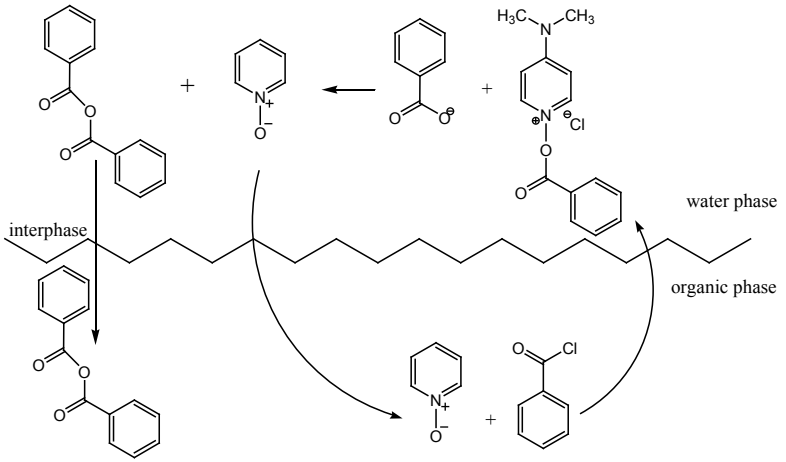
$$PhCOONP^{+}Cl_{org} \rightleftharpoons PhCOONP^{+}Cl^{-}_{aq} \quad 13$$

$$PhCOONP^{+}Cl^{-}_{aq} + PhCOO^{-}_{aq} \longrightarrow (PhCO)_2O_{aq} + PNO_{aq} + Cl^{-}_{aq} \quad 14$$

$$PhCOONP^{+}Cl^{-}_{aq} + H_2O \longrightarrow PhCOOH_{aq} + PNOH^{+}Cl^{-}_{aq} \quad 15$$

$$PhCOCl + H_2O \longrightarrow PhCOOH + HCl \quad 16$$

Reaction 16 can take place in both the organic and aqueous phases and in the interfacial region.



Scheme 4. Inverse phase transfer catalysis: the PNO-catalyzed reaction of benzoyl chloride and benzoate ion

It was generally observed that the reaction rate was independent of the agitation speed beyond 1100 rpm in H_2O – CH_2Cl_2 system, but with agitation speed below 1100 rpm the reaction rate decreased with decreasing of agitation speed. The PNO-catalyzed IPTC reactions of benzoyl chloride and benzoate ion produced a prime product (benzoic anhydride) and by product of a hydrolysis (benzoic acid). Under suitable reaction conditions, the reaction followed pseudo-first-order kinetics as shown in equation 1:

$$-\frac{d[PhCOCl]_{org}}{dt} = k_{ef}[PhCOCl]_{org} \qquad 1$$

The effective pseudo-first-order rate constant (k_{ef}) depended linearly on the initial concentration of PNO in the aqueous phase ($[PNO]_{aq,0}$) and could be expressed as:

$$k_{ef} = k_h + k_c[PNO]_{aq,0} \qquad 2$$

In equation 2, k_h and k_c were the uncatalyzed (or hydrolysis) rate constant and catalyzed rate constant, respectively. Since, in general $k_h \gg k_c$, then reaction of PhCOCl with PNO in the organic phase are the rate-determining act in the reaction of production as prime product the benzoic anhydride catalyzed by PNO. For obtaining the value of k_h were measured values of k_{ef} for reactions with various initial concentration of catalyst. From this data were obtained equation of linear regression, which represented dependence of k_{ef} from $[PNO]_{aq,0}$. Free factor in obtained equation were numerically equal k_h. The value of k_h obtained from the dependence of k_{ef} from $[PNO]_{aq,0}$ was generally consistent with that obtained in the uncatalyzed reaction. Therefore, reaction 16 was the main act in the uncatalyzed (hydrolysis) path, which led to the production of benzoic acid.

Effects of the substrates structure

Generally, in the IPTC system the rate-determining elementary act is the reaction of hydrophobic substrate and catalyst in which produce ionic intermediate. In case of the PNO-catalyzed reaction of benzoyl chloride with benzoate ion in H_2O – CH_2Cl_2 medium, the reaction of benzoyl chloride and PNO in the CH_2Cl_2 phase was the rate-determining act. Therefore, it was worthwhile investigating the effects of the substrates structure on this system. The substrates structure was varied by means of substituent's, which included H_3C-, $(CH_3)_3C$-, CH_3O-, F-, Cl-, Br-, and I- groups. Similar to the PhCOCl/PhCOONa system,

these reactions followed pseudo-first-order kinetics with the effective pseudo-first-order rate constant, which submit to Eq. 18. The values of the catalyzed rate constant (k_c) for different $X_nC_6H_{5-n}COCl$ and $Y_kC_6H_{5-k}COONa$ are summarized in Table 1.

Table 1. Effects of substituent's on catalyzed rate constant (k_c) for PNO-catalyzed reaction of benzoyl chloride ($X_nC_6H_{5-n}COCl$) and benzoate ion ($Y_kC_6H_{5-k}COONa$) in $H_2O - CH_2Cl_2$ system

X	Y	k_c, $M^{-1}\cdot s^{-1}$	T, ºC	Ref.	X	Y	k_c, $M^{-1}\cdot s^{-1}$	T, ºC	Ref.
H	H	3.60	22	39	4-Cl	4-Cl	5.37	22	44
2-CH_3	2-CH_3	1.49	10	49	4-Cl	H	5.43	22	44
3-CH_3	3-CH_3	2.53	20	49	2-Br	2-Br	7.10	15	45
4-CH_3	4-CH_3	1.53	20	49	2-Br	H	7.37	15	45
3-CH_3O	3-CH_3O	3.40	20	49	3-Br	3-Br	6.10	15	45
3-CH_3O	H	1.83	20	49	3-Br	H	6.21	15	45
4-CH_3O	4-CH_3O	0.712	20	49	4-Br	4-Br	5.80	15	45
4-CH_3O	H	0.640	20	49	4-Br	H	5.61	15	45
4-$C(CH_3)_3$	H	1.87	20	47	2-I	2-I	17.5	20	49
2-F	2-F	9.10	20	47	2-I	H	10.9	20	49
2-F	H	10.4	20	47	4-I	H	6.83	20	49
3-F	3-F	6.10	20	47	2,3-Cl_2	2,3-Cl_2	15.6	22	48
3-F	H	6.80	20	47	2,3-Cl_2	H	11.6	22	48
4-F	4-F	3.40	20	47	2,4-Cl_2	2,4-Cl_2	11.1	22	48
4-F	H	3.93	20	47	2,4-Cl_2	H	12.4	20	48
2-Cl	2-Cl	8.10	22	44	3,4-Cl_2	3,4-Cl_2	15.4	22	48
2-Cl	H	10.1	22	44	3,4-Cl_2	H	7.98	20	48
3-Cl	3-Cl	6.43	22	44	3,5-Cl_2	3,5-Cl_2	57.3	22	48
3-Cl	H	6.37	22	44	3,5-Cl_2	H	10.5	22	48

For more detail analysis, the Hammet correlations were constructed. Good correlations were obtained for the meta- and para- substituent's in the coordinate of $\log(k_c/k_{cH})$ in the X-direction and σ in the Y-direction, where σ was the substituent constant and k_{cH} was the catalyzed rate constant of the benzoyl chloride [49]. The reaction constant (ρ) as slope were obtained from with correlation. The value of ρ was +1.3. Therefore this reaction is a nucleophilic

substitution reaction and it can be accelerated by the electron-withdrawing substituent and retarded by the electron-donating substituent. This completely agrees with data which were observed in these reactions. It is well known that the application of the Hammett equation to the ortho-substituent is usually poor, because of the steric effect. However, the inductive and resonance effects, the electron-withdrawing ortho-substituent (F-, Cl-, Br-, or I-) also facilitates the reaction considerably by complexing with the positively charged nitrogen atom of the pyridinium moiety. In contrast, the electron-donating ortho-substituent (H_3C- or CH_3O-) also retards the reaction via the steric effect.

The course of investigations of the substrates structure effects, the effects of carboxylate ions were also examined. Various mono- and dicarboxylate were taken for exploration. The effects of carboxylate [RCOONa] ions on the PNO-catalyzed IPTC reactions of PhCOCl and sodium carboxylates in H_2O – CH_2Cl_2 system were investigated for selected carboxylate ions (Table 2) [42]. These results were rationalized by the good correlations of the distribution of PNO in the CH_2Cl_2 phase and the carboxylate ions in the aqueous phase [42, 46].

Table 2. Effects of carboxylate ions on PNO-catalyzed reaction of benzoyl chloride with sodium carboxylates in H_2O – CH_2Cl_2 system

Carboxylate ion	k_c, $M^{-1}\cdot s^{-1}$	Carboxylate ion	k_c, $M^{-1}\cdot s^{-1}$
$HCOO^-$	3.50	n-$C_4H_9COO^-$	3.83
CH_3COO^-	3.55	n-$C_5H_{11}COO^-$	3.75
$C_2H_5COO^-$	3.52	n-$C_6H_{13}COO^-$	3.83
$(CH_3)_2CHCOO^-$	3.77	n-$C_7H_{15}COO^-$	3.35

$[PhCOCl]_{org,0}$ = 0.0100M, $[RCOONa]_{aq,0}$ = 0.500 M, at 18^0C.

The effects of dicarboxylate [$R(COONa)_2$] ions on the composition of products of the PNO-catalyzed IPTC reactions of PhCOCl and sodium dicarboxylates in H_2O – CH_2Cl_2 system were analyzed on several dicarboxylate ions. There are oxalate, malonate, maleate, fumarate, succinate, adipate, nonanedioate, phthalate, isophthalate, and terephthalate among them [43]. The compositions of the obtained products depended on the molecular structure of the dicarboxylate ion. The reaction rates depended significantly on the type of dicarboxylates analogously to the effects of monocarboxylates (Table 3).

These results were also rationalized by the good correlations of the distribution of PNO in CH_2Cl_2 and the dicarboxylate ions, with the exception of the nonanedioate ion, due to interference by the emulsion phenomenon [42]. Generally, products included mono- and bis-(benzoyloxycarbonyl) compounds,

benzoic anhydride, and benzoic acid. Accordingly to distribution of products the four types of dicarboxylate ions were classified (Table 4).

Table 3. Effects of dicarboxylate ions on PNO-catalyzed reaction of benzoyl chloride with sodium dicarboxylates in $H_2O - CH_2Cl_2$ system

Dicarboxylate ion	k_c, $M^{-1}\cdot s^{-1}$	Dicarboxylate ion	k_c, $M^{-1}\cdot s^{-1}$
malonate	4.10	nonanedioate	4.08
succinate	4.02	phthalate	3.80
maleate	3.83	isophthalate	2.73
fumarate	3.03	terephthalate	2.72
adipate	4.27		

$[PhCOCl]_{org,0} = 0.0100M$, $[R(COONa)_2]_{aq,0} = 0.500$ M, at 18^0C.

Elementary acts for the generation of mono- and bis-(benzoyloxycarbonyl) were proposed as follows:

Aqueous phase reaction:

$$PhCOONP^+_{aq} + (RCOO^-)_{2aq} \rightarrow PhCOOCORCOO^-_{aq} + PNO_{aq} \quad 17$$

$$PhCOONP^+_{aq} + PhCOOCORCOO^-_{aq} \rightarrow R(COOCOPh)_{2aq} + PNO_{aq} \quad 18$$

Organic phase reaction:

$$PhCOONP^+_{org} + (RCOOH)_{2org} \rightarrow PhCOOCORCOOH_{org} + PNO_{org} \quad 19$$

$$PhCOONP^+_{org} + PhCOOCORCOOH_{org} \rightarrow R(COOCOPh)_{2org} + PNO_{org} \quad 20$$

Interfacial reaction:

$$PhCOONP^+_{if} + (RCOO^-)_{2if} \rightarrow PhCOOCORCOO^-_{if} + PNO_{if} \quad 21$$

$$PhCOONP^+_{if} + PhCOOCORCOO^-_{if} \rightarrow R(COOCOPh)_{2if} + PNO_{if} \quad 22$$

Table 4. Types of dicarboxylate ions

Type	Main products	Minor product	Dicarboxylate
I	PhCOOH, (70-80%)	$(PhCO)_2O$	oxalate, malonate, maleate, and succinate
II	PhCOOCORCOOH	$(PhCO)_2O$, PhCOOH	phthalate
III	$R(COOCOPh)_2$, (70-88%)	$(PhCO)_2O$, PhCOOH, PhCOOCORCOOH	fumarate, isophthalate, terephatate, and nonanedioate
IV	PhCOOH $R(COOCOPh)_2$	$(PhCO)_2O$, PhCOOCORCOOH	adipate

Type I dicarboxylates ion tend to exist in the aqueous phase due to their low organophilicities. The steric difficulties because of the nearby second carboxylate group, reduce to inhibition of the reactions 17 and 18 or 21 and 22. That is the reason that the reaction was dominated by the hydrolysis path (reactions 15 and 16) to produce PhCOOH.

Type II dicarboxylates ion includes the conjugate acids such as phthalate. The PhCOOCORCOOH is the main product for this type of dicarboxylates, because of they had higher organophlicities than those of the Type I dicarboxylates. The observed main product could be generated by reactions 17, 19, and 21. However, reactions 18, 20, and 22 were inhibited by the steric effect of the second carboxylato group at the ortho-position, that explain why no $C_6H_4(COOCOPh)_2$ was detected.

In type III dicarboxylates ion the steric hindrance of the second carboxylato group is absence. That is why, the main products were the bis(benzoyloxycarbonyl) compounds [$R(COOCOPh)_2$] for this type of dicarboxylates. The main product generated in the various reactions, against of dicarboxylate, e.g. for isophthalate and terephthalate systems, reactions 17-22 were involved in the generation of $C_6H_4(COOCOPh)_2$, for the fumarate system, trans-$C_2H_4(COOCOPh)_2$ was generated mainly via reactions 17 and 18, 21 and 22, for the nonanedioate system, $(CH_2)_7(COOCOPh)_2$ was produced mainly by reactions 21 and 22 due to its surfactant property.

For type IV dicarboxylates ion, such as succinate, a wide distribution of products was observed, because of their properties seemed to occur at an intermediate position.

Solvent effects

PNO is polar substance and so, thermodynamically, the distribution of PNO in the organic phase is favored by the polarity of the organic solvent. Kinetically, the reaction is also more favorable to take place in polar organic solvent, since the transition state formed by PhCOCl and PNO (reaction 12) is more ionic than both PhCOCl and PNO. The order of relative reaction rates with respect to the polarity of organic solvents was cyclohexanone > CH_2Cl_2 » $CHCl_3$ > CCl_4, which was consistent with the order of polarities. In case of non-polar solvent such as benzene the benzoic acid was obtained, instead of anhydride. Similar results were generally observed for other benzoyl chlorides and carboxylate ions [44,45,47-49].

Apparently from the Table 5, addition of an inert organic substance, which have larger polarity than solvent reduce to increasing of the reaction rate and vice versa, addition of an inert organic substance, which have smaller polarity

than solvent reduce to decreasing of the reaction rate. That was obtained in the H_2O – CH_2Cl_2 system with, keeping the volume of organic phase constant.

Table 5. Effect of organic phase composition on PNO-catalyzed reaction of benzoyl chloride with sodium benzoate in H_2O – organic solvent system

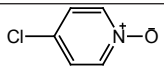
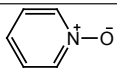
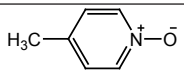
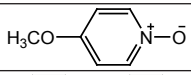
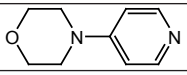
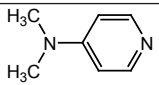
Organic solvent	$k_{ef}\cdot10^4$, s^{-1}	Organic solvent	$k_{ef}\cdot10^4$, s^{-1}
Cyclohexanone	17.7	CH_2Cl_2 (0,5 M PhCN)	10.1
$CHCl_3$	3.25	CH_2Cl_2 (0,5 M $PhNO_2$)	9.50
CCl_4	2.70	CH_2Cl_2 (1,5 M $PhNO_2$)	10.1
CH_2Cl_2	8.08	CH_2Cl_2 (0,5 M CCl_4)	7.08
CH_2Cl_2 (0,5 M $PhCH_2CN$)	10.2	CH_2Cl_2 (1,5 M CCl_4)	4.75
CH_2Cl_2 (1,5 M $PhCH_2CN$)	12.0		

$[PhCOCl]_{org,0} = 0.0100M$, $[PhCOONa]_{aq,0} = 0.500$ M, and $[PNO]_{aq,0} = 2.00\cdot10^{-4}$ M, at 18^0C.

Catalysts effects

There are many various pyridine derivatives, which can be use as IPTC catalysts. Therefore, it is important to know dependence of effective rate constant from parameters of catalyst. Recently, this dependence was investigated on reaction benzoyl chloride with sodium benzoate in the H_2O – CH_2Cl_2 system [50]. The pyridine derivatives, which were used as IPTC catalysts, are shown in Table 6.

