FIRST SUPRAMOLECULAR FLUORESCENCE-BASED ASSAY FOR CARBONIC ANHYDRASE INHIBITORS

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ABSTRACT

Prof. Pavel Anzenbacher, Jr., Advisor

Our global society has reached a breaking point facing severe issues involving pollution of the environment, treatment of various diseases and nutrition. Overcoming these challenges can be tackled with scientific and technological progress and new discoveries. As a central science with its astonishing ability to analyze and develop novel substances of unprecedented properties, Chemistry has always stood at the very forefront of the scientific progress dramatically enhancing the quality of our lives. A qualitative chemical analysis and determination of quantity of chemical species has been one of the major areas of chemistry having the power to bring the answers and to solve the challenges we are facing. Thus, the development of new chemical sensors capable to analyze the quality and quantity of various chemical species found in the environment and involved in biological processes is of the greatest interest. Being highly sensitive and relatively simple, chromogenic and luminescent molecular sensors are one of the popular tools used in the optical detection of minute amounts of various analytes. The optical sensors utilize a molecular recognition in order to provide an analyte-specific response in the form of a color change or a luminescence. This work presents the design and development of supramolecular optical sensors and their use in the state-of-the-art sensing approach employing pattern recognition analyses for the detection of environmentally and physiologically relevant analytes. This work is a contribution to the development of a new approach helping to address some of the environmental and medical challenges of our world.
To my Family
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CHAPTER I. INTRODUCTION

1.1 Luminescence

_Luminescence_ (from Latin *lumen* meaning “light”) is defined as an emission of light by a substance in ultraviolet, visible or infrared region of the spectrum. It is a result of a decay of electronically excited states of a substance not resulting from heat. There are various types of luminescence known based on the mechanism of excitation: photoluminescence (the emission is a result of absorption of light), chemiluminescence (the emission is a result of a chemical reaction), electroluminescence (the emission is a result of an electric current passing through a substance), mechanoluminescence (the emission is a result of a mechanical stress on a substance), thermoluminescence (the emission is a result of a substance being heated), sonoluminescence (the emission is a result of ultrasound) and radioluminescence (the emission is a result of absorption of ionizing radiation), etc. Various types of compounds and materials have been described to exhibit luminescence including organic compounds (naphthalene, fluorescein, amino acids, etc.), inorganic compounds (lanthanide ions, ZnS, UO$_2^+$, etc.), and organometallic compounds (Ln-based complexes, Ru complexes, 8-hydroxyquinoline complexes, etc.).

_Fluorescence_ and _phosphorescence_ are types of luminescence and are of particular interest.$^{1,2}$ Both phenomena originate in the absorption of a photon forming an electronic excited

![Figure 1.1. Possible pathways of depopulation of the excited state of a molecule.](image)
state of the absorbing species. There are several ways the substance in the excited state might return to the ground state. This return leading to the emission of photons is called photoluminescence (fluorescence, phosphorescence or delayed fluorescence), which is one of the possible physical effects resulting from interaction of light with matter (Figure 1.2). The properties of excited states of a molecule according to their spin multiplicities and the transitions processes between them and the timescales are conveniently illustrated in the so called Jablonski diagram (Figure 1.1).

Figure 1.2. Generalized Jablonski diagram for an organic molecule illustrating excited states and their transition processes.
1.2 Chemical Sensors

The field of *supramolecular chemistry* has made a very dramatic and exciting progress since J.-M. Lehn, D. J. Cram, and C. J. Pedersen were awarded the Nobel Prize in Chemistry in 1987 “for their development and use of molecules with structure-specific interactions of high selectivity”. The famous Prof. Lehn’s definition of “supramolecular chemistry” defines it as the domain of chemistry “beyond that of molecules”. Nowadays, the detection of various analytes of interest (neutral molecules, radicals, or ions) in diverse environments has been one of the major tasks of modern *supramolecular analytical chemistry*. A broad range of disciplines of human activities demands suitable and reliable tools for the detection of various analytes. Such a requirement is crucially important in areas such as health care, food processing, agriculture, detection of prohibited substance (drugs, explosives), forensics, environmental protection, cosmetics and drugs industry and water treatment. An increasing level of regulation and legislation for the presence and the detection of the analytes of interest necessitates the development of tools capable of reliable qualitative and quantitative analysis. Finally, the development of sophisticated analytical tools contributes to our understanding of fundamental chemical mechanisms and principles observed in the Nature and helps us to preserve the environment.

An unprecedented progress has been achieved in the past 40 years in the arena of host-guest chemistry which brought *de novo* designs of abiotic receptors and gave rise to a wider utilization of supramolecular chemistry. In a broader sense, the term *chemical sensor* is a device which responds to a particular analyte in a selective way and can be used for a qualitative or quantitative determination of the analyte. According to Ingman and coworkers, a chemical sensor is a device that transforms chemical information, ranging from the concentration of a specific
sample component to total composition analysis, into an analytically useful signal. The chemical information may originate from a chemical reaction of the analyte or from a change of physical property of the system investigated.\textsuperscript{13} Cattrall defines chemical sensor as “a device that responds to a particular analyte, i.e. ion or molecule of interest, in a selective way through a physical or chemical interaction, and can be used for qualitative or quantitative determination of the analyte.”\textsuperscript{14} In the field of analytical chemistry, the term \textit{sensor} is commonly defined to be a chemical indicator in an instrument that produces a signal indicative of the presence of an analyte.\textsuperscript{15} In supramolecular analytical chemistry as well as in this dissertation, the term \textit{molecular chemical sensor} (chemosensor) is used to refer to a chemical entity which is capable of reversibly changing its chemical or physical properties upon the presence of an analyte of interest generating a signal. Another term used in the area of chemical sensing is \textit{chemical dosimeter} (chemodosimeter) which is a used to describe a chemical entity which transforms a certain chemical information into an analytically useful signal irreversibly. A \textit{chemical indicator} is any chemical entity which possesses a capability to provide a useful signal upon a chemical stimulus. Finally, if the analyte-responsive moiety is of biotic origin (biomacromolecule, e.g. protein), the sensor molecular device is then called \textit{biosensor}. This is in a contrast with conventional chemosensors where the sensing moiety is of the abiotic origin.

The form of the sensor device output signal depends on the recognition mechanism and operating principle of the transducer. As a result, a change in optical (absorbance, photoluminescence, reflectance, etc.),\textsuperscript{16,9} electrochemical (redox potential, conductivity),\textsuperscript{17–19} or mechanical properties (density, temperature, etc.),\textsuperscript{20,21,15} can be observed upon the interaction of analyte with the receptor. This dissertation, however, describes the utilization of sensors based on the optical response and fluorescence in particular. Fluorescence-based sensors and methods
provide unique sensitivity, and are well suited for the utilization in high-throughput screening (HTS) assays.