Table 6. Effect of catalysts on IPTC catalyzed reaction of benzoyl chloride with sodium benzoate in H_2O – CH_2Cl_2

№	Catalyst	k_c, $M^{-1}\cdot s^{-1}$	pK_{BH+}
1		2.90	0.33
2		4.37	0.79
3		10.67	1.29
4		19.95	2.05
5		5.17	3.25
6		3.19	3.88

For catalysts №1-4 good correlation of effective rate constant and pK_{BH+} were obtained. In contrast, more based pyridines demonstrated smaller values of effective rate constant than it was possible to expect from the obtained correlation (Figure 3). Also, the reactions have a zero-order of reaction on benzoate or on benzoyl, if they were catalyzed by catalysts №1-4 or №5, 6 respectively. These facts were explained by changing of the rate determining elementary act. In case of catalysts №1-4 the rate determining elementary act was the reaction of ionic

Intermediate formation in an organic phase, but with increasing of catalysts basicity it changed by reaction of ionic intermediate and benzoate ion in a water phase.

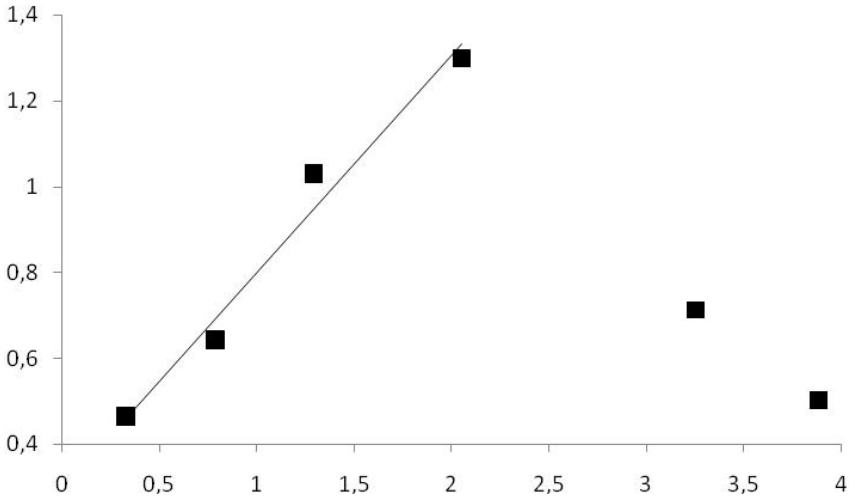


Figure 3. Dependence of lgk_c – pK_{BH+} for reaction of benzoyl chloride with sodium benzoate (catalysts №1-№6)

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Chapter 5

The potential usage of MALDI Q-TOF technique for determination of peptides in cosmetics formulation

Anna Olejnik, Izabela Nowak and Grzegorz Schroeder
Adam Mickiewicz University, Faculty of Chemistry, Grunwaldzka 6, 60-780 Poznań, Poland

Every component of cosmetic formulation such as oils, emulsifiers, emollients, humectants has an influence on the skin or hair. Currently specific active ingredients are added to those formulations to obtain special effects that increase the value of the product. The cosmetic products with specific bioactive substances have become more and more popular in dermatology and in the skin care industries. The demands for these novel substances have created a diverse new field devoted to designing molecules for the application in these industries (businesses). This, in turn, has created an urgent search for new quantitative and qualitative analytical methods for determining the active ingredients in cosmetics formulation. Nowadays, the most common substances are bioactive peptides, which are important in many natural processes with relevance to skin care. Although protein hydrolysates (protein fragments that are also called "peptides") had been used since the '60s, the idea of using specifically chosen peptide sequence which may act at extremely low levels (in the ppm range) and still show measurable cosmetic activity in formulas is new and quickly gains general approval. Numerous biologically active peptides do exist in the human organism and many of them have properties useful for the skin, the scalp, nails and the hair, and now fin their place in cosmetic formulations. Especially creams with peptides are the new cosmetics with the potential to improve the appearance of aging skin and are known as anti-age or anti-wrinkle cosmetics. In the case of the skin care industry, peptides seem to be stable, do not exhibit toxicity, irritation or allergy and have a positive impact on the skin and body. Thanks to new experimental techniques, there are more and more methods for the synthesis of peptides, which are capable of reaching their desired targets intact and in their active form. These bioactive molecules can be formulated in such a way as to be compatible with other components and can be delivered to the skin more effectively than proteins [1, 2].

The activity of peptides in cosmetic formulations can be compared with how a messenger works. Every peptide, depending on its amino acid sequence, transports a special message to the skin cells and thus has an influence on their functioning. The type, number and sequence of the 20 basic amino acids are the factors that determine all of their biological properties. Usually significant differences (quantitative and qualitative differences and effects on the skin and hair) are related to the type, number and sequence of the amino acids. The long-term research and analysis of the biochemical mechanisms of wrinkle formation resulted in the invention of special peptides and suggested their application in cosmetic industry, that has revolutionized the cosmetics world. It is thought that peptides are able to reduce the depth of wrinkles on the face, especially in the forehead and around the eyes. The synergetic effect induced by the combined influences of several peptides has also a significant meaning [3]. Additionally, it is postulated that peptides are safer, gentler and more inexpensive than Botox®, which affects the same mechanism of wrinkle formation, however Botox® is more toxic and invasive.

The peptides applicated in cosmetic formulations can be divided into three groups [4]:

1. Signal peptides – stimulated – peptides with the ability to improve the appearance of fine and coarse wrinkles by increasing fibroblast production of collagen or decrease collagenaze breakdown of existing collagen or elastin production. Actually, the first peptide released, with huge fanfare, was Matrixyl® with five amino acids in a chain, INCI name: Palmitoyl Pentapeptide-4 (pal-Lys-Thr-Thr-Lys-Ser) made by Sederma. However, the most widely used and popular signal peptide in the cosmetics industry has the sequence H-Ser-Leu-Ile-Gly-Lys-Val-NH_2 (hexapeptide-10, also known as Serilesine®, Lipotec SA) (Fig. 1).

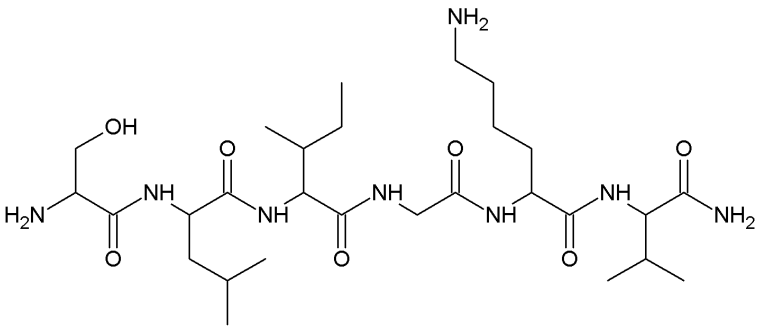

Figure 1. Chemical formula of hexapeptide – stimulated peptide Serilesine®

The hexapeptide mentioned above can have a positive influence on the cohesion between the dermis and epidermis, due to the increase of hemidesmosomes production. Additionally, induces the production of novel skin cells and causes the increasement in the coherence, density and smoothness of the skin. The hexapeptide (Serilesine®, Lipotec SA) stimulates the production of α6-integrin and laminin-5, the protein involved in cell adhesion, signal transduction and differentiation of keratinocytes. Hexapeptide-10 is able to have an influence on strengthening the dermo-epidermal junction and for that reason, can be incorporated into cosmetics formulations such as emulsions, gels, and serums in order to regenerate the skin. [2, 5] One have to acknowledge that the most widely published study on signal peptide is mentioned before the sequence lysine-threonine-threonine-lysine-serine (KTTKS) found on type I procollagen. This pentapeptide has been demonstrated increase production of extracellular matrix proteins such as types I and II collagen and fibronectin. KTTKS is also combined with palmitic acid in order to enhance delivery through the epidermis. This pentapeptide is known in cosmetics industry as palmitoyl pentapeptide-4 or Matrixyl® (Sederma) [6].

Another signal peptide used in cosmetics is the sequence of valine-glycine-valine-alanine-proline-glycine. This bioactive compound is able to stimulate human dermal skin fibroblast production and simultaneously down-regulate elastin expression. The peptide was combined with palmitic acid in order to aid peptide penetration through the epidermis and is known in many cosmeceutical products as palmitoyl oligopeptide (Dermaxyl®, Sederma). On the other hand the sequence of tyrosine-tyrosine-arginine-alanine-aspartame-aspartamealanine inhibits procollagen-C proteinase, which cleaves C-propeptide from type I procollagen, thus leading to decrease collagen breakdown.

Lipospondin, a tripeptide combined with elaidic acid (elaidyl-Lys-Phe-Lys) activates latent transforming growth factor-beta, inhibits matrix metalloproteinase mRNA: messenger ribonucleic acids (MMP) and is able to upregulate collagen and tissue inhibitor of metalloproteinase (TIMP)-1 production. Another tripeptide, lysyl-valine-lysine, has been shown to have similar effects on the up-regulation of transforming growth factor-beta when combined with palmitic acid and bistrifluoroacetic acid and is currently marketed under the name of palmitoyltripeptide-3/5 or Syn®-Coll (Pentapharm) [7].

2. Neurotransmitter - inhibiting peptides (neuropeptides) – are able to function as neuromodulators, neurotransmitters or neurohormones and exhibit anti-wrinkle activity. Their effect is connected with their ability to block the entrance of calcium ions, and in this way muscular contraction is attenuated. The neurotransmitters were developed as topical mimics of the botulinum

neurotoxins. The neuropeptides transfer the signal to skin cells in order to inhibit muscle movement. Potentially they are able to decrease facial muscle contraction, reducing lines and wrinkles. It has been published that the neuropeptide Leuphasyl® (pentapeptide with the sequence *Tyr-Ala-Gly-Phe-Leu*, Lipotec SA*)* reduces the depth of wrinkles caused by the muscle contraction associated with facial expressions, especially in the forehead and around eyes (Fig. 2) [8].

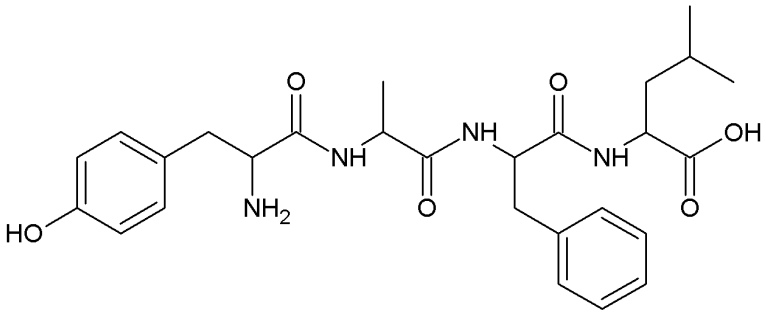

Figure 2. Chemical formula of pentapeptide - neuropeptide Leuphasyl®

The most widely used neuropeptide in cosmetic formulation is acetyl hexapeptide-3 (Ac-Gly-Glu-Met-Gln-Arg-Arg-NH_2 known as Argireline® (McEit [Tianjin] International Trade Co., Ltd.). This synthetic peptide is patterned from the N-terminal end of the protein SNAP-25 that is able to block vesicle docking by preventing formation of the ternary SNARE complex and catecholamine release.

The observation of nature caused the discovery of an interesting source of bioactive peptides. It was observed that the temple viper *Tropidolaemus wagler* produces neurotoxin, which blocks the release of acetylocholine thus inhibits muscle contraction and paralyzes the victim. The research proved that the venom of the temple viper consist of protein Walglerin-1 (Pentapharm). Thanks to these observations a new neuropeptide - tripeptiede-3 (beta-Ala-Pro-Dab-NH-benzyl × 2 AcOH), which is proposed to act similarly to Walglerin-1, is synthesized. This peptide is currently marketed as Syn®-Ake (Pentapharm).

3. Carrier peptides - the main role of carrier peptides is to stabilize and deliver an important trace elements, such as copper which is necessary for wound healing and enzymatic processes. The tripeptide Gly-His-Lys (GHK) is able to spontaneously complex with copper and thus facilitate its uptake by cells and exhibits also an important functions in the skin. It is postulated that carrier peptides can improve skin firmness and texture [9, 10].

The quantitative and qualitative analysis of peptides in cosmetics formulation seems to be really challenging, especially if the assay is made without any initial processes such as separation, extraction or inspissations of the component to be determined. The conventional methods for determining proteins in cosmetics form actually depend on protein identification. The main aim of protein profiling is to identify as many peptides in a sample as possible. A typical technique of profiling a population of peptides is by two-dimensional electrophoresis. However, mass spectrometry based on analytical methods has become standard in cosme- and pharmaceutical research and product development laboratories. Specifically, liquid chromatography (LC) combined with tandem mass spectrometry (MS/MS) is commonly used for the analysis of compounds in active components/drug discovery. The widespread use of MS is a result of its high sensitivity, rapid analysis time, and the high specificity gained through employing MS/MS by using triple quadrupoles, Paul and Penning ion traps, and quadrupole TOF mass spectrometers. A major feature of the LC-MS/MS methodology is the requirement for relatively involved, destructive, and laborious sample preparation. For the analysis of cosmetic samples, these steps include extraction of the target analyte(s). However, it can now be characterized directly without initial steps by using matrix assisted laser desorption ionization (MALDI) mass spectrometry [11]. The combination of MALDI MS with a time-of-flight mass analyzer (TOF) is a particularly valuable technique which is often used in biochemistry to analyze peptides, nucleotides, proteins, lipids and oligosaccharides [12]. MALDI-TOF (Matrix-Assisted Laser Desorption Ionization-time of flight) technology has been adopted rapidly by the global proteomics community, as its tandem mass analyzers provide a leap in the throughput rates achieved in protein analysis.

A series of commercially available cosmetics peptides were analyzed using the method mentioned above. The main goal of the research was to investigate if MALDI Q-TOF mass spectrometry could be an appropriate method for the analysis of peptides in cosmetics formulations. The main aim of current investigation is the analysis of the commercially available bioactive peptides such as pentapeptides and hexapeptides, determination of their molecular weight and selection of the appropriate experimental conditions for these measurements. In addition the detection limit of peptides in cosmetics formulation is verified with usage of MALDI Q-TOF mass spectrometry.

The procedures of experimental

The Waters MALDI Q-TOF Premier was used to carry out the analyses of peptides. Every mass spectrometer can be divided into five fundamental

elements, which are presented in Fig. 3.

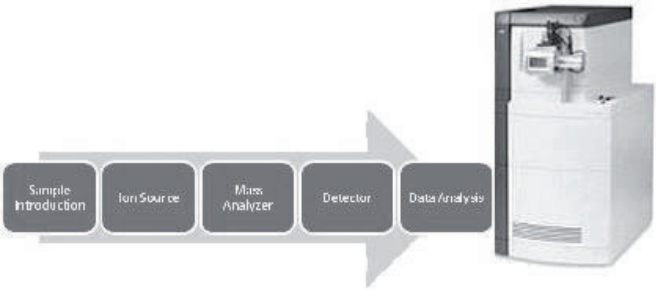

Figure 3. Fundamental parts of the mass spectrometer together with the picture of the Waters MALDI Q-TOF Premier equipment

In case of a MALDI Mass Spectrometer, the sample introduction has to be made with the use of MALDI target plates (Fig. 4) The prepared sample is directly inserted into the high vacuum of the mass spectrometer (below 10^{-6} mbar). The ionization process is caused by a laser beam, that excites the matrix, which transfers energy to the analytes. This results in the ionization and desorption of the analytes, mainly as singly charged ions (species) [13]. The charged ions are accelerated and brought to the time-of-flight (TOF) mass analyzer where the ions are easily separated [13, 14].

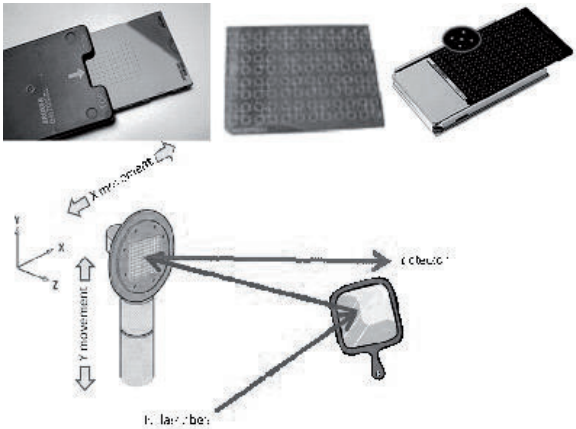

Figure 4. The sample target for matrix-assisted laser desorption ionization mass spectrometry (top) and the alignment of the sample target (bottom) [13, 14]

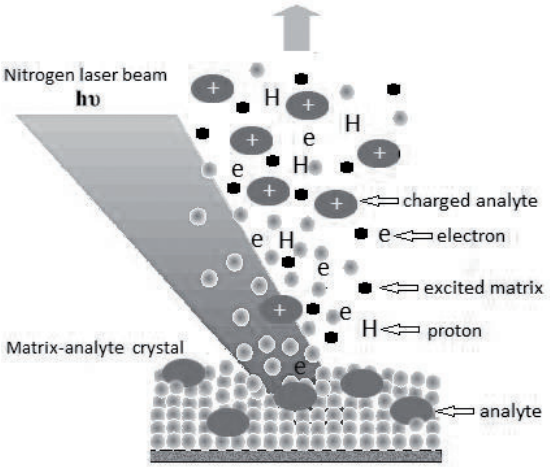

Figure 5. MALDI ionization process [13]

Firstly, the sample was dissolved in a suitable and volatile solvent. Subsequently, the prepared solution was mixed with an excess of an appropriate matrix. In some preparations, the small amounts of substances, which facilitate formation of ions, were added (e.g., trifluoroacetic acid, sodium or copper salts). In the experiments, 2,5-dihydroxybenzoic acid (2,5-DHB) was used as the matrix. The prepared mixture (1 µl) was residued on a MALDI plate and air-dried. Under these circumstances, the sample was co-crystallized with the matrix. This step of the preparation is fundamental for the quality of the spectrum (Fig. 6) [9].