In a classical description, a chemosensor consists of two distinct parts: receptor and transducer. The receptor acts as a binding unit providing specific moieties for an interaction with an analyte. The transducer (aka signaling unit, signal transduction unit, reporter) is an entity capable of translating the chemical information about the analyte into a useful analytical signal (Figure 1.3). The transducer itself does not show selectivity but may exhibit cooperativity.13,22

1.3 Nature of Supramolecular Interactions

The receptor-analyte interactions represent the energies that hold supramolecular complex together. The term non-covalent interactions (Table 1.1) describes a wide range of attractive (or repulsive) forces which range between 2-300 kJ mol\(^{-1}\), thus being substantially weaker than typical covalent bonds (150-450 kJ mol\(^{-1}\) per bond). The most common non-covalent (supramolecular) interactions are electrostatic (ionic) interactions, van der Waals forces (dipolar interactions, \(E \propto \frac{1}{r^6}\)), hydrogen bonds, cation-π interactions, π-π (aromatic) stacking, halogen bonding,23,24 hydrophobic (solvophobic) effects,15 and coordination bonds (Figure 1.4).25 Some authors also consider reversible covalent bonds (dynamic covalent bond) as a source of reversible interaction.
Such systems utilize cyclic boronates formation, imines formation, acylhydrazone formation, disulfides exchange, olefin metathesis, Diels-Alder reaction, acetal/hemiacetal formation, or aminal/hemiaminal formation, etc.\textsuperscript{26,27} A significant effort has been recently devoted to the development of new elegant multicomponent dynamic assemblies for sensing applications. For example, James and Bull developed an NMR shift reagent based on imines and boronate esters for the determination of chiral amines.\textsuperscript{28,29} Other systems employing dynamic supramolecular assemblies have been recently reported by Anslyn,\textsuperscript{30} Matile,\textsuperscript{31} Nitschke,\textsuperscript{32} Leigh,\textsuperscript{33} and Anzenbacher.\textsuperscript{26}

**Figure 1.4.** Examples of supramolecular (non-covalent) and dynamic covalent interactions.

Table 1.1. Summary of common supramolecular interactions.\textsuperscript{34,35}

<table>
<thead>
<tr>
<th>Interaction</th>
<th>Bond energy (typical) (kJ mol\textsuperscript{-1})</th>
<th>Example</th>
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<tr>
<td>Coordination bonds</td>
<td>20-630</td>
<td>Cu(bipy)\textsubscript{2}</td>
</tr>
<tr>
<td>Ion-ion</td>
<td>200-300</td>
<td>tetrabutylammonium chloride</td>
</tr>
<tr>
<td>Ion-dipole</td>
<td>50-200</td>
<td>Na\textsuperscript{+}···15-crown[5]ether</td>
</tr>
<tr>
<td>Dipole-dipole</td>
<td>5-50</td>
<td>acetone dimer</td>
</tr>
<tr>
<td>Hydrogen bonding</td>
<td>4-120</td>
<td>water dimer</td>
</tr>
<tr>
<td>Cation-\pi</td>
<td>5-80</td>
<td>K\textsuperscript{+}···benzene</td>
</tr>
</tbody>
</table>
1.4 Optical Chemical Sensors

Compounds incorporating a binding site, a fluorophore, and a mechanism for communication between the two are called fluorescent chemosensors. Historically, the first fluorescence chemosensor system were reported by Sousa in 1977, who described fluorescent crown-ether-decorated naphthalene derivatives 1 and 2 as sensors for alkali metal ions (Figure 1.5). In 1994, Vance and Czarnik published preparation of anthrylpolyamines (e.g., 3) exhibiting chelation-enhanced fluorescence upon the complexation of phosphate and sulfate anions. The sensor design is of particular importance especially in the fields like analytical and environmental chemistry, medicine and clinical biochemistry. A number of various analytes are suitable for the detection by fluorescence-based methods: neutral molecules and gases (saccharides, glucose,}

<table>
<thead>
<tr>
<th>Anion-π</th>
<th>&lt;60</th>
<th>trichlorotriazine···Cl⁻</th>
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<tr>
<td>π-π stacking</td>
<td>&lt;50</td>
<td>benzene and graphite</td>
</tr>
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<td>Halogen bonding</td>
<td>4-42</td>
<td>C-Br···Cl⁻</td>
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<td>Van der Waals forces</td>
<td>&lt;5</td>
<td>argon</td>
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<td>depends on solvent-solvent interaction energy</td>
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**Figure 1.5.** Examples of early supramolecular optical sensor designs: 2,3- and 1,8-crown[6]ether-decorated naphthalenes 1 and 2 for alkali metal ions (M⁺) presented by Sousa and 1,8-disubstituted anthracene based polyamine 3 reported by Czarnik capable to bind phosphate with a very high selectivity over other anions.
oxygen, carbon dioxide, nitrous oxide), cations (H\(^+\), Na\(^+\), K\(^+\), Ca\(^{2+}\), Mg\(^{2+}\), Zn\(^{2+}\), Hg\(^{2+}\), Cd\(^{2+}\), Pb\(^{2+}\), etc.) and anions (F\(^-\), Cl\(^-\), Br\(^-\), I\(^-\), phosphate, pyrophosphate, nucleotides, DNA, RNA, carboxylates, etc.). The unprecedented success of optical (fluorescent) sensors over other designs can be attributed to several major advantages given by its high sensitivity, rapidity of analysis, portability of instrumentation, and overall cost-effectiveness.\(^{38}\) In general, three main functional designs of optical molecular sensors can be found throughout the literature (Figure 1.6):\(^2\)

**Group 1**: Fluorescence of the reporter is quenched by a collision with the analyte.

**Group 2**: Reporter is equipped with receptor moiety and can reversibly bind the analyte of interest. The fluorescence is either quenched (CEQ: chelation enhancement of quenching) or enhanced (CEF: chelation enhancement of fluorescence) upon the binding event. If the analyte is a proton, the term fluorescent pH indicator is often used. If the analyte is an ion, the term fluorescent chelating agent is used.

**Group 3**: Reporter is attached to fluorophores via a spacer or directly incorporated in the fluorophore structure (integrated sensors). This type of sensor design requires special attention in order to meet the criteria for desired affinity and selectivity of the sensor.

**Figure 1.6.** Main types of fluorescent supramolecular sensors for neutral molecules and ions (green ball). Group 1: non-associating sensors, group 2: sensors with complexing fluorophores, group 3: sensors with fluorophores linked to a receptor.
Fulfilling these aspects is one of the most challenging areas of modern supramolecular chemistry and molecular design of functional molecules.

The changes in photophysical properties of the reporter upon the interaction with the analyte are given by the perturbation of electronic and steric aspect of the molecular sensor most commonly by photoinduced processes such as electron transfer, charge transfer, energy transfer, excimer or exciplex formation or disappearance, etc. and the study of the exact mechanism involved in these processes is relevant to the field of photophysics.

1.5 The Binding Constants and Complex Formation

In general, the formation of a host-guest complex is a fundamental process in supramolecular chemistry and binding (affinity) constant \( K (K_a) \) (Eqn. 2). The binding constant has been used as a general measure to assess the affinity of a particular guest (G) to a host (H) in a given complexation process which forms a supramolecular complex (C). Dissociation constant \( K_d \) is widely used especially in biological applications (\( K_d = K_a^{-1} \)) to describe protein-ligand binding processes.\(^\text{39} \) There is a number of methods used for binding constant determination based on a particular host-guest system design.\(^\text{40-43} \) In supramolecular chemistry, selectivity is usually defined as a ratio between two independent equilibria \( K_1 \) and \( K_2 \) (Eqn. 5).

\[
\begin{align*}
\text{Equation 1} & \quad a \cdot H + b \cdot G_1 \rightleftharpoons C_1 \\
\text{Equation 2} & \quad K_1 = \frac{[C_1]}{[H]^a \cdot [G_1]^b} \\
\text{Equation 3} & \quad a \cdot H + b \cdot G_2 \rightleftharpoons C_2 \\
\text{Equation 4} & \quad K_2 = \frac{[C_2]}{[H]^a \cdot [G_2]^b} \\
\text{Equation 5} & \quad \text{Selectivity} = \frac{K_1}{K_2}
\end{align*}
\]
Over past three decades of research, a lot of effort has been devoted to the development of various sensor systems providing a high selectivity and sensitivity towards the analyte of interest allowing qualitative and quantitative detection even in complex environments. A magnitude of response of given sensor is dependent on the concentration of an analyte while the slope of the calibration curve is related to the sensitivity of the sensor. Therefore, a sensor with a large binging affinity to a given analyte provides larger response while a sensor with small binging affinity provides relatively small response. A highly sensitive sensor allows low limit of detection (LOD) defined as a concentration of analyte which gives an instrument signal significantly different from the ‘blank’ or ‘background’ signal with a given degree of confidence.

The temperature dependence of binding constant (van’t Hoff equation, Eqn. 8) can be obtained using equation 6 and Gibbs-Helmholtz equation (Eqn. 7) as a function of thermodynamic parameters enthalpy (ΔH), entropy (ΔS) and Gibbs free energy (ΔG).

\[ \Delta G = -RT \ln K \]  \hspace{1cm} (Equation 6)

\[ \Delta G = \Delta H - T\Delta S \]  \hspace{1cm} (Equation 7)

\[ K = \exp \left( -\frac{\Delta H}{RT} + \frac{\Delta S}{R} \right) \]  \hspace{1cm} (Equation 8)

The van’t Hoff equation allows to calculate the binding energy (ΔG) at given temperature T using parameters ΔH, ΔS.
1.6 Signaling Recognition Mechanisms in Optical Sensing

Changes in both the absorption and emission of light can be used as signals in the case sensor is equipped with suitable chromo- or fluorophore. Both spectrophotometry and fluorimetry are relatively technically simple methods, the results can be obtained in Changes in both the

Absence of the Analyte: Oxidative PET (no emission)

Absence of the Analyte: Reductive PET (no emission)

Presence of the analyte: Emission

Figure 1.7. Frontier molecular orbitals representation of the mechanism of oxidative and reductive photoinduced electron transfer (PET).
absorption and emission of light can be used as signals in the case sensor is equipped with suitable chromo- or fluorophore. Both spectrophotometry and fluorimetry are relatively technically simple methods, the results can be obtained in a short time, they are both non-destructive and suitable for analysis of complex mixtures. Fluorimetry provides substantially higher sensitivity over spectrophotometry. While spectrophotometry can detect concentrations down to 100 nM, fluorimetry can measure concentrations $10^6$ times lower. A superior advantage of fluorimetry is, however, that the various analytes can be distinguished by time-resolved methods.

The response (a change of optical properties) of a fluorescent molecular sensor is usually measured as a change in fluorescence intensity, fluorescence lifetime, or a shift of fluorescence wavelength (ratiometric response). The major mechanisms responsible for a change of optical properties of a fluorophore to the binding of an analyte are photoinduced electron transfer (PET), fluorescence (Förster) resonance energy transfer (FRET), electronic energy transfer (EET), excimer/exciplex formation (the formation of a dimer or complex between an excited excited state and a ground-state species), and photoinduced charge transfer (PCT). Kim and coworkers presented a fluoride-selective PCT chemosensor based on formation of pyrene excimer. The sensor 5 (Figure 1.8) was based on calix[4]arene decorated with pyrene fluorophores in 1,3 positions of the macrocycle. The authors observed change in emission wavelength and a formation of excimer feature in the fluorescent spectrum which was apparently facilitated by the addition of fluoride. The PET, however has been the most widely used fluorescence signal transduction mechanisms. It has been utilized to create a variety of turn-on fluorescent sensors. The situation involving PET-based electron donor–acceptor systems can be illustrated using frontier molecular orbital theory (Figure 1.7). If no analyte is present in the receptor, the LUMO of electron-poor acceptor (A) is energetically lower so that the acceptor
effectively de-populates excited state of the donor ($D^*$) and no fluorescence is observed (oxidative PET). If an electron-rich acceptor is present in the structure, the HOMO of the acceptor can donate electron to HOMO of $D^*$ resulting in fluorescence quenching (reductive PET). The situation changes, however, if the analyte is bound to the receptor. The HOMO energy level of the $A$ is lowered and LUMO level is unavailable as well. This diminishes the ability to transfer an electron to/from the acceptor, resulting in fluorescence recovery (turn-on response). This principle has been widely utilized in so called turn-on fluorescence sensors and pioneered by Czarnik, de Silva, and Shinkai.$^{11,62,63}$ An intramolecular (internal) partial charge transfer (IPCT) is another signal transduction mechanism resulting in a change of color based on charge transfer taking place upon the binding of the analyte (usually a charged species). In order to obtain a change in optical signal, the IPCT requires the chemosensor to contain an electron-donating group (EDG) acting as charge transfer donor and electron-withdrawing group (EWG) acting as charge transfer acceptor. Such a push-pull chemosensor design was studied by Anzenbacher, Jr. and co-workers,$^{64,65}$ and was

**Figure 1.8.** Structure of octamethylicalix[4]pyrrole optical sensor 4 decorated with an electron-poor dye moiety. Below is a schematic depiction of ICPT mechanism originating from anion hydrogen-bonding to electron-rich pyrrole unit (left). Calixarene-based chemosensor 5 with two fluorogenic pyrene units conjugated to amide groups as guest recognition sites showed excimer emission upon the complexation of fluoride (right).
extensively utilized in octamethylcalix[4]pyrrole-based optical sensors decorated with electron-poor indanylidene dyes (e.g., 4) for anions sensing (Figure 1.8).

1.7 Indicator-Displacement Assay

The molecular sensors utilize the transducer–spacer–receptor (TSR) design where the transducer (reporter) is covalently attached to the receptor via a linker (Figure 1.3). Binding of an analyte to the receptor moiety modulates fluorescence or absorbance signal of the dye (reporter). The main disadvantage of this traditional design, however, is often the need of complicated synthesis required to covalently attach the indicator to the receptor and cost of the final sensor. The indicator–displacement assay (IDA),\textsuperscript{66–75} overcomes this obstacle in elegant way: In the IDA, the dye (indicator) is first allowed to bind to the receptor forming a reversible noncovalent complex. Afterwards, an introduction of competitive analyte displaces the indicator from the receptor thereby changing its optical properties (Figure 1.9). In this setup, the binding affinity of indicator–receptor must be sufficiently lower than the one of analyte-receptor complex. Also, the complexation equilibrium of the dye must give a rise to significant (measurable) change in its optical properties. In general, several mechanisms can be employed in the signal modulation when designing an IDA: fluorescence resonance energy transfer (FRET),\textsuperscript{76} photoinduced electron transfer (PET),\textsuperscript{7,24} electronic energy transfer (EET),\textsuperscript{25,77} or a change of ionic strength or pH.\textsuperscript{78} To facilitate the complex formation between the dye and the receptor, hydrogen bonding and

![Figure 1.9. Schematic of the indicator–displacement assay](image-url)
electrostatic interactions, has been widely employed.\textsuperscript{74,79–83} Complexation with transition metal centers has also been investigated.\textsuperscript{84–87} Both colorimetric as well as fluorescence-based IDA systems have been reported.