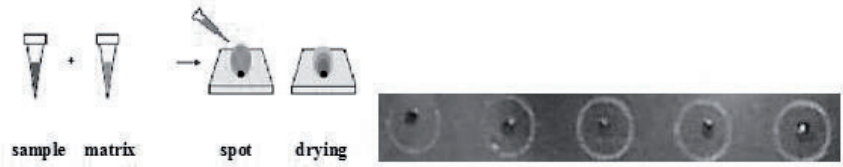

Figure 6. The sample preparation – dried-droplet method [14]

Results

The analyses of the two parent peptides - pentapeptide (Leuphasyl® - Tyr-Ala-Gly-Phe-Leu) and hexapeptide (Serilesine® H-Ser-Leu-Ile-Gly-Lys-Val-NH_2) were carried out using the MS MALDI technique. In the MALDI TOF

spectrum, the signals were collected as a function of intensity. In the Fig. 7 the spectra of pentapeptide MW=569 and hexapeptide MW=615 are presented. The positive ion mode of pentapeptide (TOF MS LD+) registers a protonated molecular ion $[M+H]^+$ at m/z 570.6 and a molecular ion with an associated sodium $[M+Na]^+$ at m/z 592.6. In the negative ion mode (TOF MS LD-), the analyzed molecule is deprotonated $[M-H]^-$ matching to the signal at m/z 568.4. A similar pattern was also obtained in the case of hexapeptide. The protonated molecular ion $[M+H]^+$ at m/z 616.7 and the molecular ion associated with sodium $[M+Na]^+$ at m/z 638.7 were detected.

Subsequently the parent peptides were added to a newly prepared cream, and their detection limit by the usage of MALDI MS was verified. The researches proved that the peptides could be detected if the cream contains at least 0.05 % of the peptides. One has to keep in mind that the percentages of peptides in cosmetic forms are in the ingredient list mid-level, to high minimums. Usually, contents of the peptides which must be present in a formulation to achieve actual results are rather high (e.g., Matrixyl – 5 wt.%; Argireline – 10 wt.%). In the Fig. 8 the signals of the pentapeptide $[569.6+Na]^+$ and the hexapeptide $[615.7+Na]^+$ were registered. Additionally, a series of masses (m/z 693.8, 737.9, 782.0, 826.0, 870.0), corresponding to a detergent, were observed.

The MALDI-TOF MS technique was also used to identify peptides in commercial cosmetics formulations. In the Fig. 9 the spectrum of a cream containing acetyl hexapeptide-8 with the sequence - acetyl-Glu-Glu-Met-Gln-Arg-Arg-NH_2 (marketing name Argireline®, known as acetyl hexapeptide-3,) is presented. The signal at 911 m/z provides specific evidence for the identification of acetyl hexapeptide-8.

Next spectrum of commercial available cosmetic is presented in the Fig. 10. The analysis of registered signals proves that the cream contains two different peptides. The signal at 606 m/z is the ion associated with sodium of caproyl tetrapeptide-3 [MW=582] and the other signal at 911 m/z is the ion associated with sodium of acetyl hexapeptide – 8 [MW=888].

In order to improve the results, the analyses were carried out under various experimental conditions, i.e., with and without the presence of sodium chloride. The spectrum in the Fig. 11 confirms that even a small addition of a sodium salt facilitated the formation of ions, thereby the signals of the ions coupled with sodium were more intense.

Furthermore, the influence of the matrix on the mass spectra quality was also checked. For this purpose the same experiments were carried out in the presence of the matrix (Fig. 12) and without the matrix (Fig. 13). The analysis of the MALDI-TOF mass spectrum of hexapeptide with the usage of the matrix,

2,5-dihydroxybenzoic acid (2,5-DHB), confirms the molecular weight of Serilesine® (616.6). In the case of the negative ion mode (TOF MS LD$^-$), the detected m/z 614.4 might be the deprotonated molecular ion $[M-H]^-$. A positive ion mode (TOF MS LD+) spectrum shows a protonated molecular ion $[M+H]^+$ at m/z 616.6 and molecular ions associated with sodium $[M+Na]^+$and potassium $[M+K]^+$ ions at m/z 638.6 and 654.6, respectively.

In the mass spectrum of hexapeptide (Serilesine®) without a matrix (2,5-DHB) (Fig. 13), neither the signal of a quasi molecular ion $[M+H]^+$ nor molecular ions associated with sodium $[M+Na]^+$ or potassium $[M+K]^+$ ions were observed although these species were detected in the spectra Fig. 12. The results confirm the importance of a matrix in the MS MALDI technique. The energy cannot be transferred to the macromolecules without a matrix, and thereby the ionization is impossible.

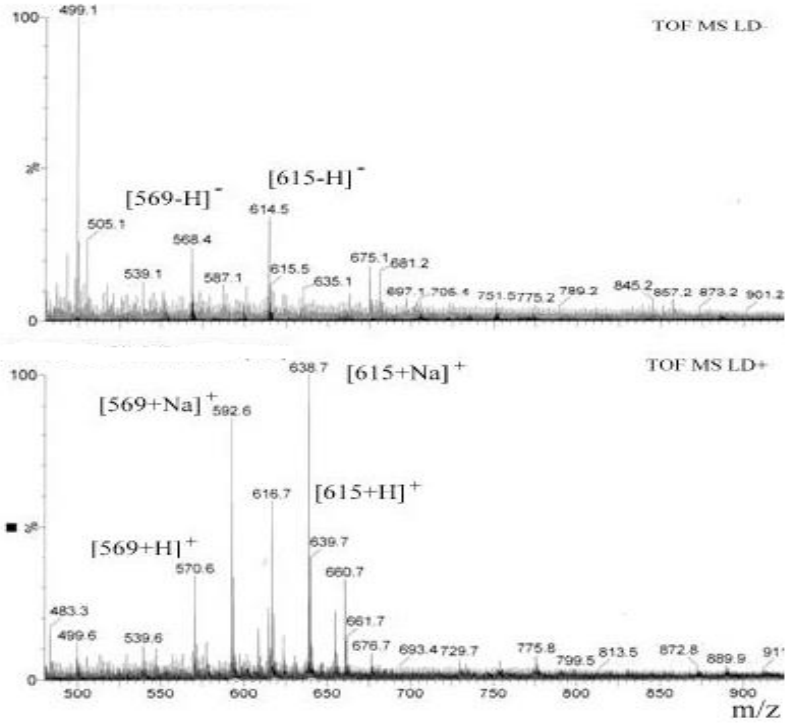

Figure 7. MALDI-TOF mass spectrum of pentapeptide and hexapeptide

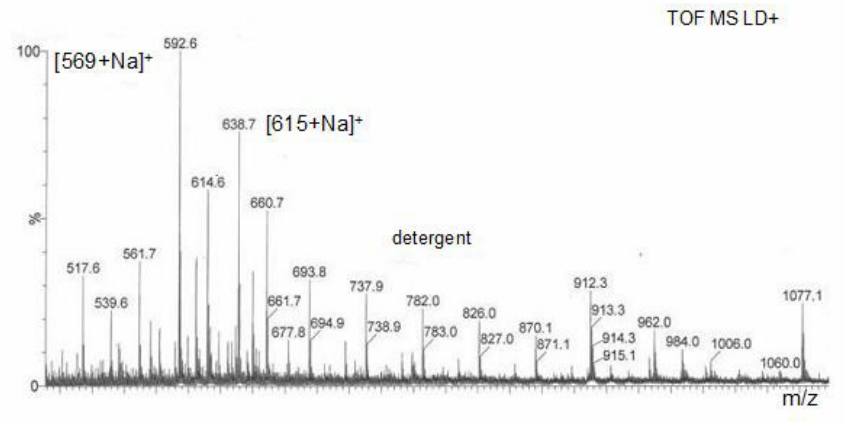

Figure 8. MALDI-TOF mass spectrum of a cream containing the pentapeptide and hexapeptide

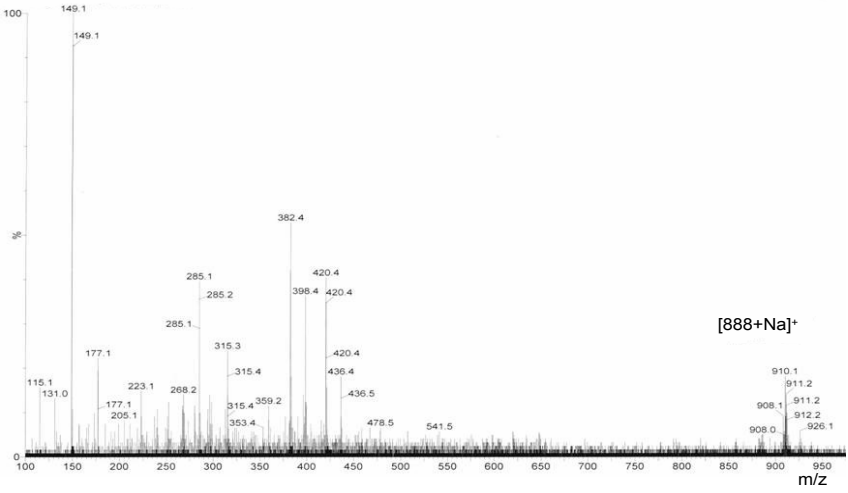

Figure 9. MALDI-TOF mass spectrum of a cream containing hexapeptide

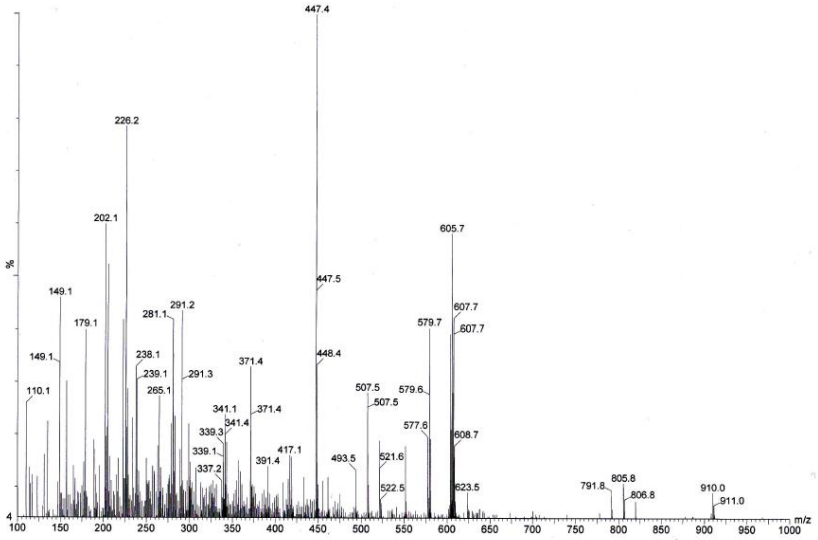

Figure 10. MALDI TOF spectrum of a cream containing tetrapeptide and hexapeptide

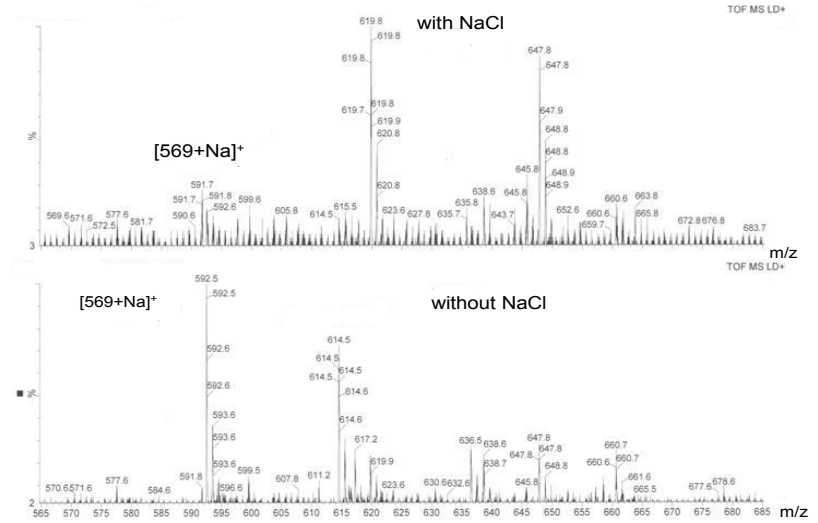

Figure 11. MALDI-TOF mass spectrum of hexapeptide (Serilesine®)

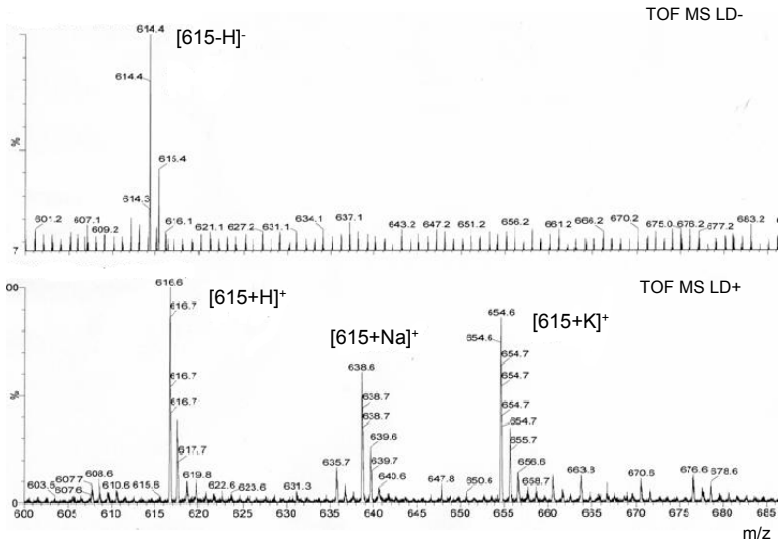

Figure 12. MALDI-TOF mass spectrum of hexapeptide (Serilesine®) with matrix (2,5-DHB)

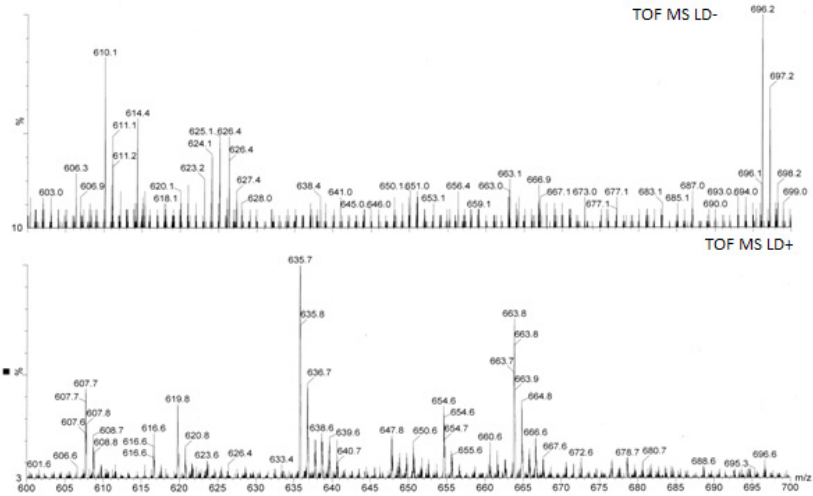

Figure 13. MALDI-TOF mass spectrum of hexapeptide (Serilesine®) without matrix (2,5-DHB)

As many proteins, especially these in low abundance, are quite scarce owing

to the difficulties of sample isolation or due to peptide digest prior to MALDI-TOF MS, the analysis therefore requires that peptide preparations undergo an enrichment step beforehand. However, conventional methods for concentrating peptides by simple evaporation result in sample loss through protein adsorption onto the surface of the container. An additional problem with evaporation is the simultaneous concentration of buffer components (e.g., salts) and other contaminants. Alternative methods for peptide enrichment by reversed-phase resin are complex and are typically suitable only for hydrophobic peptides. Therefore a universal and simple peptide enrichment method is in high demand. Zeolites have attracted considerable attention as a novel chromatographic carrier in the adsorption and purification of proteins [15]. However, molecular sieve particles at the nanometer scale show a number of distinct characteristics: large external surface area, high dispersibility in both aqueous and organic solutions, and a variety of tunable surface properties, such as adjustable surface charge and hydrophilicity/hydrophobicity. These properties make them promising candidates for the concentration of trace biomolecules in large solution volumes through various protein–molecular sieve-surface interactions.

Herein, we report a remarkable enrichment of low-abundance peptides on the mesoporous molecular sieve based on their strong adsorption ability and high dispersibility. More importantly, the peptides adsorbed on the solid can be directly analyzed by MALDI-TOF MS. Hence, the risk of sample loss from any elution steps is avoided and the concentration procedure is simplified. The Fig. 14 presents the spectrum of pentapeptide (Leuphasyl®), hexapeptide (Serilesine®) and octapeptide (SNAP-8®), which were adsorbed on the mesoporous molecular sieve.

Summary

Mass spectrometry (MS), with the use of matrix-assisted laser desorption/ ionization (MALDI), is a soft and rapid analytical method for the analysis of bioactive compounds such as peptides. This technique allowed us to determine the molecular weight of peptides, however the determination of the specific amino acids in the sequences of the peptides is challenging, because fragmentation peaks were not registered. A comparative analysis of the hexapeptide spectra, made in the presence and in the absence of a matrix, proves the importance of matrix in the MS MALDI technique. Without the matrix, ionization process of the analyte is impossible. Thus no signals of the quasi-molecular ions were registered without a matrix. This research indicates that, in order to determinate the peptides in cosmetics formulations, the presence of a matrix and the addition of sodium salts or molecular sieves are required. Sodium chloride was used to

facilitate the formation of the ions. In addition, the identification of peptides in cosmetics formulations is possible if the prepared cream contains 0.05% of the peptides.

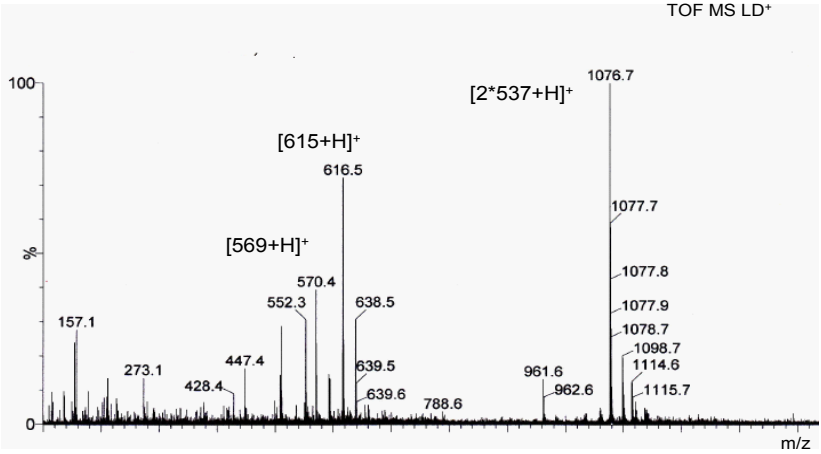

Figure 14. MALDI TOF mass spectrum of pentapeptide, hexapepide and octapeptide

Acknowledgment

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Chapter 6

Amphotericin B as a member of natural antibiotics family

Radosław Pankiewicz and Grzegorz Schroeder
Adam Mickiewicz University, Faculty of Chemistry, Grunwaldzka 6, 60-780 Poznań, Poland

The term macrolides refers to a family of natural or synthetic chemical compounds of mainly lactone structure containing a macrocyclic lactone ring with methyl and hydroxyl substituents and from 1 to 3 sugar residues of which one is amino-sugar. A characteristic feature of this group of compounds, following from a large number of oxygen atoms in their molecular structure, is the ability to form host-guest type complexes.

Another group of compounds capable of forming complexes of this type are ionophores. The molecule of ionophore contains a lipophilic skeleton and hydrophilic groups (–OH, C=O, C–O–C). Thanks to this structure, the ionophore can induce conformational changes upon complex formation that involve the ion capture in a hydrophilic cage (Fig. 1). In this way the ionophore can release (or dissolve) a charged ion inside the lipid membrane. The mechanism of the ion transportation is as follows. The host (ionophore) molecule dissolved in the hydrophobic phase captures the ion at the interface and then the complex diffuses in the lipid membrane. When the complex reaches the other side of the membrane, the host opens and the ion is released into the water phase. The empty host diffuses back to the other side of the membrane and the cycle is repeated until reaching equilibrium.

Because of their bacteriostatic and bactericidal properties the majority of macrolides and ionophores are antibiotics. They have a wide range of activity and are widely used as medical and veterinary drugs or as additives to animal fodder.

The ionophore ability to transport ions and the high selectivity of their complex formation has been used in construction of ion-selective electrodes. Their specific chemical affinity not only to ions but also to neutral molecules indicates them as an important group among chemical receptors. According to the results of our study performed for lasalocid acid, the chemical affinity can be relatively easily modified by attachment of oxaalkyl chains of different lengths

to the ionophore molecule. The attachment of such chains has practically no influence on the complex forming abilities of the ionophore but it considerably improves the solubility of the ionophore itself and the host-guest complexes it makes in the lipid membranes. The modification has been found to change not only chemical but also microbiological properties of the compounds. As proved by our results, the bactericidal or bacteriostatic properties of this group of compounds can be enhanced or weakened depending on the length of the oxaalkyl chain.

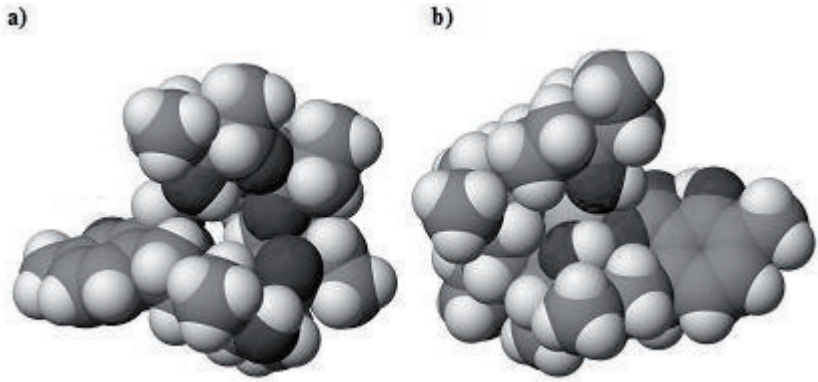

Figure 1. Calculated by PM5 semiempirical method structures of a) lasalocid acid b) lasalocid sodium salt

Lasalocid

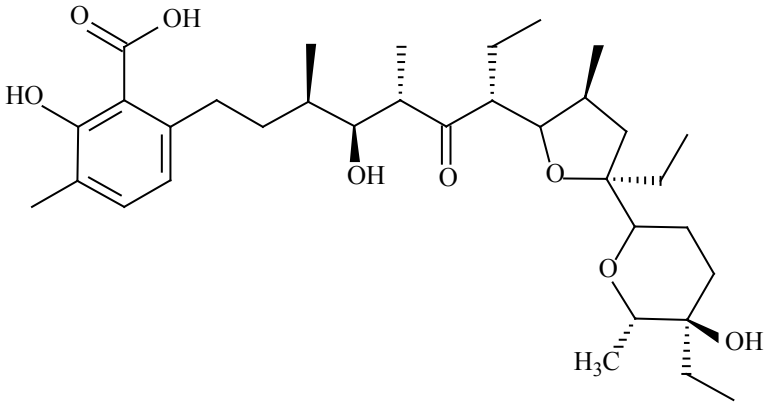

Figure 2. Lasalocid

Lasalocid (Fig. 2) was for the first time isolated in 1951 from *Streptomyces lasaliensis* by Berger et al. [1]. For the last three decades the subject of particular interest has been the interaction of lasalocid acid with alkali metals investigated by many methods including NMR, FT–IR and circular dichroism (CD). The interaction of lithium ions with lasalocid acid in acetonitrile has been studied by CD, ^{1}H, ^{13}C and ^{7}Li NMR [2-6]. The results indicated the formation of a mixture of 1:1 and 1:2 sandwich type complexes.

The properties and stabilities of the complexes of lasalocid acid (and its bromo-derivative) with a number of alkali metals in methanol have been studied by UV–vis spectrometry and calorimetry [7]. Results were interpreted in the conditions in which only neutral 1:1 complexes were forming (in these complexes the carboxyl group was not dominant in the metal ion bonding. The mode of complexation changed regularly with the ionic radius of the cation. It suggested that lasalocid acid was able to adapt to the size of the cation and does not show specific selectivity related to its size. Lasalocid acid is able to transport a wide range of cations (mono, di and trivalent included) through lipid membranes [8-12]. Like all ionophores it forms ion transfer complexes, which differentiates it from the other classes of antibiotics that only increase the permeability of biological membranes for this class of compounds [13-16].

Salinomycin

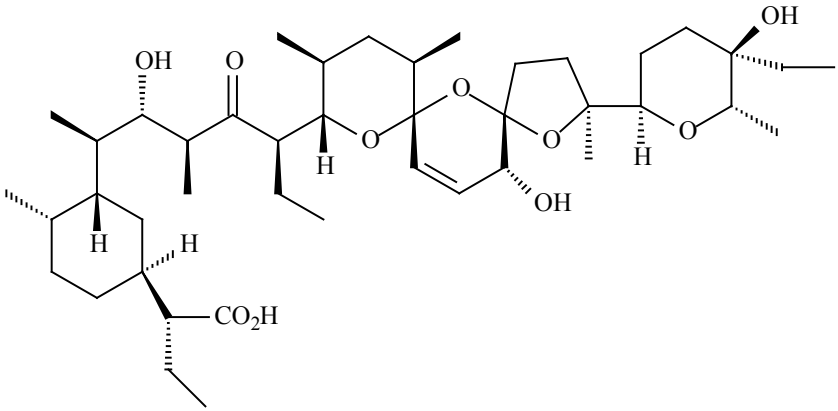

Figure 3. Salinomycin

Salinomycin (Fig. 3) isolated from *Streptomyces albus* is an effective antibiotic against gram-positive bacteria, mycobacteria and fungi. It has been widely used as a coccydiostatic in rearing of cattle and poultry. Coccydiostatics

are a group of chemotherapeutic substances that can be administered to animals, mainly to poultry, in prophylactic or treatment of coccidiosis caused by protozoa *Eimeria.*

Biological activity of salinomycin (likewise that of other ionophores) is strictly related to its physico-chemical properties. Salinomycin is able to dynamically adapt to the cation with which it is to make a complex. However, thanks to the ability to make a pseudo-ring, like many ionophores, on complexation it prefers closing the best geometrically fitted cation in the pseudo-ring, which is the potassium cation. The pseudo-ring is formed thanks to the formation of a hydrogen bond of head-to-tail type between the carboxyl group and the hydroxyl group attached to the last pyrane ring. Investigation of this structure is difficult because of the problems with getting the crystalline form of salinomycin. Well-developed crystals were obtained only of its derivatives [17]. Computer simulations permit visualisation of the structures of the complexes (Fig. 4) and determine their structural parameters.

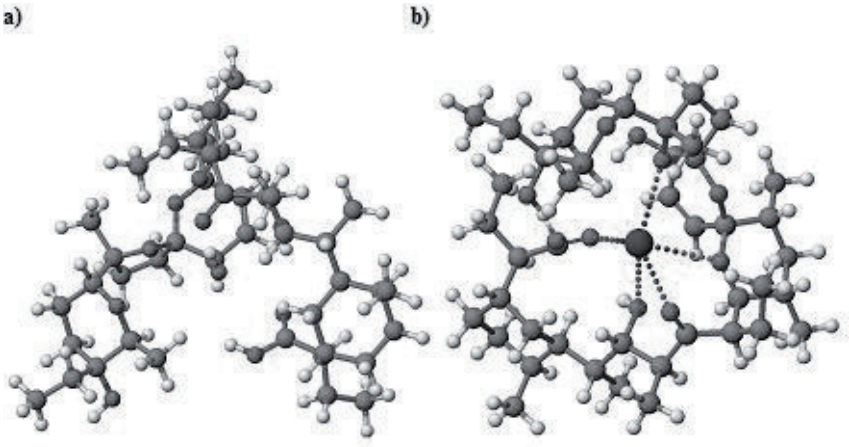

Figure 4. Calculated by PM5 semiempirical method structures of a) salinomycin acid b) salinomycin potassium salt

Erythromycin

Macrolides make a group of antibiotics of bacteriostatic activity. The name comes from the words macro (large) and oligo (lactone), as the molecules of these antibiotics have a 12-16 atom lactone core. Erythromycin is the best known natural macrolide antibiotic obtained from *Streptomyces erythreus*. Erythromycin is a polyhydroxylactone containing two sugar rings (Fig. 5.).

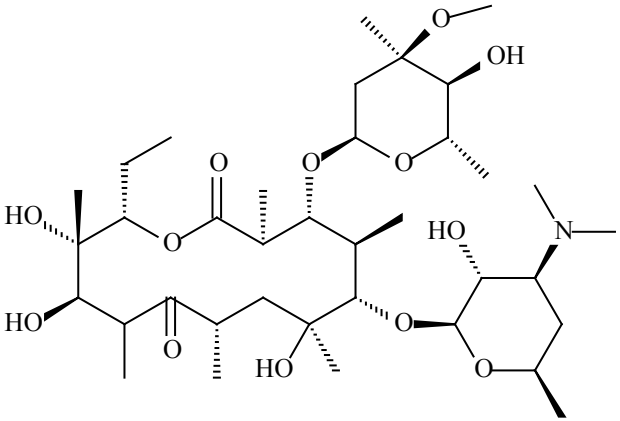

Figure 5. Erytromycin

The aglyconic fragment of the molecule is a 14-membered lactone ring. The basic character of the whole molecule (pK_a=8.8) is determined by the amino sugar attached to the lactone ring through the ß-glycoside bond [18]. The other sugar attached by the ß-glycoside bond is L-cladynose, unique for erythromycin (Fig. 6).

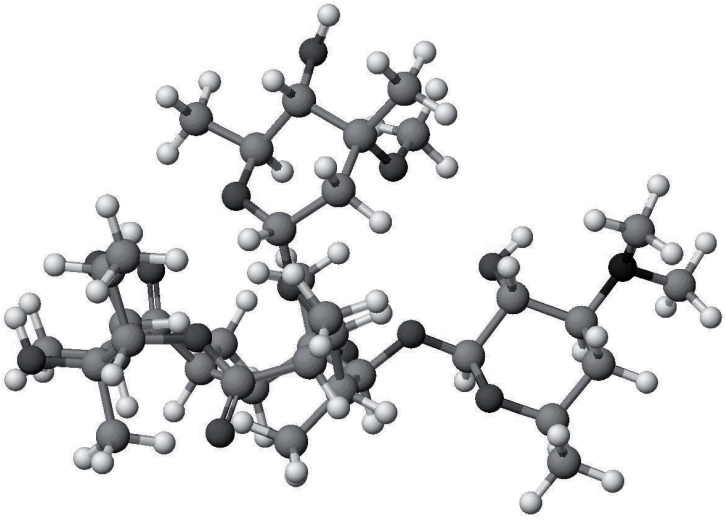

Figure 6. Calculated by PM5 semiempirical method structure of erytromycin

The bactericidal activity of erythromycin is related to its ability to inhibit biosynthesis of proteins [19]. This inhibition is realised by the attachment to ribosomal RNA, leading to off dissociation of *t*-RNA upon translocation [20].

Erythromycin, like many macrolides, is the most effective against Gram-positive, partly Gram-negative bacteria, *Mycoplasmas, Chlamydia* and *Rikettsia*. It is applied as an antibiotic to patients allergic to penicillin.

Amphotericin B

Amphotericin B (Fig.7) is a natural antibiotic obtained from the gram-positive bacteria *Streptomyces nodosus*. Amphotericin B was isolated in 1955 and already in 1960 it was introduced to therapy as the only effective means for treatment of systemic mycosis. Spatial structure of this antibiotic was established in 1970 [21], and then attempts of its chemical synthesis have been made Amphotericin B shows a wide range of antimycotic activity, it is effective against prostatic hypertrophy and hypercholesterolemia. It is produced in many formulations by many firms under the commercial names of Fungillin, Amphotericin B, Amphozone, Ampho-Moronal, Wypicil and Ampho-Moronal V.

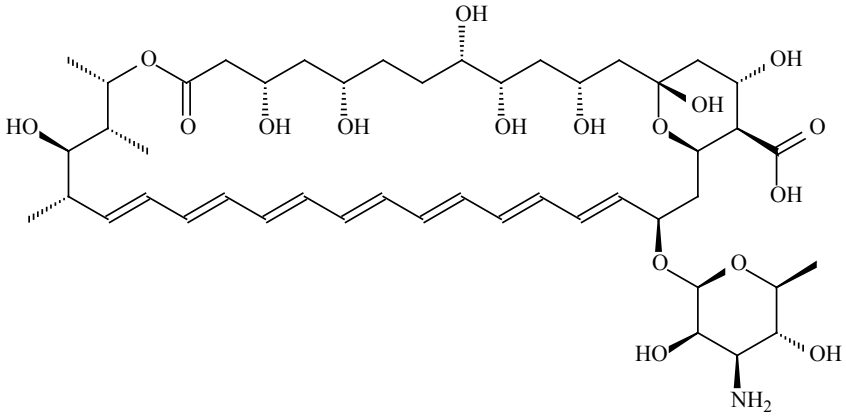

Figure 7. Amphotericin B

This antibiotic has a wide antimycotic activity and is also used as fungicide in infections of AIDS patients. It is supposed that the toxicity of Amphotericin B in the interaction with sterols of the lipid cell membrane is related to formation of molecular aggregates in the form of pores disturbing physiological transport

of K^+, Na^+, H^+, Cl^- ions, leading to the cell death. According to the most popular sterol hypothesis, sterol molecules are indispensable for AmB action in the membrane. It is also regarded that chemotherapeutic application of the antibiotic is based on the higher affinity/activity of AmB towards membranes containing ergosterol (in fungal membranes) than cholesterol (in mammalian membranes) [22]. Unfortunately, the application of amphotericin is limited because of its poor solubility in the majority of organic solvents and practical insolubility in water, less than 1 mg/L at physiological pH (pH 6-7) [25-31]. Due to its amphipathic nature, AmB forms aggregates in water at concentrations about 2x10-7 M. They are formed at 3 μM, well below the critical micellar concentration, by interaction between neighboring polyene chains [32].