The idea of IDA was first illustrated in 1998 by Anslyn and coworkers,\textsuperscript{82} using tris(imidazolium)-decorated 1,3,5-triaminomethyl-2,4,6-triethylbenzene scaffold 6 as a receptor and 5-carboxyfluorescein 7 as a dye for the detection of citrate 8 (\textit{Figure 1.10}). Interestingly, the assay was also capable to quantify citrate concentration in popular soft drinks using UV/Vis spectroscopy. Importantly, IDA-based sensing systems possess several advantages over the conventional receptor-linker-reporter designs: (i) simpler (cheaper) sensor synthesis, (ii) IDA allows to employ various indicators, hence providing access to a library of different sensing ensembles and (iii) a large number of indicators are commercially available. The main disadvantages of the IDA-based sensing, however, is an excess of the dye needed to accomplish binding of the dye to the receptor (since the binding affinity is low) which effectively decreases the sensitivity of IDA sensor systems. Additionally, rather inherent cross-reactivity of the IDA receptors may be a disadvantage as well since the receptor must be capable to provide binding to both dye and analyte(s). To overcome the above disadvantages, Anzenbacher, Jr. and coworkers in 2014 introduced so called \textit{intramolecular indicator displacement assays} (IIDAs).\textsuperscript{88} They

\textbf{Figure 1.10.} First IDA system presented by Anslyn and coworkers in 1998 for citrate fluorescence sensing in various beverages.
presented sensors 9 and 10 (Figure 1.11) capable to recognize phosphates over other biologically and environmentally relevant anions featuring thiourea and amide groups as anion recognition moieties and naphthyl-carboxylate as an anionic chromophore. The authors were able to utilize sensors 9 and 10 in a polymer-based microchip array for qualitative and quantitative recognition of the anions of interest.

Figure 1.11. IIDA sensors 9 and 10 developed by Anzenbacher, Jr., and coworkers in 2014 for fluorescence sensing of glyphosate 11.

A plethora of fluorescence-based indicator-displacement assay sensing ensembles have been reported for sensing of biomolecules. Kowalczykowski and coworkers described a monitoring of *E. coli* Rec A protein, a helicase functioning in unwinding DNA during the

Figure 1.12. Thiazole orange 12 was used to monitor *E. coli* Rec A protein activity by Kowalczykowski and coworkers. Sulfonate 13 was utilized in competitive IDA-type binding of biotine 14 to avidin.
replication process to produce single-stranded DNA (ssDNA), using thiazole orange 12 (Figure 1.12). The dye’s fluorescence intensity is increased when it is bound to double-stranded DNA (dsDNA) and decreased when only ssDNA is present. Thus, the system could be used to monitor the Rec A protein activity. 89 Similar method was utilized by Mock and coworkers to investigate the environment of biotin 14 binding site on avidin. The authors revealed that 2-anilinonaphthalene-6-sulfonate binds to avidin binding site accompanied with a dramatic increase in fluorescence intensity. They were also able to completely displace the dye from the binding site of avidin by competitive titration with biotin and confirm stoichiometry of the biotin-avidin complex ($K \approx 10^{15} \text{ M}^{-1}$). 90 The IDA concept and its application in fluorescence sensing of biologically relevant species and biomacromolecules will be further discussed in Chapter II.

1.8 Optical Sensor Arrays and Multivariate Analysis

Two principally distinct sensing approaches are presented in this dissertation. The first one is based on the Emil Fischer’s hallmark of specific binding from nature, the *lock-and-key paradigm* (Schlüssel-Schloss-Prinzip). 91–94 This approach is known for a large degree of complementarity between the host and guest. In the field of supramolecular sensing, a highly specific sensor binds the analyte of interest with a unique selectivity and high affinity (Figure 1.13). Chapter II of this dissertation describes in details such an approach in the design of fully synthetic high affinity sensors for the detection of carbonic anhydrases and its application in fluorescence-based competitive assay for carbonic anhydrase inhibitors. Yet, there is another nature’s method of molecular recognition utilized for example in the mammalian senses of gustation and olfaction. The method relies on *differential sensing* where an array of differential (rather than specific or selective) sensors is employed in order to obtain a response signal which can be evaluated and
The "lock and key" paradigm:

Interpreted by pattern recognition protocols. The cross-reactive sensor array contains at least two rather non-specific sensing elements (chemosensors) with different affinity towards various analytes. This provides a complex response pattern created by a single analyte or by a mixture of analytes. Each sensor-analyte generates a distinct analyte-specific response patterns, or fingerprint, based on sensor’s selectivity (binding affinity) and sensitivity towards each analyte (Figure 1.13). The composite multi-variate response obtained as fluorescence intensity or absorbance read-out is then evaluated employing pattern recognition techniques allowing to interpret, identify and classify the analytes of interest.

Figure 1.13. Two distinct approaches of molecular recognition found in nature: the lock and key and the differential sensing scenarios. Adapted from reference.°
The selection of the competent chemosensors to be used in an array is a crucial task. The individual chemosensors selection largely depends on the knowledge of the target analyte, and whether a qualitative or quantitative analysis is sought. Generally, the analyte concentration to be investigated must be within the dynamic range of the chemosensor elements. Additionally, highly cross-reactive (non-specific) chemosensors tend to generate a low information density response pattern (low discriminatory power). This can be circumvented by employing selective sensors with different degree of sensitivity for each analyte. Additionally, the discriminatory power an array can be dramatically improved by increasing the number of read-out channels and number of conditions at which the array is performed. This procedure is commonly used and usually generates a large multi-dimensional-response space.96

A pattern recognition binding scheme capable of recognizing and discrimination of an immense amount of odor and smell molecules is found in the mammalian senses of gustation and olfaction (taste and smell).97,98 Given the concept of arrays of differential receptors, this approach provides a number of advantages. Unlike the traditional rational design leading to highly selective chemosensors which are usually difficult and expensive to synthesize, the concept of arrays of differential receptors requires a very little chemical design needed for the creation of the cross-reactive sensor array.99 Another very important feature of pattern recognition based cross-reactive sensor arrays is the possibility to simultaneously identify and quantify mixtures of multiple chemically and biologically relevant species. The cross-reactive sensor array approach for the simultaneous detection of multiple chemically or biologically relevant species has been widely exploited in the last three decades. Methods for the detection of plethora of analytes in a wide range of various environments has been reported since the introduction of the “high-density array” in 1995.100 The application of sensor arrays allowing an implementation of HTS has spread to
perhaps all fields of human’s activity. The differential sensing (array) has become a standard strategy and has been extensively studied by the groups of Anslyn, Anzenbacher, Jr., Severin, Suslick, and Rotello, who made significant contributions to the development of this field in the area of sensing of cations, anions, small neutral molecules, and proteins, cells and bacteria.