The physico-chemical properties of this antibiotic are related to its structure comprising two main elements: the chain of coupled double bonds and the alkyl chain with hydroxyl groups. The rigid olefin chain (Fig. 8) prevents the molecule to coil up [fold up] in the process of ion complexation, which is so characteristic of ionophores such as lasalocid acid or monensin.

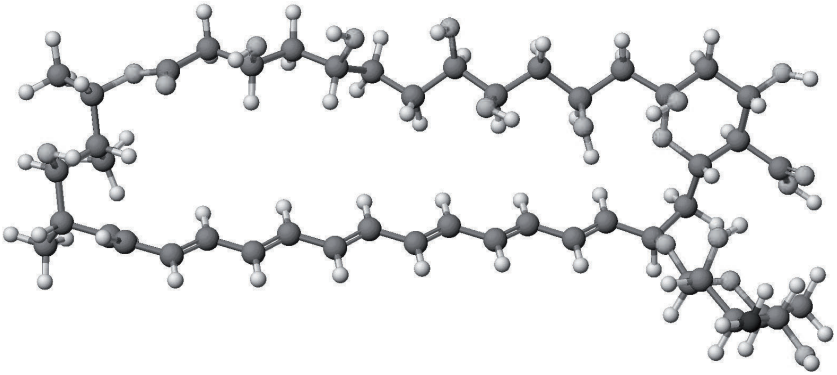

Figure 8. Calculated by PM5 semiempirical method inflexible structure of Amphotericin B

Amphotericin makes ionic channels in the cell membranes [21, 33, 34]. Such structures are made of eight molecules of amphotericin with the olefin fragments directed towards the lipophilic cell membrane and the lipophobic fragment directed to the centre, making a hydrophilous, water filled, channel. A single amphotericin molecule is only about as long as half the thickness of a bilayer membrane, so two such aggregates associate in an end-to-end fashion to create a membrane-spanning channel. Amino and carboxyl groups of AmB molecules

form a chain of intermolecular hydrogen bonds. These strong intermolecular AmB–AmB interactions within the channel are responsible for its stability. This facts are in good agreement with the experimental data which suggested that modification of either the carboxyl or the amino group modifies the activity or selectivity of a particular AmB derivative [35]. Analysis of computational data also revealed significant differences between AmB-ergosterol and AmB-cholesterol (Fig. 9) channels.

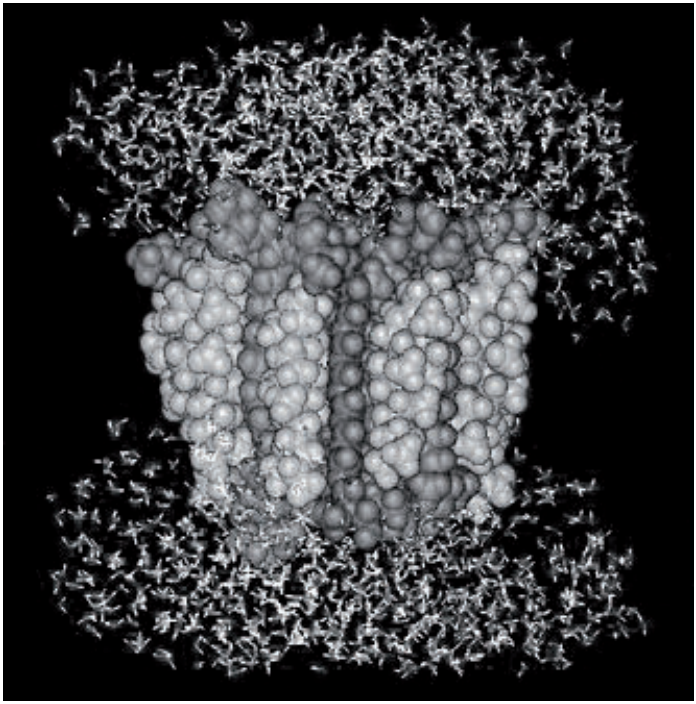

Figure 9. Lateral view of the AmB/cholesterol channel. Green AmB, violet cholesterol, red/white water molecules. With kind permission [36]

The first one is wider and more stable because the chain of hydrogen bonds between AmB molecules in the channel is more efficient. This may explain why AmB channels are more effective in membranes containing ergosterol molecules [29]. Through this channel the sodium and potassium ions can be transported freely according to the gradient of their concentrations (Fig. 10), which disturbs the cell functioning leading to its death.

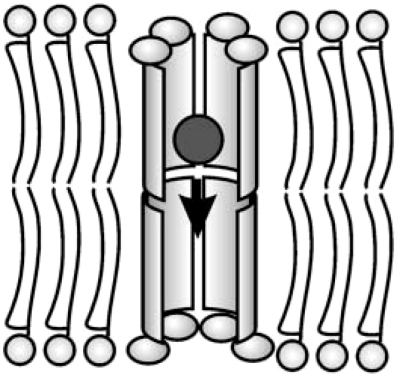

Figure 10. Schematic structure of Amphotericin B canal in bilayer lipid membrane [37]

This antibiotic is not specific and interacts also with cholesterol, the component of biological membranes of mammals [23]. The toxic effect is the strongest in the central nervous system, kidneys and liver, which considerably restricts the use of this antibiotic [24].

The mechanism of aggregation of the antibiotic molecules is strictly related to the capture of Na^+ and K^+ by the molecules of amphotericin B. Analysis of the absorption and emission spectra and fluorescence excitation spectra of amphotericin B in water solutions indicate a higher level of aggregation of the compound in the presence of potassium ions thanin the presence of sodium ones. The most important spectral changes typical of aggregated amphotericin B take place in the range of physiological concentrations of hydrogen ions (pH~7). The explanation of mechanisms of the molecular aggregates formation is of key importance for alleviation of the primary toxic effects occurring immediately after amphotericin administration [25].

Acknowledgements

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Chapter 7

Self-assembled monolayers - chemical modification, characterization and applications

Paweł Niedziałkowski, Tadeusz Ossowski, Justyna Czupryniak and Dorota Zarzeczańska
University of Gdańsk , Faculty of Chemistry, Sobieskiego 18/19, 80-952 Gdańsk, Poland

Self-assembled monolayers SAMs have aroused much interest due to their potential applications in biosensors, biomolecular electronics and nanotechnology. This has been largely attributed to their inherent ordered arrangement and controllable properties. SAMs can be formed by chemisorption of organic molecules containing groups like thiols, disulphides, amines, acids or silanes, on desired surfaces and can be used to fabricate biomolecular electronic devices. Due to high affinity of SH groups to metals, thiol-terminated SAMs have attracted tremendous attention for construction of biomolecular electronic devices. Thiols form SAMs on gold, silver, copper, platinum, palladium and copper, but thiols on gold make the strongest bond and are known to be very stable.

Particularly SAMs have recently become very important due to their potential applications to biosensors, in nanotechnology and biomolecular electronics. Recent developments in self-assembled monolayers, nanoscale science, nanotechnology and microsystems have led to the evolution of biomolecular electronics [1].

SAMs are one of the most important systems for investigating contributions of molecular structure and composition to macroscopic properties of materials. They provide organic surfaces whose structure and properties can be varied. Control over dimensions and properties make SAMs excellent systems for understanding fundamentals of many natural phenomena occurring in the surroundings [2]. The properties of SAMs can be easily manipulated by changing their end group, making them compatible for desired applications [3], such as molecular and biomolecular recognition, lithography resists, sensing and electrode modification, corrosion prevention, or other areas where tailoring the

physicochemical properties of an interface is required.

Self-assembled monolayers

The interest of self-assembled films focuses on a number of systems including carboxylic acids on metal oxides (aluminum, silver), alcohols and amines on platinum, organosilicon compounds and related systems on hydroxylated surfaces, and organosulfur compounds on gold, silver, copper, nickel, and semiconductor surfaces [4]. Most studies of self-assembled monolayers to date have been made on alkanethiol monolayers on gold surfaces because this system probably offers the best available combination of high structural order, flexibility in the structure of groups exposed at the extreme surface, and ease of preparation and analysis.

In 1983 Nuzzo and Allara published the first paper about these systems, showing that dialkyl disulfides form well-ordered oriented monolayers on gold surfaces [5]. The reason for adsorbing thiols, sulfides or disulfides on gold as a preferred substrate is based on two considerations [6]: first, gold is a relatively inert metal and does not form stable oxides on its surface. Second, it has a strong specific interaction with sulfur, which allows the formation of stable monolayers.

The construction of organized monolayer films of sulfur-containing compounds on gold surfaces provides a unique opportunity for a rational manipulation of the architecture of organic surfaces. Such assemblies at solid surfaces allow the fabrication of interfaces with a well defined composition and structure. By varying the terminal functional group of the thiol chain, organic surfaces having a wide range of structures and properties can be created. More complex systems can be constructed by coadsorbing several thiols with different terminal functional groups or different chain length. Controlled degrees of order or disorder can be thus introduced into model surfaces on the atomic scale. The ability to control interfacial processes has important implications from the point of view of both fundamental and technological advances. Physical and chemical properties of surfaces indeed influence many phenomena such as catalysis, corrosion, lubrication, adhesion, wettability, electrochemistry, and biocompatibility [7].

Self-assembled monolayers SAMs are typically formed from the exposure of a surface to molecules with chemical groups that possess strong affinities for the substrate or a material patterned on it. How well these assemblies order is a function of the nature of the chemical interaction between substrate and adsorbate, as well as the type and strengths of intermolecular interactions between the adsorbates that are necessary to hold the assembly together. Molecules ‘‘binding to’’ surfaces are either described in terms of physisorption, in which

the enthalpies of interactions are rather low (considered to be DH < 10 kcal/mol, typically from van der Waals forces), or in terms of chemisorption with DH > 10 kcal/mol. Strengthening interactions between molecules and substrates and between molecules themselves include phenomena such as hydrogen bonding, donor–acceptor and/or ion pairing, and the formation of covalent bonds, rendering the assemblies more stable than their physisorbed counterparts [8].

SAMs of organosulfur compounds (thiols, disulfides, sulfides) form on substrates by spontaneous adsorption from either the liquid or the vapor phase. To form an alkanethiol monolayer, a clean gold substrate, freed from organic impurities, is simply immersed into a dilute (1-10 mM) solution of the thiol molecule in an organic solvent at room temperature. The immersion time varies from a few minutes to several hours, or even days, depending on the system. This procedure is widely used and originates from early studies of SAMs. The experimental details resulted from a combination of studies designed to optimize the reproducibility of the SAMs produced and convenience. There are, a number of experimental factors that can affect the structure of the resulting SAM and the rate of formation: solvent, temperature, concentration of adsorbate, immersion time, purity of the adsorbate, concentration of oxygen in solution, cleanliness of the substrate, and chain length (or more generally, structure of the adsorbate [9-13].

SAMs of alkanethiols can also be prepared from the gas phase. The substrate is located in an UHV (Ultra High Vacuum) chamber, which allows surface cleaning by, e.g., ion sputtering and annealing, and the molecules are dosed through a valve with a controllable flux from a little container or glass bulb. The substrates usually used for the monolayer growth are gold deposited onto mica or silicon, and gold single crystals, the choice of which depends on the final monolayer quality required for a specific study or application. The chemisorption of thiol (RSH) on gold implies the formation of a RS–Au bond.

The mechanism involved in the reaction of thiol with gold is not completely understood, but it is clear from X-ray photoelectron spectroscopy measurements, the species chemisorbed on the surface is a thiolate. This requires the departure of the sulfur-bound hydrogen, probably as molecular hydrogen H_2. The kinetic behavior during the formation of the film consists of two distinct phases: a very fast step (adsorption of the molecules onto the substrate), which takes a few minutes, and a slower one (organization and structuration), which lasts several hours. During the first step, the contact angles with water and the thickness are close to their maximum, and at the end of the second step, they reach their final values (Figure 1).

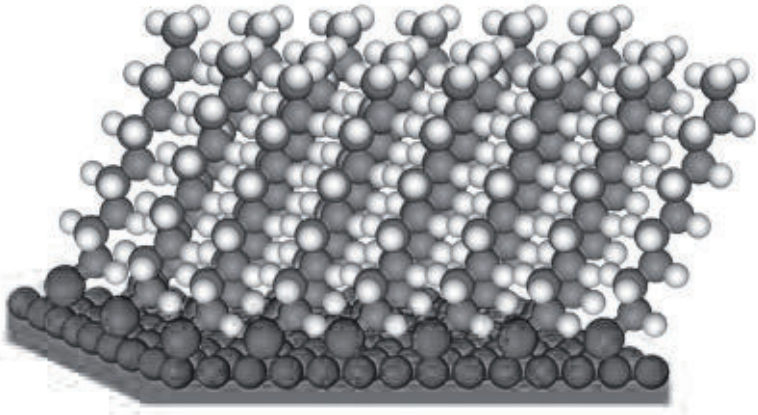


Figure 1. Schematic of an n-alcanethiolate monolayer self-assembled on gold surface

The densely packed, pin-hole-free, oriented films that result from this self-assembly are thermodynamically stable and mechanically robust. The hydrocarbon chains are trans-extended and tilted approximately 25–30° from the normal to the surface, as a result of reestablishment of van der Waals contact between the chains in an assembly with ~5Å S–S distance, larger than the distance of ~4.6Å for perpendicular alkyl chains in a close packed layer [**7**, 14, 15]. After molecules are put in place on the surface, a closely packed well-ordered assembly starts to form. There is probably some surface mobility prior to final pinning, which could explain the formation of quasi-crystalline monolayers. When the molecules are close enough together, short-range dispersive van der Waals forces can establish themselves between the chains [7].

Methods of SAMs characterization

A variety of experimental methods have been used to probe the quality and chemical nature of SAMs. The SAMs can be characterized by a variety of methods like contact angle (CA) measurements, electrochemical investigations like cyclic voltammetry (CV), electrochemical impedance spectroscopy (EIS). These can also be characterized using infrared, X-ray photoelectron spectroscopic methods (XPS), Scanning probe microscopes like scanning electron microscopy (SEM), scanning tunneling microscopy (STM), atomic force microscopy (AFM), ellipsometry, surface plasmon resonance (SPR) [1].

Contact angle goniometry (CA) has been often used to examine the general hydrophilicity or hydrophobicity of a surface of SAMs. The concept of CAG is to place a drop of water (or other liquid) into contact with the surface, and the angle

between the film and liquid droplet is measured. The measured angle reflects the degree of surface order, and can indicate the incorporation of functional groups, for the contact angle changes with varying film composition. While CAG cannot determine the exact molecular composition of a self-assembled monolayer, it can provide a rough estimate of both film quality and overall hydrophilic character.

Fourier transform infrared (FT-IR) spectroscopy is a powerful tool for studying the molecular orientation and ordering in a self-assembled monolayer. (FT-IR) has long been used to measure the vibrational frequencies of bonds within molecules. The vibrational modes of molecules attached to surfaces can also be probed, however specific surface selection rules exist, and can be used to advantage. Only molecules whose vibrations are perpendicular to the surface will be detected, as the oscillations running parallel to the surface are effectively cancelled out by the dipole symmetry between the molecules in the film and the metallic substrate. FT-IR has been used to characterize the vibrational modes of SAMs, it is most recognized for characterizing the general order within the alkyl matrix of the molecular backbone.

Raman spectroscopy has also been used to study SAMs. It provides important information about adsorbate orientation through measured vibrations in the m(C–S), m(C–C), m(C–H), and m(S–H) regions, which are often too weak to be detected in IR spectra[16, 17]

X-ray photoelectron spectroscopy (XPS) has been used to probe the chemical nature of the SAMs. Initial studies of SAMs using XPS showed that a covalent bond exists between the sulfur headgroup and the gold substrate, defined the chemical species and oxidation states of constituent atoms in the SAM, and demonstrated that the film is of single monolayer thickness [8, 18, 19,20] X-rays bombard the sample, and electrons are ejected from the core shells of the atoms within the SAM. Those electrons are collected and dispersed in an analyzer by measuring the kinetic energies of the electrons entering the analyzer, the binding energies are calculated. These are specific to each element and give indications of the oxidation states of the elements as well. Through angular dependent sputtering experiments, in which the X-rays are focused to destroy through the SAM down to the substrate beneath, the thickness of the SAMs can be calculated based on ratios of the substrate signal before and after the presence of the SAM [21].

Surface plasmon resonance (SPR) is a well-known optical technique based on the excitation of surface charge–density waves propagating along metal/dielectric interfaces. SPR technique is a very sensitive technique based on the change of local refractive index of the surface, it can be used to detect the binding of thin films to metallic surfaces and can be utilized for the detection and

characterization of SAM formation [1].

The combination of SAMs and electrochemistry provides for many sophisticated analyses of the film, as well as for controlling the reactivity of the SAM interface by modifying the molecules at the surface. In electrochemical characterization, electrochemical impedance spectroscopy (EIS) and cyclic voltammetry (CV) are the most common techniques. The electrochemical impedance spectroscopy and cyclic voltammetry are the sensitive tools for studying the electron transfer processes and for the detection of pinholes and defects in SAMs.

The quality and uniformity of a monolayer at various lengths (from macro to nano) can be assessed by directly imaging the surface topography by atomic force microscopy (AFM), scanning electron microscopy (SEM) and scanning tunneling microscopy (STM). The strength of scanning probe microscopes stems in providing a direct image of the structure, including defects or mixtures of different structures during growth, which have made them irreplaceable tools. These techniques have found many applications, by this method was imaged a variety of materials, not only SAMa, such as metals, semiconductors, insulators, organic molecules and biomolecules.

Mechanism of self-assembled monolayers formation

Developing a comprehensive understanding of the assembly of SAMs requires careful considerations of both kinetic and thermodynamic factors. Although the dynamics of the assembly remain incompletely understood, it is clear that the process leading to the formation of SAMs involves a subtle interplay of the energetics of the metal sulfur bonds and (typically)

noncovalent lateral interactions among the organic groups. In most cases, the specific ordering of the sulfur moieties on the metal lattice defines the free space available to the organic components. The organization of the organic layer results from maximizing the attractive lateral interactions (van der Waals, hydrogen bonding) within the geometric constraints imposed by the structure of the adlayer. The organic groups, however, can also restrict the density of coverage: steric crowding of the organic groups can limit the arrangement of the sulfur atoms to one that is less dense than that exhibited by elemental sulfur on a given substrate (for example, the ($\sqrt{3}$ x $\sqrt{3}$) R30^{o} structure for sulfur on gold) [22].

Factors affecting the formation of SAMs and their defects

The structures of SAMs are generally regarded as if they contained few defects. A point of fact, they are substantially more complex than the highly

ordered arrangements that are commonly assumed (Figure 2). The causes of defects in SAMs are both intrinsic and extrinsic: external factors, such as cleanliness of the substrate, methods for preparing the substrates, and purity of the solution of adsorbates, are responsible for some defects in SAMs, but some result simply because SAMs are, in fact, dynamic systems with complex phase behaviors.

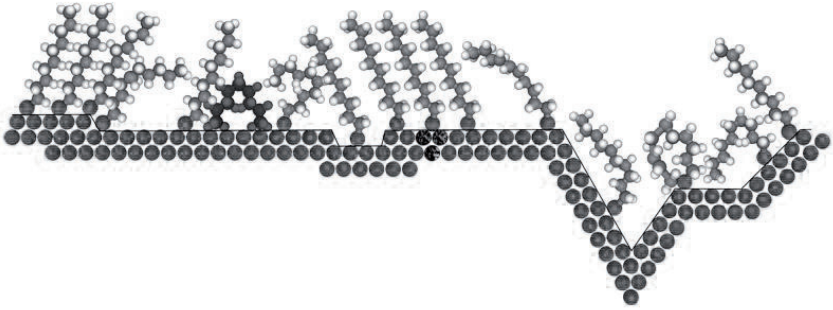


Figure 2. Schematic illustration of some of the intrinsic and extrinsic defects in SAMs

There are several reason formation of defects SAMs, mainly caused by: defects at gold step edges, impurities on surface, defects at SAM crystal edges, vacancy islands, defects at gold grain boundaries, or caused by exposed chain at gold step edges.

Removing SAMs from Surfaces

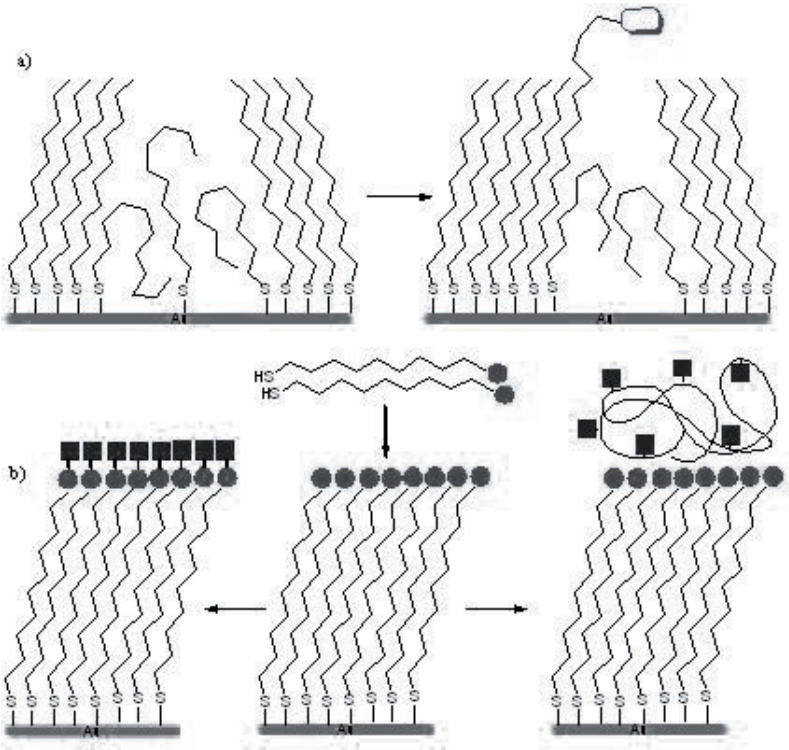
There are a number of different techniques for removing SAMs from gold, silver, and other substrates. Thermal desorption [23] or ion sputtering [24] are convenient techniques for removing SAMs from single-crystal substrates in UHV (Ultra High Vacuum) environments. SAMs are mechanically fragile surfaces, and thus, techniques for polishing or roughening surfaces of metals can remove the SAM and expose a clean surface on bulk metal substrates. Chemical oxidants or reductants such as concentrated acids or bases or "piranha" solutions (H_2O_2:H_2SO_4) [25] also are effective for cleaning substrates. Another method for removing SAMs from metal substrates is plasma oxidation. In some substrates such as patterned thin films or suspensions of nanoparticles (colloids, rods, other structures) can be damaged by harsh mechanical or chemical treatments [22].

Modifications of Self-assembled monolayers

Self-assembled monolayers formed from alkanethiols make it possible to

generate organic surfaces that present a wide range of organic functionalities (nonpolar, polar, or electroactive, biologically active). There are three general strategies for engineering the composition of the exposed surface: (1) synthesis of functionalized thiols for forming single-component or mixed SAMs by (co-) adsorption, (2) insertion of synthesized thiols into defect sites of preformed SAMs (Scheme 1a) and (3) modification of the surface composition of a preformed SAM (Scheme 1b).

Both covalent reactions and noncovalent interactions (van der Waals forces, hydrogen bonding, metal ligand bonding) can generate new interfaces for SAMs [22].



Scheme 1. Strategies for modifying the interfacial composition of SAMs after formation [22]

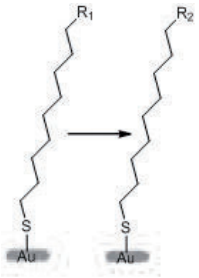
Modification of the exposed surface of a SAM after formation offers four advantages:

- it uses common synthetic procedures and thus simplifies the preparation

of functionalized surfaces,
- it enables the incorporation of ligands into SAMs that are not compatible with thiols or the synthetic methods for preparing them,
- it can generate multiple samples with different types of ligands in a short period of time (SAMs are easy to prepare),
- it preserves the ordered underlying structure of the SAM.

There are also disadvantages of modifying the composition of the SAM after formation:
- the extent of surface coverage is usually unknown,
- the reactions can produce a mixture of functional groups on the surface,
- the structure of the resulting surface is unknown.

Many classes of organic reactions have been explored for modifying the surfaces of SAMs, such as oxidation, nucleophilic substitutions, nucleophilic addition esterification, acylation, or Diels – Alder reaction [26]. Functional groups occurring on SAMs can be modify by direct reactions by immersed in a solution of ligands, which react directly with the molecules present in solution, that is why many direct immobilization techniques have been adapted from methods for immobilizing DNA, amino acids, polypeptides, and proteins on surfaces (Scheme 2).



Scheme 2. Direct interfacial reactions of exposed functional groups

Examples of reaction of exposed functional groups on SAMs are collected in table 1 [22]. The Mrksich et al. have shown that SAMs presenting maleimide functional groups react in good yield with biologically active ligands having thiols[27]. Also disulfide thiol exchange is another method used to attach thiol-modified DNA, [28] peptides, [29] and carbohydrates [30] to SAMs on gold. The exchange process appears to occur more readily than displacement of the thiols on the surface. The steric bulk of the thiol-modified biomolecules may hinder their transport into defect sites on the surface.

Michel et al. used [31] isothiocyanate group to immobilization of antibodies

on a photoactive self-assembled monolayer on gold by the forming thiourea group.

Ruthenium-catalyzed olefin cross-metathesis is a versatile method for forming carbon carbon bonds, which was used by Choi et al. for attaching acrylamide, acrylic acid, and methyl acrylate to vinyl-terminated SAMs [32].

Triazoles formed by 1,3-dipolar cycloadditions of acetyl groups to azides so-called "click" chemistry [33] provide thermally and hydrolytically stable linkage between two molecules. Collman et al. showed that azides attached to undecanethiolates reacted readily with ferrocene molecules modified with acetylenes [34]. There different way of use "click" chemistry, by cetylenyl-terminated SAMs, which form triazoles upon reaction with azide compounds [36].

Another type of reaction that uses terminal azides is the Staudinger reaction: substituted phosphanes react with azides to form amide bonds. This reaction can modify the surfaces of cells [36] and immobilize small molecules on glass slides. The advantage of both 'click' chemistry and the Staudinger ligation is that the reactions are highly selective, that is, the reaction is not sensitive to the presence of other functional groups, such as amines, hydroxyls, or thiols, in solution or on the surface [22].

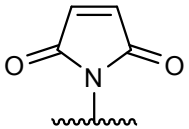
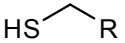
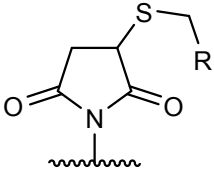
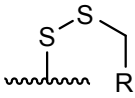
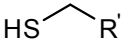
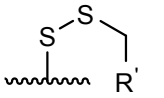
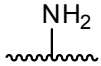
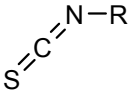
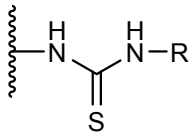
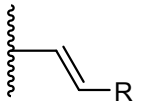
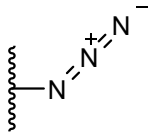
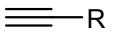
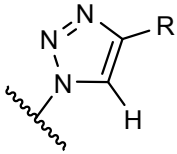
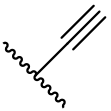
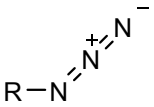
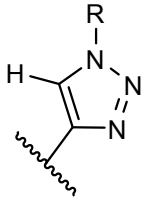
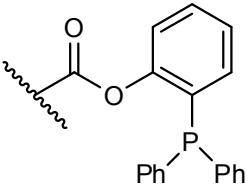
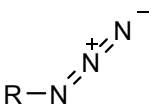
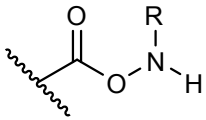
Actually the "click" chemistry is very popular, not only in organic synthesis but also to synthesize functionalized of oganothiols, which can be assembled to form SAMs with the appropriate surface. The "click" chemistry can be used in three different ways by the reaction in solution with appropriate thiols and then SAM is formed, or by the formation of SAM modified by alkyne terminated group or azide terminated group and then the "click" reaction is running on surface (Scheme 3).

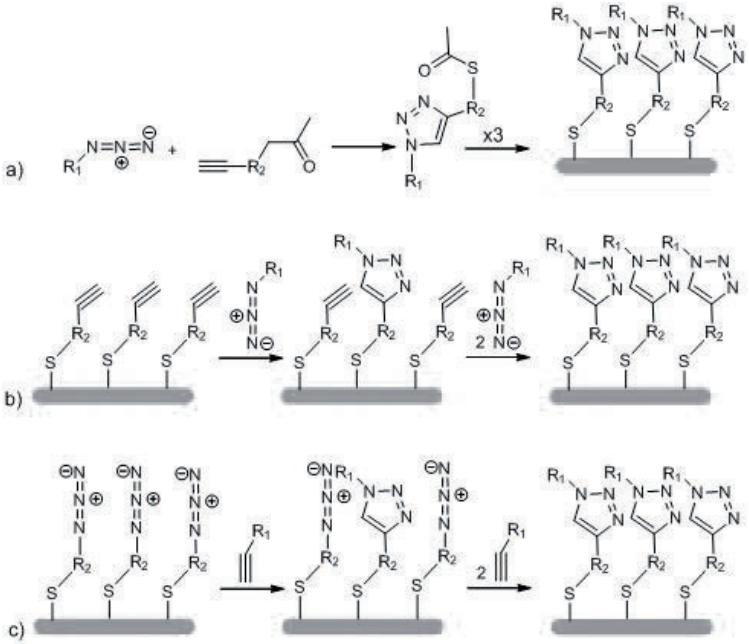
Different approach to the functionalization of the surfaces of SAMs is to form a reactive intermediate, which is then coupled to a ligand (Scheme 4). There are two primary advantages of this strategy:

- the common intermediate can react with a variety of ligands,
- it allows, in principle, spatial discrimination of active and inactive regions, that is, the reactivity of regions on the surface can be turned 'on' or 'off'.

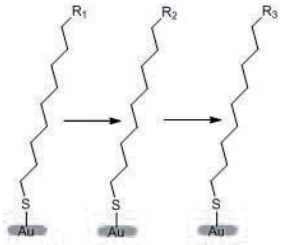
The combination of activated intermediates with methods for spatial patterning, microcontact printing and scanning probe lithography, make it possible to attach ligands in specified locations [38], what found many industrial application.

Table 1. Reaction of the terminal groups assembled on surface with selected functional organic groups

Surface group (R_1)	Ligand	Formed complex (R_2)	References
			[27]
			[28-30]
			[31]
			[32]
			[33]
			[34, 35]
			[36]



Scheme 3. Schematic view of processes leading to SAM over "click" reaction: (a) the reaction in solution with subsequent SAM formation, b) SAM formation, and then click reaction for alkyne-terminated and (c) azide-terminated SAMs [37]



Scheme 4. Interfacial reactions involved intermediate functional groups

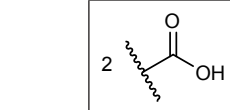
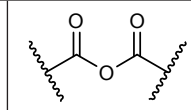
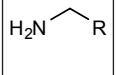
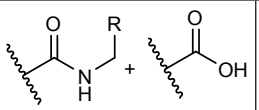
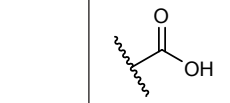
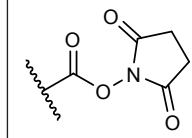
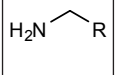
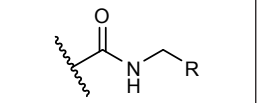
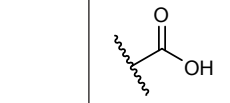
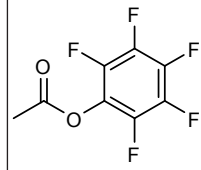
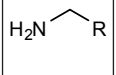
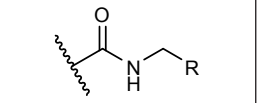
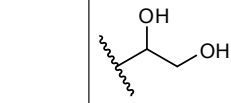
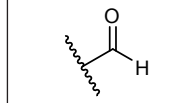
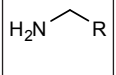
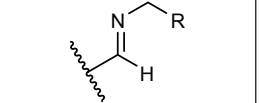
One of the simplest and most broadly applicable methods developed for modifying SAMs is the formation of amide linkages via an anhydride intermediate [39]. Three factors make this reaction very useful for screening structure property relations for surfaces:

- the simplicity and rapidity of the method,

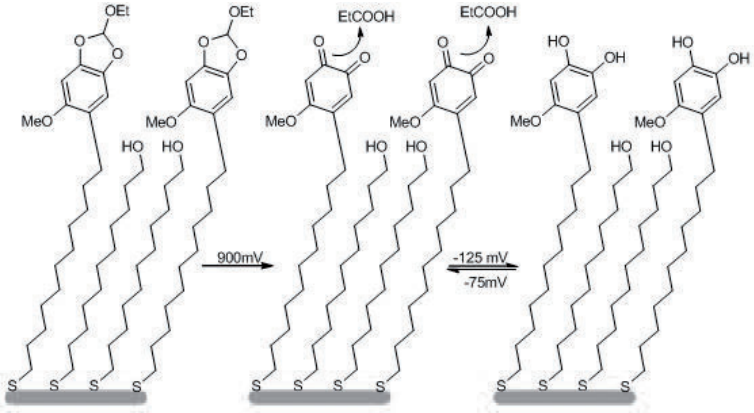
- the large number of amine-containing organic and orga nometallic ligands that are available commercially or that can be synthesized easily,
- the high yield normally observed for the coupling reaction.

The free carboxylic acid groups can be also activated by the N-hydroxysuccinimidyl (NHS) esters or pentafluorophenol ester which increas the reactivity of the activated ester on the surface [40]. Using this method, it is possible in very easy way attach ligands or proteins on SAMs (Table 2).