Array experiments frequently provide an immense amount of data that is impossible to interpret using for example a simple linear regression. Hence, the data acquired from fluorescence-based cross-reactive sensor array experiment obtained from microwell plate or glass microchip are routinely processed using chemometric pattern recognition protocols. In general, these chemometric methods are applied to reduce the obtained data set dimensionality (dimensionality reduction) and present it in graphical form for visual interpretation. Variety of statistical analysis methods for pattern recognition exists. In this dissertation, only linear discriminant analysis (LDA), hierarchical clustering analysis (HCA), and a type of artificial neural network (ANN) termed support vector machine (SVM) for linear regression analysis, were used. Based on data classification algorithm, two main groups of statistical methods exist. Unsupervised methods require just independent variable information (i.e., sensor responses) to be provided in order to classify data set to be analyzed. On the other hand, supervised methods also require dependent variable information (e.g., analyte classes) to be provided. The choice of suitable statistical method to be used largely depends on more specific details of the application and operator preference. For qualitative analysis applications, the unsupervised methods are best suited. Supervised methods are used for quantitative applications, such as determining which class a particular observation belongs to.
Linear discriminant analysis (LDA) is a supervised method that has been successfully used in supramolecular analytical chemistry to assign an unknown analyte to its appropriate class and to formulate boundaries between components of different classes based on responses from sensor arrays. The data obtained from the array experiment (e.g., fluorescence intensities) and the analyte classes (e.g., analytes identities) are provided in the input data set. Linear discriminant functions are then calculated from these input data provided so that the separation between individual classes is maximized while the variation within the individual groups is minimized. The LDA algorithm first develops a model (model prediction) which is later on tested to how well the model predicts the data set (model validation). The most common cross-validation method is leave-one-out (or jackknife) method and the identity of an unknown analyte can be assigned to one of the classes based on the similarity of the response pattern.

Hierarchical clustering analysis (HCA) is a popular unsupervised method of multivariate analysis used for clustering analysis that clusters data points based on relative distances in the $n$-dimensional space ($n$ usually being the number of different sensor responses) to one another and groups them in hierarchical manner. The observations are clustered according to similarities (or distances) in their features. HCA is very sensitive as it uses the entire data set dimensionality to represent the patterns in two-dimensional representation. The results of the clustering depend on the clustering metric used and the similarity measure applied to the input data set. The most commonly used distance metric in sensor array applications is the Euclidean distance metric which calculates the distance between two data points with $n$-dimensions. Also, there are several methods available to define the linkage between the clusters calculated. A number of studies use Ward’s (minimum variance) method, which considers the minimum amount of the variance between the samples and analytes to define a cluster. The graphical result of HCA is
a dendrogram showing the quantitative differences (or similarities) between the individual analytes in a hierarchical manner based on how different the response patterns are for each analyte and group.

**Support vector machine** (SVM), has proved to be capable to resolve even difficult analytical problems, such as multianalyte samples and has been found to be very suitable for quantitative analysis of the analytes of interest. It is a set of supervised methods that are based on a statistical learning theory that can be used for both, classification and regression analyses of the data.\textsuperscript{149–152} They are capable to separate analyte classes by mapping the input into an $n$-dimensional vector space using kernel functions (linear, polynomial, radial basis function, etc.). Basically, the SVM is an advanced chemometric method and a detailed explanation of its function goes far beyond the scope of this dissertation. In this dissertation, the SVM was used for a linear regression analysis of data obtained from quantitative assays. In this application, the data are used to train the SVM leading to regression (calibration) model. Based on the kernel function selected for the SVM regression algorithm, the model produces the *root mean square error of calibration* (RMSEC) and after the model is created and validated using $n$-fold cross-validation approach one can obtain *root mean square error of cross-validation* (RMSECV). At the end, the model’s predictive ability is validated using an independent data set (“unknown samples”) which produces *root mean square error of prediction* (RMSEP). *Root mean square deviation* (RMSD or RMSE) quantifies how different are the values predicted by the regression model from the actual (real) values that are being modeled.
1.9 References


(152) Williams, G. In *Data Mining with Rattle and R*; Use R; Springer New York, 2011; pp 293–304.
CHAPTER II. FIRST SUPRAMOLECULAR FLUORESCENCE-BASED ASSAY FOR CARBONIC ANHYDRASE INHIBITORS

2.1 Abstract

Here, we present supramolecular fluorescence probes S1-S7 capable of specific detection of carbonic anhydrases (CAs), a biologically and clinically important family of metalloenzymes implicated in certain pathological health conditions in humans. The probes, comprising 1,3,4-thiadiazol-5-yl-2-sulfonamide high-affinity moiety, were employed in fluorescence titrations with CAs and showed highly variable analyte-dependent change in fluorescence intensity. Moreover, the probes were utilized in competitive fluorescence-based assay to investigate affinity of model carbonic anhydrase inhibitors towards CAs. The probes were utilized in a competitive sensor array using simple instrumentation under high throughput screening settings. The excellent capability of recognizing a large number of structurally distinct CAIs at various concentrations was demonstrated in qualitative as well as quantitative assays. The throughput, sensitivity and limit of detection surpass the current state-of-the-art methods that generally require enzyme-linked immunosorbent assay based protocols and/or computational modeling. This study opens a new avenue for the development of simple high-throughput assays for the drug development and drugs candidates structure optimization in the near future. To the best of our knowledge, this study presents the first supramolecular fluorescence-based assay for carbonic anhydrase inhibitors.

2.2 Carbonic Anhydrases: Structure, Distribution and Physiological Function

Carbonic anhydrases (also known as carbonate dehydratases or carbonate hydrolyases, EC 4.2.1.1) are ubiquitous metalloenzymes,\textsuperscript{1-3} discovered in 1933 by Meldrum and Roughton in red
blood cells.\textsuperscript{4,5} They are present in prokaryotic and eukaryotic organisms and encoded by six evolutionary distinct gene families: $\alpha$-CAs (expressed in vertebrates, bacteria, algae and cytoplasm of green plants); $\beta$-CAs (predominantly in bacteria, algae and chloroplasts of monodicotyledons and dicotyledons); $\gamma$-CAs (mainly found in archaea and some bacteria); $\delta$-CAs (found in marine diatoms), $\zeta$-CAs (found exclusively in several chemolithotrophic bacteria and marine cyanobacteria) and recently identified $\eta$-CA (found in parasitic protozoa plasmodium).\textsuperscript{6} The $\alpha$, $\beta$- and $\delta$-class of CAs were found to contain a Zn(II) ion at its active site, the $\gamma$-CAs contain Fe(II) ion (but show certain catalytic activity with Zn(II) or Co(II) ions as well). The $\zeta$-class of CAs was found to contain Cd(II) in its catalytic center. The sequence length of CAs ranges from 260 to 459 amino acid residues and the X-ray crystallography data shows that the active center is localized in at the bottom of a 15 Å deep and about 15 Å wide (at its mouth) catalytic cleft.\textsuperscript{7-9} All CAs catalyze hydration of carbon dioxide to bicarbonate ion and proton in a reversible two-step mechanism. The process is schematically summarized in the following equations:

\[
\text{EZn}^{2+}–\text{OH}^{-} + \text{CO}_2 \rightleftharpoons \text{EZn}^{2+}–\text{HCO}_3^{-} + \text{H}_2\text{O} \rightleftharpoons \text{EZn}^{2+}–\text{OH}_2 + \text{HCO}_3^{-} \quad \text{(Equation 1)}
\]

\[
\text{EZn}^{2+}–\text{OH}_2 \rightleftharpoons \text{EZn}^{2+}–\text{OH}^{-} + \text{H}^{+} \quad \text{(Equation 2)}
\]

The proton transfer reaction (Eqn. 2) is facilitated by histidine residues located near the entrance of the cavity (His-64) and at its rim (His-3, His-4, His-10, His-15 and His-17). This ensemble of amino acid residues capable to facilitate fast proton exchange is often called the \textit{proton shuttle}.\textsuperscript{10} The reaction regenerating the zinc-bound hydroxide ion (Eqn. 2) is the rate-limiting step and was shown to be a diffusion-controlled process.\textsuperscript{11} The reaction turnover rate $k_{\text{cat}} = 10^4–10^6 \text{ s}^{-1}$ found in the catalytically most active isoforms (such as CA II, CA IV, CA V, CA VII, and CA IX) makes CAs the most active enzymes known.\textsuperscript{12–14} A detailed catalytic mechanism (the Lindskog’s mechanism) is shown in \textit{Figure 2.1}.\textsuperscript{14–17} In the hydration direction, the metal ion
center significantly increases acidity of the metal-bound water molecule ($pK_a = 7$), hence enabling its deprotonation by nearby histidine moiety giving metal-bound hydroxide ion. The nucleophile (OH$^-$) is further stabilized by hydrogen bond with Thr-199 which is in turn bridged to Glu-106. This unique hydrogen bond network within the hydrophobic pocket enhances the resulting nucleophilicity and orients the substrate molecule (CO$_2$) to a favorable position for nucleophilic attack. A new water molecule arriving into the cavity then displaces formed bicarbonate anion and liberates it into bulk solution. The structure of hCA II with carbon dioxi

**Figure 2.1.** The catalytic mechanism according to Lindskog (with zinc active center) of $\alpha$-carbonic anhydrase (hCA I amino acid sequence), similar to all CA isozymes, showing reversible hydration of carbon dioxide molecule at the Zn$^{2+}$ active center.

**Figure 2.2.** X-ray diffraction analysis of hCA II depicting (A) carbon dioxide (PDB code 2VVA) and (B) bicarbonate (PDB code 2VVB) binding in the active site. The Zn$^{2+}$ center and participating amino acid residues are also shown.
and bicarbonate were recently obtained by Sjöblom and co-workers (Figure 2.2).\textsuperscript{20} The metal cofactor ($K_{Zn} = 4 \times 10^{12} \text{ M}^{-1}$ at pH 7.5, 25° C, for human apo-CA II)\textsuperscript{21} is essential for the catalytic activity of CAs and its removal leads to a catalytically inactive apo-CA.\textsuperscript{22}

There have been fifteen human CA (hCA) isozymes identified up to now which have different molecular features, display different catalytic activity, subcellular localization, oligomeric arrangement, distribution in tissues and organs, expression levels, kinetic properties and response to different classes of inhibitors (Table 2.1).\textsuperscript{23,7,24} CA isozymes I, II, III, VII and XIII are found in cytosol, CA IV, IX, XII, and XIV are membrane-bound, CA VA and VB are localized in mitochondria. CA VI is a secreted CA isoform and is found in saliva and milk (Figure 2.3). Structures of all hCA isoforms except CA VB have been confirmed by X-ray or neutron diffractometry and the analysis of these data shows, regardless of their subcellular localization, the isoforms show a very high sequence homology and similar structural features.\textsuperscript{9,25–35}

In this study we have used predominantly CA isozyme II, which represents most of the CAs since it provides a significant sequence homology and structural similarity with all other CAs of $\alpha$-class family.\textsuperscript{26} For example, the sequence homology between human and bovine CAs was

\textbf{Figure 2.3.} Schematic illustration of subcellular localization of human $\alpha$-CAs in human cell: CAs I, II, III, VII and XIII are found in cytosol and the mitochondrial CA VA and VB are located in mitochondria; CAs IV, IX, XII, and XIV are membrane-associated proteins. CA VI is a secreted CA isozyme.
found to be 79.5%. All α-class CAs feature a typical ellipsoidal fold (Figure 2.4A) with tetrahedral Zn$^{2+}$ active site coordinated with three histidine residues (His-94, His-96 and His-119) residing at the bottom of the conical cavity. The tertiary structure of the CAs consists of six right-handed α-helices surrounding a twisted plane formed by ten β-strands complemented by a number of β-turns. The catalytic cleft is divided in two parts based on its nature. Residues Phe-91, Ala-121 and Ala-135, Leu-131, Leu-138, Leu-146, Leu-109, Val-207, and Pro-201, Pro-202 delimit hydrophobic character while residues Tyr-7, Val-62, His-64, 67, 200, Asn-69, Gln-92 and Thr-199 located on the opposite side of the catalytic cleft provide hydrophilic character (Figure 2.4B). This dual character of the active center site was found to be essential for catalytic activity of CAs.\textsuperscript{17,37–42}

CA isozymes are among the most important enzymes since they facilitate the transport of CO$_2$ in and out of the cell and are involved in various other physiological processes.\textsuperscript{43} Some of the human (α-class) isozymes are ubiquitous and their function is not yet fully understood.\textsuperscript{44}

**Figure 2.4.** A: Ribbon diagram of the hCA II (PDB code 3HS4) depicting secondary-structure domains (β-sheet: yellow, α-helix: red, β-turn: green) as well as His-94, His-96 and His-119 residues and zinc(II) center (magenta). B: Solvent accessible surface of hCA II (PDB code 3HS4) using surface hydrophobicity mapping. Residues determining the hydrophobic half of the active cavity are shown in red (Ile-91, Phe-131, Val-121, Val-135, Leu-141, Val-143, Leu-198, Pro-202, Leu-204 Val-207 and Trp-209), while residues determining the hydrophilic character are shown in grey (Asn-62, His-64, Asn-67 and Gln-92). Zinc center is shown in magenta.
Table 2.1. Subcellular localization and distribution, relative catalytic activity, affinity to sulfonamides of human α-CA isoforms and their involvement in diseases.2,52