Table 2. Activation of the terminal carboxyl groups and the surface reaction products with ligands

Sufrace group (R_1)	Intermediate (R_2)	Ligand	Formed complex (R_3)	References
				[39]
				[40]
				[40]
				[38]

A third strategy for modifying the interfacial composition of a SAM is the cleavage of covalent bonds of a terminal surface group. Mrksich et al. [41] demonstrated that SAMs can be prepared from two or more alkanethiols. The self-assembled monolayer of alkanethiolates on gold can release attached groups when an electrical potential was applied to the gold. Under the conditions of the experiment, the obtained catechol orthoformate was stable and did not undergo hydrolysis or decomposition (Scheme 5).



Scheme 5. Design of monolayers that present the catechol orthoformate group. An applied potential causes irreversible oxidationof the group to give a monolayer terminated in the orthoquinone. Reversible reduction gives the catechol-terminated monolayer [41]

Another set of methods for modifying the composition of preformed SAMs use either the intrinsic properties of the surface (hydrophobicity, electrostatics) or selective interactions with the preformed chemical functional groups on the surface to promote adsorption of materials from solution. These methods use noncovalent interactions rather than covalent reactions to stabilize the adsorbed materials. Surfactants, polymers, polyelectrolytes, proteins, organic dyes, and colloidal particles are examples of the types of materials that can adsorb onto SAMs. Hydrophobic SAMs, such as ones formed from n-alkanethiols, readily adsorb amphiphilic molecules some polymers, and most proteins. One disadvantage of this method is that there is limited control over the thickness of the adsorbed layer and the orientation of the functionalities of the adsorbed material [22].

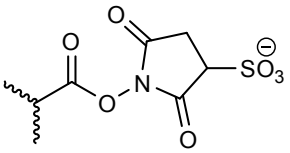
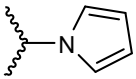
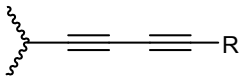
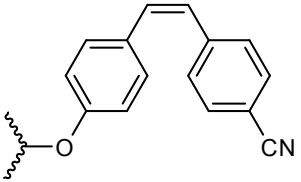
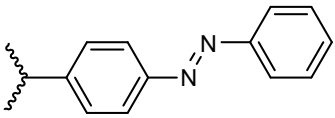
Applications of self-assembled monolayers

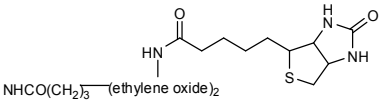
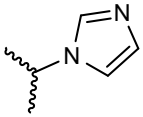
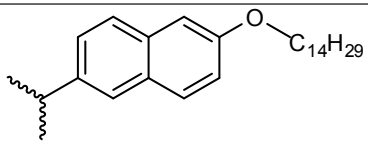
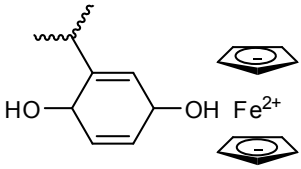
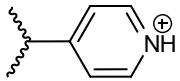
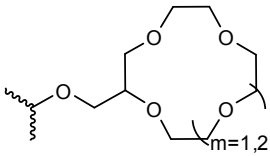
SAMs provide a basis for many important scientific and technological applications, ranging from micropatterning methods, through sensing, to biological recognition. SAM found many application as electrochemical sensors, in soft lithography, there are used as molecular lubrication and as quartz crystal microbalance (QCM).

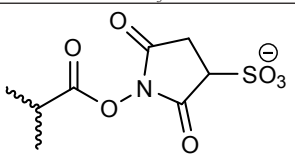
The most frequent application of self-assembled monolayers in electroanalytical chemistry is in the development of sensors where the SAMs are used to impart selectivity onto an electrode for a particular analyte. Sensors

using SAMs are ussually used to monitor pH, inorganic species and organic molecules using both chemical and biological recognition elements [42]. Some of the many applications of SAMs have been presented in the table 3 below [43].

Table 3. Examples of applications of SAMs of alkyl thiols and/or disulfides

Area	Application	End Group(s) R (HS-(CH_2)n-R)	n
Soft Lithography	Etching	CH_3	>10
	Control of Surface	OH, COOH, $CONH_2$	15
	Wettability	$O(CH_2)mCH_3$ m=3,4	2,11,15,16
	"Sticky" surfaces	SH	8
Surface Reactions	Surface Activation	 (NHSS), COO-NHS	>7
	Surface polymerization		2,4,6
			10
		$Si(OMe)_3$	3
Surface Reactions	Photoswitchable Surfaces		10
			6,10,12

Biocompatible Coatings	Protein-resistant surfaces	(ethylene oxide)mOH, m=2-6	11
		O(Maloze)	10
Immobilization of Biomolecules.	Covalent Immobilization	Cl, Br, -OSO_2CH_3	16
		ethylene oxide 3/ ethylene oxide 5-maleimide disulfide	11
		CHO	4
	Noncovalent Immobilization		14
			10
		COOH and NH_2	11, 16
Molecular Electronics	Elecron Tunelling	CH_3	7-11
		SH	8-12
	Current Rectification		3
	Probing Specific Chemical Bond	COOH, NH_2	10
Electrochem. Sensing	pH Sensing		8, 11
	Voltammetric Sensing	COOH	10
			2
	Impedance Spectroscopy		1, 6
		COOH	5

Mass Detection	Cantilever sensors QCM	COOH	10
		CH_3	11
		OH and OCH_2Ph	6
		CH_3	17
			10
Molecular Lubricants	Tailoring Frictional Forces	CH_3	1-18
		CF_3, $CH(CH_3)_2$	10-12
		OH, CH_3	12
Corrosion Protection	Corrosion Protection	CH_3	7, 11, 15, 17, 19, 21, 28
		$F(CF_2)$mCONH m = 6, 7, 8	2
		$(CH_3O)_3Si$	3

Conclusions

In summary, it can be stated that the generation of organic surfaces, based on organothiol, forming SAMs on gold still offers tremendous potential. Analyses on such model systems, during last decade have given rise to significant progress in understanding of the chemical and physico-chemical properties of organic surfaces as well as the possibility of manipulating them by controlled modification in application purpose. While the fundamental structural characteristics of these organic model substrates are now understood relatively well, in particular the analysis of the chemical properties of organic surfaces is currently a significant research area to develope new application of SAMs.

Acknowledgements

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Chapter 8

Some anion receptors in chemistry

Bogusława Łęska
Adam Mickiewicz University, Faculty of Chemistry, Grunwaldzka 6, 60-780 Poznań, Poland

The aim of this short review article is to present the knowledge in anion receptor chemistry. This review consists of three sections. The first discusses the anions role, their type (also size and shape) and which factors play the main role in complexation anion process. The second one reviews information of some natural and synthetic anion binding agents.

Nature of anions

An area of interest in supramolecular chemistry that continues to attract attention is the coordination of anions. The rapid growth in this area is due to the realization of the many roles that anions play in biology, medicine, catalysis and the environment [1, 2]. It is interesting to note that anion binding by proteins is most often achieved by way of neutral amide functions employing the hydrogen-bond acceptor properties of the amido NH group [3]. The anions are ubiquitous in the nature. Chloride anions are present in large quantities in the oceans; nitrate and sulfate are present in acid rain; and carbonates are key constituents of biomineralized materials.

The anions, including pertechnetate, a radioactive product of nuclear fuel reprocessing, and phosphate and nitrates from agriculture and other human activities, constitute major pollution hazards. Anions are necessary to the maintenance of life and in almost at some level every conceivable biochemical operation because of the recognition, transport, or transformation of anions is involved [4].

The design of anion receptors (and receptors for ion-pairs) is particularly challenging when compared to the design of receptors for cations. There are a number of reasons for this. Anions are larger than the equivalent isoelectronic cations (Table 1) [4, 5] and hence have a lower charge to radius ratio. The more diffuse nature of anions means that electrostatic binding interactions are less

effective than they would be for the corresponding isoelectronic cation.

Table 1. The difference in radii for typical isoelectronic cations and anions (in octahedral environments) serves to underscore the more diffuse nature of the anionic species [4, 5]

Group 1 (cations)		Group 17 (anions)		Δr
Na^+	1.16 Å	F^-	1.19 Å	0.03 Å
K^+	1.52 Å	Cl^-	1.67 Å	0.15 Å
Rb^+	1.66 Å	Br^-	1.82 Å	0.16 Å
Cs^+	1.81 Å	I^-	2.06 Å	0.25 Å

The chemistry medium (*e.g.* pH, type of solvents …) are very important for anions, because in particular situations anions may be sensitive. They would be protonated because of the low pH and after losing their negative charge. Thus, receptors must function within the pH window of their target anion. This is a particular problem when designing protonated receptors for anions (*e.g.*, ammonium containing receptors) as the protonation window of the receptor (and the anion) must also be considered. It is, of course, less of a problem for neutral receptors, or those containing permanent built-in charges, designed to operate in aprotic media [4].

Anionic molecules are challenging targets for recognition studies as they possess a wide range of sizes and shapes The anions are characterized by a wide range of geometries (Figure 1) and one of the problems which most plagues chemists involved in the design and synthesis of anion hosts, however, is that of introducing anion selectivity. Factors such as anion size and topology, charge density, hydrogen-bond donor/acceptor properties and Lewis-basic character must all be considered and such properties are often less easily defined than in analogous examples in cation complexation [6]. Because of these facts, the higher degree of design and adapting is required to make receptors that are selective for a particular anionic guest than for most simple cations [4].

The solvent in which the anion-binding event occurs plays a vote role in controlling anion-binding strength and selectivity. Electrostatic interactions generally dominate over other recognition forces and are particularly important in stabilizing anions in solution. The hydroxylic solvents are ability to form strong hydrogen bonds with anions. A potential anion receptor must compete effectively with the solvent environment in which the anion recognition event is to take place. The neutral receptor that binds anions solely through hydrogen-bonding interactions is less likely to be capable of competing with the polar protic solvation shell surrounding the target anion in a hydroxylic solvent and hence may only function as an anion receptor in aprotic organic solvents.

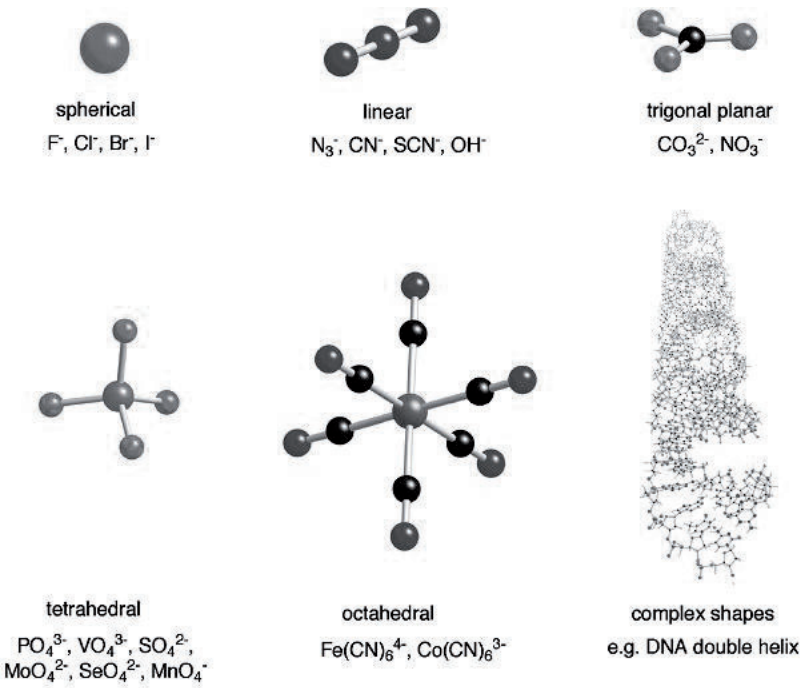


Figure 1. Anions shapes and sizes [4]

A charged receptor can benefit from electrostatic effects and thus may compete more effectively with polar protic solvents. Protonated polyammonium macrocycles are capable of binding anions in water. The anion receptor must not just compete with the solvent but also with the counter cation that is necessarily paired with the targeted anion [4].

When we think about planning the geometry of receptor in non-polar solvents, the anion may be weakly solvated but there may be significant ion-pairing. In more polar solvents, the solvation may be stronger – but solvation of both the cation and anion will reduce the strength of ion-pairs in solution. It is important to noted that binding experiments in solution always include an element of competition whether from solvent or from counter ion. The problems can arise when attempts are made to contrast quantitative data derived from analyses carried out using different instrumentation or from experiments carried out in, *e.g.*, different solvents, at different concentration regimes, or using different counter cations [4, 7].

Hydrophobicity can also influence the selectivity of a receptor and, as such, any relative assessment of its anion-binding characteristics and it may therefore be used by chemists in the design of anion receptors to bias selectivity towards larger anions with low charge Hydrophobicity effects and the Hofmeister series are particularly relevant to the solvent extraction of anions from aqueous solution [4, 8].

The Hofmeister series: organic anions > ClO_4^- > SCN^- > I^- > salicylate$^-$ > NO_3^- > Br^- > Cl^- > HCO_3^- > $H_2PO_4^-$ > F^-, SO_4^{2-} > HPO_4^{2-}. It was first established through studies on the effect of salts on the solubility of proteins, orders anions by their decreasing hydrophobicity (and therefore increasing degree of aqueous solvation).

Why anion receptors?

Anion binding continues to attract growing interest among supramolecular chemists [9-18]. There is a very considerable range of possible applications of synthetically created receptors with affinities and selectivities that rival biological anion receptors [19] particularly in a sensing context [20] either using colorimetric methods or in systems with appended redox-active or fluorescent groups [21–32].

Host–guest systems for ionic species have played an important role in the development of the field of supramolecular chemistry, the chemistry of the non-covalent interactions. One of the earliest reports of anion complexation dates back to 1967, when the research of Pedersen on the complexation of alkali metal ions by crown ethers initiated the development of many other neutral host species for metal ions [33, 34]. Compared with the cation receptors, anion receptors were developed much later, although already in 1968 the first synthetic receptor for inorganic anions was reported [35] (size selective binding of Cl_2 anions was described with diprotonated 1,11-diazabicyclo- [9.9.9]nonacosane (Figure 2). The field started to develop in 1976 when Graf and Lehn reported that protonated cryptate (Figure 2) encapsulates F_2, Br_2 and Cl_2 anions [36]. Since then several other positively charged anion receptors have been developed that have protonated nitrogen atoms or metal ions [37-41]. In these receptors mainly coulomb interactions contribute to the attractive force.

With the advent of macrocyclic ligands such as the crown ethers, cryptands and an enormous range of other multidentate hosts, supramolecular cation coordination chemistry has progressed rapidly.[42-45] In comparison, progress in the non-covalent complexation of anions has been more slow, probably as a consequence of the large ionic radii of anions, high free energy of solvation and the wide variety of topologies encountered, resulting in great difficulty in

designing multidentate receptors with appropriately situated Lewis-acidic or other acceptor sites.

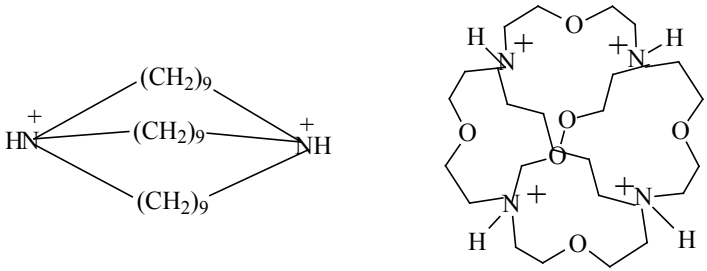


Figure 2. Diprotonated 1,11-diazabicyclo-[9.9.9]nonacosane and protonated cryptate

Anion binding, the coordination of chemical species by virtue of their anionic nature, plays a central role in biological processes. For instance, a large variety of substrates and cofactors engaged in biological processes are anions. They are present in roughly 70% of all enzymatic sites, play essential structural roles in many proteins, and are critical for the manipulation and storage of genetic information (DNA and RNA are polyanions). Anions are also involved in regulating osmotic pressure, activating signal transduction pathways, maintaining cell volume, and in the production of electrical signals. Not surprisingly, therefore, the disruption of anion flux across cell membranes (especially chloride, present in cells at the 5–15 mM concentration level [46] is increasingly recognized as being the primary determinant of many diseases, including cystic fibrosis [47], Bartter's syndrome [48], Dent's disease [49], Pendred's syndrome [50, 51], and osteopetrosis [52]. In fact, the transport of anions through cell phospholipid bilayers is known to be mediated by a variety of channels and anion transport proteins with at least 14 mitochondrial anion transport systems having been identified so far [53] These include (among others) systems responsible for the trafficking of ADP, ATP, phosphate, citrate, maleate, oxaloacetate, sulfate, glutamate, fumarate, and halide anions.

But the presence of anions in biological systems is also problems, because in recent years, however, increasing attention has focused upon supramolecular anion complexation [5] perhaps not in little part because of the important environmental consequences of the presence of excess nutrients such as nitrate and phosphate [54-56]. Also relevant are anionic products of nuclear fuel reprocessing such as $^{99}TcO_4^-$, [57, 58] as well as the extreme importance of anionic substrates in biochemistry [6, 59-67].