<table>
<thead>
<tr>
<th>Isoform</th>
<th>Organ / tissue of distribution</th>
<th>Subcellular localization</th>
<th>Catalytic activity (CO₂ hydration)</th>
<th>Rel. affinity to sulfonamides</th>
<th>Drug targets in disease</th>
</tr>
</thead>
<tbody>
<tr>
<td>CA I</td>
<td>erythrocytes, gastrointestinal tract, eye</td>
<td>cytosol</td>
<td>low (10% of that of CAII)</td>
<td>medium</td>
<td>cerebral edema⁵³</td>
</tr>
<tr>
<td>CA II</td>
<td>erythrocytes, eye, gastrointestinal tract, bone osteoclasts, kidney, lung, testis, brain</td>
<td>cytosol</td>
<td>high</td>
<td>very high</td>
<td>glaucoma,⁵⁴ epilepsy,⁵⁵,⁵⁶ edema,⁵⁷ altitude sickness⁵⁸</td>
</tr>
<tr>
<td>CA III</td>
<td>skeletal muscle, adipocytes</td>
<td>cytosol</td>
<td>very low (0.3% of that of CAII)</td>
<td>very low</td>
<td>oxidative stress⁵⁹</td>
</tr>
<tr>
<td>CA IV</td>
<td>kidney, lung, pancreas, brain, colon, myocardium, eye liver</td>
<td>membrane-associated</td>
<td>medium</td>
<td>high</td>
<td>glaucoma,⁵⁰ stroke⁶¹</td>
</tr>
<tr>
<td>CA VA</td>
<td>myocardium, muscles, pancreas, kidney, spinal cord, gastrointestinal tract</td>
<td>mitochondria</td>
<td>low</td>
<td>high</td>
<td>obesity⁶²,⁶³</td>
</tr>
<tr>
<td>CA VB</td>
<td>kidney, lung, pancreas, brain, colon, myocardium, eye liver</td>
<td>mitochondria</td>
<td>high</td>
<td>high</td>
<td>obesity⁶²,⁶³</td>
</tr>
<tr>
<td>CA VI</td>
<td>salivary and mammary glands</td>
<td>saliva, milk, tears</td>
<td>low</td>
<td>very high</td>
<td>cariogenesis⁶⁴</td>
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<td>CA VII</td>
<td>central nervous system</td>
<td>cytosol</td>
<td>high</td>
<td>very high</td>
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<tr>
<td>CA VIII</td>
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<td>cytosol</td>
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<td>not determined</td>
<td>neurodegeneration, cancer⁶⁶,⁶⁷</td>
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<td>high</td>
<td>high</td>
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<tr>
<td>CA X</td>
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<td>cytosol</td>
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<tr>
<td>CA XI</td>
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<td>kidney, intestine, reproductive epithelia, eye, tumors</td>
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<td>low</td>
<td>very high</td>
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<td>high</td>
<td>sterility⁷⁰</td>
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<tr>
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<td>membrane-associated</td>
<td>low</td>
<td>high</td>
<td>epilepsy,⁷¹ retinopathy⁷²</td>
</tr>
</tbody>
</table>

CA I is expressed mainly in red blood cells and after hemoglobin it is the most abundant protein found in erythrocytic cytosol.⁴⁵ CA isozyme II was found to have the widest tissue and organ distribution out of all CAs. It is expressed in osteoclasts in bone, choroid plexus epithelia, retinal Müller cells, hepatocytes, kidney, oligodendrocytes in brain, salivary glands, erythrocytes and platelets.⁴⁶ The major importance of CA II is its participation in acid–base homeostasis,⁴⁷–⁵⁰ but
was also shown to function in other processes such as anti-reflux defense, bone resorption, production of aqueous humor, sperm motility, urine acidification, gas exchange and nasal chemosensitivity to carbon dioxide.\textsuperscript{51} CA III is released from skeletal muscle in a fixed ratio with myoglobin during cell injury,\textsuperscript{73} and is one of the most inefficient CAs in terms of its ability to catalyze CO\textsubscript{2} hydration, but its complete physiological role is not yet fully understood.\textsuperscript{74} It was found in skeletal muscles and identified as a protein regulating adipogenesis and mediating glycosaminoglycan formation in connective tissue cells.\textsuperscript{75,76} CA IV is a membrane associated isozyme expressed in gastrointestinal tract, lung and kidney tissues, pancreas, salivary glands, colon, myocardium and eye,\textsuperscript{77} and was found to be involved in a cerebral blood flow regulation among other functions.\textsuperscript{78} CA isozymes VA and VB are mitochondrial isoforms that supply bicarbonate ion for pyruvate carboxylase in the synthesis of fatty acids (lipogenesis) and have been considered as a target for obtaining anti-obesity formulations.\textsuperscript{62,63,79} CA isoform VI is expressed, for example, in pancreas and central nervous system and many other organ tissues where it is involved in pH regulation and cariogenesis.\textsuperscript{80} CA VII is found in the central nervous system where it functions in cerebrospinal fluid production and was found to be involved in certain kinds of epilepsy.\textsuperscript{18,65} CA isozyme VIII is CA-related functionless protein found in the central nervous system and has been connected to certain types of cancer and neurodegenerative conditions.\textsuperscript{66} CA IX is a membrane associated isoform found in tumors and gastrointestinal tract and has been correlated with renal carcinoma.\textsuperscript{81,82} Cytosolic and acatalytic CA isoforms X and XI are expressed in central nervous system and their role has not yet been described. Overexpression of CA XII was observed in certain human cancers for example in an invasive breast carcinoma.\textsuperscript{29,83,84} CA XIII is the most recently reported CA and studies revealed the isozyme distribution occurs in several organs, including spleen, small intestine and prostate where is known to control optimal
bicarbonate concentration and pH homeostasis.\textsuperscript{32,70} Its physiological function is yet to be fully understood.

Importantly, many of the CA isozymes were found to be implicated in various diseases and are important therapeutic targets in the treatment of a range of disorders including edema,\textsuperscript{57} glaucoma,\textsuperscript{54,85,69} obesity,\textsuperscript{62,79,63,86,87} cancer,\textsuperscript{82,88–92} epilepsy\textsuperscript{93,55,56,65,71} and osteoporosis.\textsuperscript{94,64,80} Specifically, CA I is found in many tissues and is involved in retinal and cerebral edema, and its inhibition may be a valuable tool for fighting these conditions.\textsuperscript{53,95} CA II is involved in several diseases, such as glaucoma, edema, epilepsy, and altitude sickness.\textsuperscript{96,58} CA III is implicated in oxidative stress, and takes part in a number of inflammatory diseases.\textsuperscript{97,98}

2.3 Carbonic Anhydrases: Inhibitors and Inhibition Mechanisms

Several studies demonstrated that abnormal levels or activities of CAs were found to be associated with a number of pathophysiological conditions in humans. Consequently, CAs have become an important therapeutic targets for drug development. The activity of CAs is modulated by carbonic anhydrase inhibitors (CAIs). Four main classes of CAIs are known and many of them are commonly used to treat conditions related to CAs activity disorders: ureates and hydroxamates,

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure2.5}
\caption{CA inhibition mechanism by sulfonamide and anionic inhibitors.}
\end{figure}
mercaptophenols, metal-complexing anions and unsubstituted sulfonamides (including their bioisosteres, such as sulfamates and sulfamides). Studies revealed that all types of CAIs bind to the zinc(II) center of the enzyme thus effectively inhibiting coordination of carbon dioxide. This happens either with preservation of zinc tetrahedral geometry (substitution, Figure 2.5A) or by addition to the tetrahedral center leading to the formation of trigonal-bipyramidal geometry (Figure 2.5B). Metal-complexing ions (e.g., cyanide, azide, hydrogen sulfide and trithiocarbonate) are known to be weak CAIs with affinities typically in the range of $10^3$–$10^4$ M$^{-1}$. Sulfonamides were found to be the most potent CAIs, and are the most important and clinically used zinc-binding CAIs.