One of the problems which most plague chemists involved in the design and synthesis of anion hosts, however, is that of introducing anion selectivity. Factors such as anion size and topology, charge density, hydrogen-bond donor/acceptor properties and Lewis-basic character must all be considered and such properties are often less easily defined than in analogous examples in cation complexation [5].

Natural anion receptors

The anion-bound structures of phosphate binding protein (PBP) from *E. coli* and sulfate binding protein (SBP) from *S. typhimurium* have been determined using X-ray crystallography [68,69]. PBP and SBP are members of a family of periplasmic proteins that act as initial high-affinity receptors for orthophosphate and sulfate anions, respectively, and are involved in the high affinity active transport system to uptake these essential nutrients into bacteria cells. The anion ligand is bound in a deep cleft in the protein and completely buried and desolvated. The precise arrangements of hydrogen bonding groups in the anion binding sites of PBP and SBP (Fig. 3) exactly match with the hydrogen bonding oxygen atoms of the tetrahedral ligand anions and do not allow non-specific binding of other anions. In the PBP- phosphate complex (dissociation constant K_d =1x10^{-6} M at pH 8.3), the anion is bound by 12 hydrogen bonds including an interaction between the carboxylate from Asp56 and the proton of HPO4^{2-}. As sulfate has no hydrogen bond donor to match with the carboxylate, PBP is unable to bind sulfate [70]. Although the phosphate anion makes a salt-bridge with the Arg135 guanidinium group, which is in turn bound with the carboxylate of Asp137, studies with mutant proteins showed that the phosphate binding is insensitive to the perturbation of this ion-pairing interaction [71]. Selective binding of SBP (K_d = 0.12x10^{-6} M for SO_4^{2-}, 6x10^{-2} for HPO_4^{2-} at pH 8.3 [72] can be also explained by the lack of hydrogen bond acceptors to pair with phosphate in the anion binding site, whereas sulfate oxygen atoms are bound by seven hydrogen bond donors.

Recently, several X-ray crystal structures have been solved that have allowed the direct visualization of enzyme–anionic substrate complexes that are stabilized via multiple hydrogen-bonding interactions. In particular, the structure of the DNA helicase RepA sulfate complex, solved to 1.95 Å resolution, shows six hydrogen-bonding interactions between the sulfate anion and the RepA protein scaffold. The sulfate anion is also hydrogen bonded to Asp140 via an intervening water molecule (Figure 4) [4, 73]

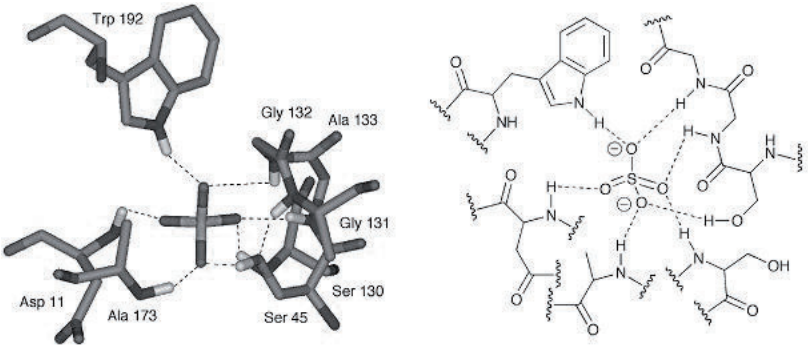


Figure 3. The X-ray crystal structure and schematic of the sulfate-binding site in the sulfate-binding protein. The anion is bound by seven hydrogen bonds from neutral NH and OH hydrogen bond donor groups [4]

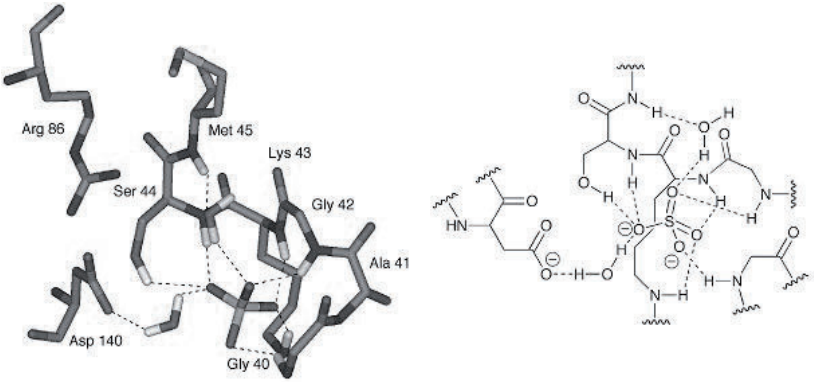


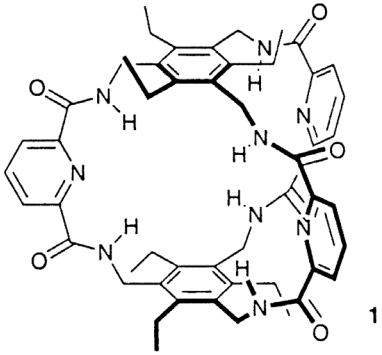
Figure 4. ATPase active site in DNA helicase Rep A showing the interaction of the bound sulfate anion with various P-loop residues [4]

Selective ion transport is crucial for a range of cellular processes and ion channels are the proteins that carry out this vital role. Ion channels must be selective for certain types of ions and transport them with a high rate rather than necessarily bind them with high affinity. Structures of two chloride channels from *E. coli* and *S. typhimurium* have been determined recently [74].

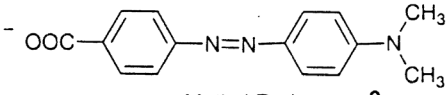
Macrocyclic receptors

Amide based anion receptors

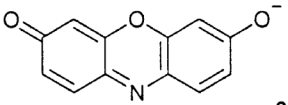
Anslyn and co-workers have extended their displacement assay paradigm [75, 76] to include a molecular ensemble consisting of a previously reported nitrate selective trigonal amide box **1** [77] and a colorimetric dye such as resorufin (**3**) or Methyl Red (**2**).



1



Methyl Red **2**



3

resorufin

Changes in the absorbance of **2** and **3** upon addition of receptor **1** are shown in Fig. **5** [78]. The equilibrium between the complex **1**–**2** or **1**–**3** and its component parts is perturbed upon addition of nitrate anions resulting in large changes in absorbance. Consequently the complexes **1**–**2** and **1**–**3** act as optical sensing assays for nitrate. Receptor **1** has also been used to compare the effects of NH-p versus hydrogen bonding effects on carbon acid pK_a shifts [79].

Pyrrole based anion receptors

Sessler, Gale and Kra´l have continued their studies of the anion binding abilities of calix[4]pyrrole macrocycles [81–87]. These tetrapyrrolic macrocycles bind anions by the formation of four pyrrole NH…anion hydrogen bonds. Sessler, Gale and co-workers synthesised a variety of calix[4]pyrrole anthracene conjugate compounds and demonstrated that these receptors can detect the presence of anions via significant perturbations in their fluorescence properties [88]. A new calix[4]pyrrole mono-acid derivative **5** was synthesised by treatment of *meso*-octamethylcalix[4]pyrrole **4** with four equivalents of *n*-BuLi in THF at -78°C followed by addition of excess solid CO_2 (Scheme 1). This acid was then coupled to 1-aminoanthracene using DCC and HOBt in DMF to afford a calix[4] pyrrole–anthracene conjugate **6** (in 34% yield from the acid) that possesses a direct conjugated link between the calix[4]pyrrole anion binding site and the anthracene fluorophore.

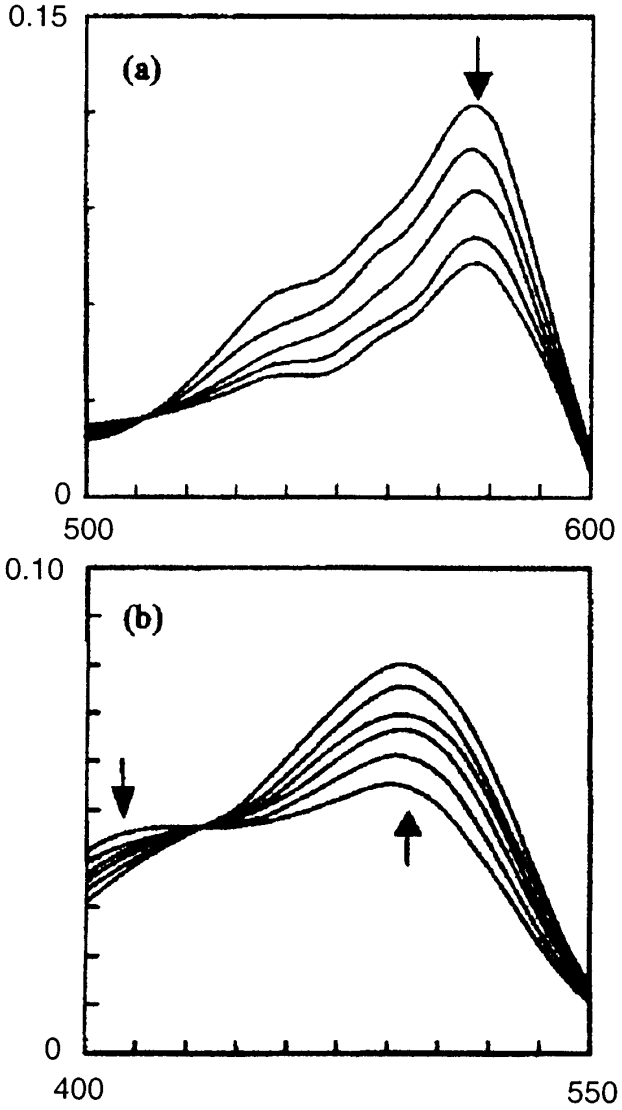


*Figure 5. UV–vis absorption spectra of: (a) resorufin (**3**); and (b) methyl red (**2**) upon addition of receptor **1** in 1:1 MeOH–CH2Cl2 (v:v). [Indicator]_2 mM, [**1**], 0–20 mM. Reproduced with permission from J. Chem. Soc. Perkin Trans. 2 (1999) 1111, Copyright 1999, Royal Society of Chemistry [80]*

Other calix[4]pyrrole–anthracene conjugates were synthesised by coupling the calix[4]pyrrole mono-acid **7** [83] with 1-aminoanthracene or 9-aminomethylanthracene using the BOP amide coupling agent to afford the conjugate compounds **8** and **9** in 63 and 51% yields, respectively. The matched set of compounds **6**, **8** and **9** therefore contain zero, one or two methylene groups between the calixpyrrole and anthracene fluorophore. Stability constants for anion binding were determined in acetonitrile-d_3 by 1H-NMR titration techniques (Table 1). The fluorescence of receptors **6**, **8** and **9** was shown to be quenched significantly in the presence of certain anionic guests (most efficiently quenched by fluoride) and that of the three compounds, receptor **6** was most sensitive, having its fluorescence quenched most efficiently by the added anions. This may be due to the electron withdrawing effect of the directly linked amide group and also to the presence of the conjugated link between the anion binding site and fluorophore.

Table 2. Stability constants of compounds 6, 8 and 9 with various anions in form of tetrabutylammonium salt determined by 1H-NMR titration in acetonitrile-d_3 (errorsB15%) [80]

Anion	Stability constant (log*K*) of compound **6** in CD_3CN	Stability constant (log*K*) of compound **8** in CD_3CN	Stability constant (log*K*) of compound **9** in CD_3CN
F^-	[a]	[a]	[a]
Cl^-	>4	>4	>4
Br^-	3.59	3.00	2.63
$H_2PO_4^-$	>4[b]	3.50	3.08
HSO_4^-	2.77	[c]	[c]

[a] - NH resonance broadened precluding a determination of this value using NMR spectroscopic methods;
[b] - Stability constant determined by following pyrrole CH resonance;
[c] - Weak interaction K<20 M^{-1}

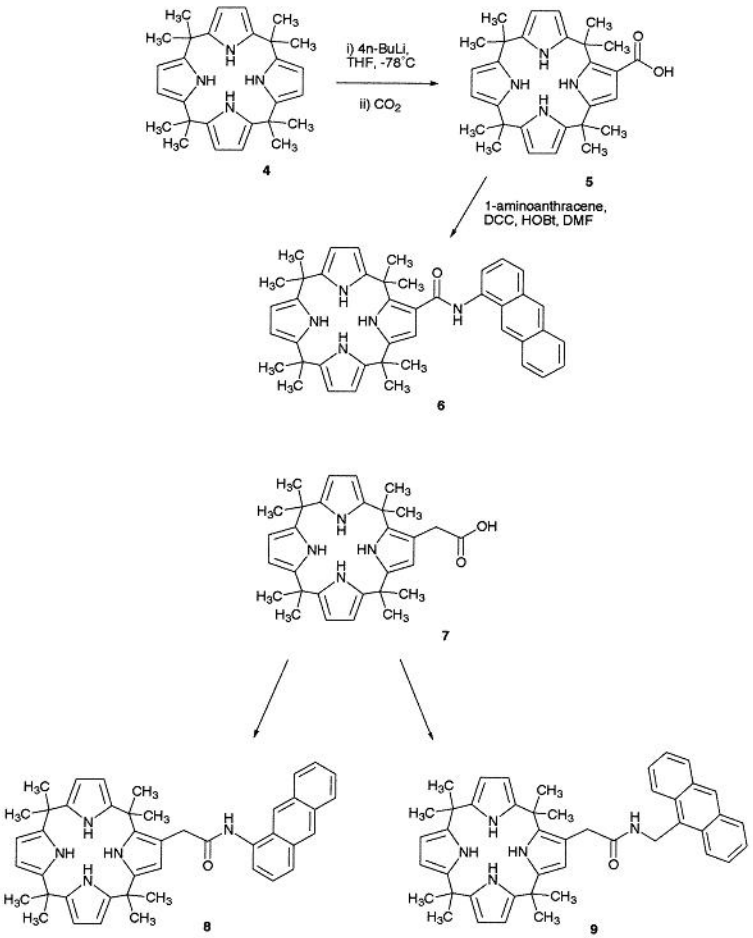
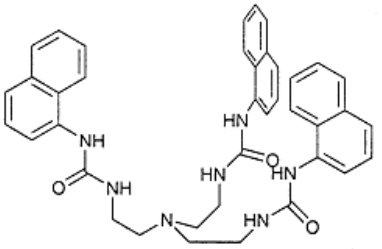


Figure 6. Synthesis of fluorescent calix[4]pyrroles [80]

Urea based anion receptors

Urea and thiourea are particularly good hydrogen bond donors and are excellent receptors for anions such as carboxylate via the formation of two hydrogen bonds. Wu and co-workers have synthesised tripodal hosts containing naphthylurea groups [89]. The anion coordination and fluorescence properties of **10** and **11** were compared with the model compounds **12** and **13**. They found that **26** is selective for $H_2PO_4^-$ in the presence of other putative anionic guest species (Fig. 7).



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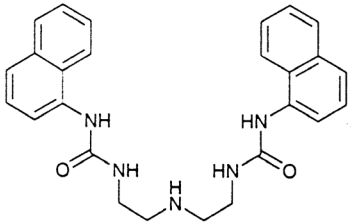

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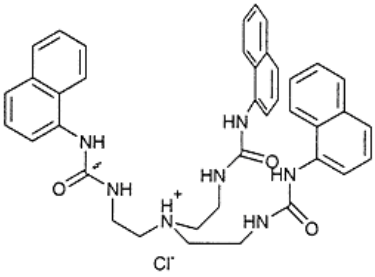

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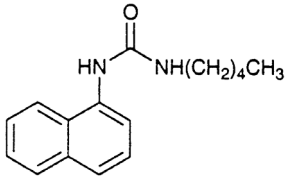
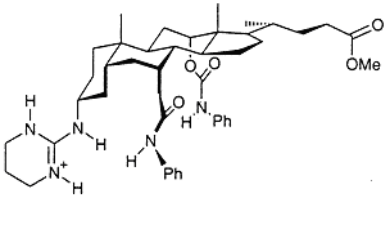


13

Figure 7. Tripodal hosts containing naphthylurea groups

Guanidinium and amidinium receptors

Davis and Lawless have synthesised two guanidinium functionalised steroids (**14, 15**) **(Fig. 8)** and studied the extraction properties of these receptors with *N*-acetyl-aamino acids [90]. **14**·Cl^- and **15**·Cl^- when dissolved in chloroform proved to be effective extraction agents for carboxylate anions from both neutral or alkaline solutions.



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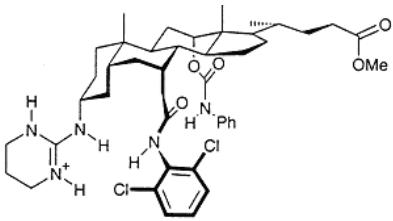

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*Figure 8. Guanidinium functionalised steroids (***14, 15***)*

Conclusion

The supramolecular chemist designs and synthesizes receptors for both cations and anions, but also evaluates their binding properties and selectivity. In the field of anion receptor chemistry, a variety of techniques have been employed to measure the stability constants of hosts with guests.

The different functional group should continue to be important in anion receptors because of its ease of synthesis and biological precedence. Future advances should focus on binding in competitive solvents, such as water, as well as the efficiency of the reporter group; in particular sensing by facile methods at low concentrations.

Acknowledgements

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