Unsubstituted sulfonamides bind to the zinc(II) center in the active site of CAs in deprotonated form following the substitution mechanism leading to the preservation of tetrahedral geometry of the zinc center. Clinically most successful and commonly used CAIs are heterocyclic sulfonamides (Figure 2.6). This includes N-acetylated thiadiazole sulfonamide known as acetazolamide (1) and marketed as Diamox. It is used to treat acute altitude sickness, glaucoma and certain types of epileptic seizures among other conditions. It also acts as a diuretic. Benzolamide (2), is an orphan drug that is widely used in many physiological and pharmacological studies. Methazolamide (3), marketed as Neptazane, is relatively new CAI structurally similar to acetazolamide and used in glaucoma treatment regimen. Ethoxzolamide (4) is a benzothiazole sulfonamide. It is mainly used in the treatment of glaucoma, duodenal ulcers, epilepsy and it has a diuretic effect. Dorzolamide (5) and brinzolamide (6), marketed as Trusopt and Azopt, respectively, are both used to treat glaucoma and to reduce intraocular pressure (IOP). Both structures contain thiophene sulfonamide moiety decorated with aliphatic ring featuring a stereogenic center. Diclofenamide (7) is a $m$-disulfamoylbenzene marketed as Fenamide and
used as an ophthalmic agent to treat certain types of glaucoma and epilepsy.\textsuperscript{115,116} Celecoxib (8) marketed as Celebrex is a popular non-steroidal anti-inflammatory drug (NSAID) that has been primarily administered as COX-2 selective inhibitor to treat pain and inflammation originating from arthritis and acute pain in adults.\textsuperscript{117} A number of studies suggest its capability to act as a potent CAI due to related binding site recognition.\textsuperscript{118,119}

Considering clinical significance of CAIs, many studies were devoted to the rational design of isozyme-specific CAIs aimed at obtaining novel classes of drugs free of undesired side-effects.\textsuperscript{6,120–123} The standard approach involved in a drug development and structure optimization to estimate an inhibitor efficiency is to measure its effect on the catalytic activity. These methods usually involve pH measurement using either a pH indicator in combination with an optical detection using suitable spectrophotometric technique or a direct pH measurement using potentiometry.\textsuperscript{124,125} In this dissertation, we decided to explore inhibitors efficacy using a supramolecular approach based on an indicator-displacement assay.

\textbf{Figure 2.6.} Clinically relevant sulfonamide-based carbonic anhydrase inhibitors.
2.4 Carbonic Anhydrases in Supramolecular Optical Sensing

CAs have been widely studied as a benchmark systems suitable for supramolecular sensing either as an analyte of interest or as the receptor moiety of the sensing ensemble. Typically, in polar or protic solvents the fluorescence signal of environmentally sensitive dyes is suppressed or broadened due to self-aggregation while the fluorescence is amplified and blue-shifted in lipophilic environments. This approach was recently investigated by Tan and coworkers who achieved a specific turn-on fluorescence detection of human CA isozyme II (hCA II).

The authors introduced environmentally sensitive benzoaxadiazole and dansyl derivatives 9-12 (Figure 2.7) conjugated to benzene sulfonamide acting as a high-affinity ligand for hCA II. Tan and coworkers observed very weak fluorescence when sensors 9-12 were dissolved in aqueous phosphate-buffered saline (PBS). The fluorescence was, however significantly amplified upon the addition of hCA II to the solution. They observed 15-fold enhancement of the fluorescence response in the case of the sensor 9 associated with a blue-shift of the emission maximum by 39 nm from 589 nm upon the addition of the sensor. Importantly, authors showed that the probes presented provided an exceptional selectivity for hCA II over other proteins.

![Figure 2.7](image.png)

**Figure 2.7.** Environmentally-sensitive fluorescent sensors for hCA II.
The utilization of CAs in supramolecular sensing has been pioneered by Hamachi and coworkers. They recently demonstrated a novel promising strategy utilizing on/off fluorescent probes for CAs sensing. They presented synthetic ligands tethering hydrophobic linkers with fluorescent probes.\textsuperscript{127} In aqueous buffer media, these amphiphilic conjugate molecules self-assemble into aggregates (nanoprobes) providing a weak or no fluorescence signal due to self-quenching. The dissociation of the aggregates is then mediated by protein recognition event leading to the emission of bright fluorescence. Hamachi and coworkers chose human carbonic anhydrase I (hCA I) as a model protein and designed probes 13 and 14 (Figure 2.8). The amphiphilic probes consisted of hydrophobic fluorophore (BODIPY or tetramethylrhodamine) tethered with benzensulfonamide acting as hCA I recognition moiety, through a hydrophobic linker. As expected, in aqueous HEPES buffer the authors observed extremely weak fluorescence ($\Phi_f = 0.1\%$). The emission intensity of 13, was however substantially amplified (38-times) upon the addition of hCA I ($\Phi_f = 4\%$). Interestingly, the authors used ethoxzolamide 4 as a competitive high-affinity inhibitor to demonstrate reversibility of the switching of the fluorescence response.

\textbf{Figure 2.8.} BODIPY- and tetramethylrhodamine-based self-assembling amphiphilic fluorescent probes used by Hamachi for hCA I.
After the addition, the inhibitor 4 competitively binds to the hCA I replacing probe 13 which in turn forms self-assembled aggregates resulting in almost complete quenching of fluorescence signal. Importantly, the authors showed a very high specificity of 13 for hCA I. This was demonstrated in the mixture of bovine serum albumin, hemoglobin, concanavalin A, and chymotrypsin. Only the presence of hCA I in the mixture induced turn-on fluorescence response. The authors also demonstrated that the hydrophobic BODIPY-based probe 13 providing a dramatic difference in intensity between on/off states was more suitable as a fluorophore rather than charged probe 14. Hamachi and coworkers very recently presented the novel sensing mechanism involving disassembly-driven fluorescence turn-on nanoprobes for specific CAs detection of intracellular proteins and cell-surface proteins under live cell conditions.128,129

The first attempt to use fluorescence in order to determine Zn²⁺ using CAs as a recognition element was presented by Chen and Kernohan in 1967.130 They noticed that the fluorescence emission of dansylamide 15 (Figure 2.9) is substantially enhanced and blue-shifted upon its binding to holo-CA. In 1993, Thompson and Jones reported that dansylamide shows a negligible affinity for apo-CA hence the fluorescence response in the mixture of CA and dansylamide comes exclusively from dansylamide complexed with holo-CA. Therefore, this approach could be used

![Figure 2.9](image)

Figure 2.9. Examples of fluorescent dyes used for CA-based biosensors for metal ions: Dansylamide (15) used by Chen, Thompson and Jones. Dapoxyl® Sulfonamide (16) used as a donor and Alexa Fluor 594 specifically labeled fluorescent variant of apo-CA II (17) used as an acceptor by Thompson and coworkers in FRET ratiometric Zn²⁺ sensing scheme.
to determine concentration of free $\text{Zn}^{2+}$ in the solution.\textsuperscript{131} CA-based fluorescence biosensors for metals were further optimized for accurate quantitation of free zinc in biological samples (neuronal zinc) using ratiometric indicators, lifetime- and polarization-based methods,\textsuperscript{21,132–134} and Förster resonance energy transfer (FRET).\textsuperscript{135} Fierke and coworkers also developed fluorescent biosensors where CAs serve as the recognition element to detect metal ions. They found that various metal ions induce changes in the fluorescence lifetime and/or fluorescence intensity of specifically labeled fluorescent variant of $apo$-$\text{CA II}$ and these changes in spectral properties of the dye can be used to determine $\text{Zn}^{2+}$, $\text{Mn}^{2+}$, $\text{Ca}^{2+}$, $\text{Mg}^{2+}$, $\text{Fe}^{2+}$, $\text{Cu}^{2+}$, $\text{Co}^{2+}$, $\text{Cd}^{2+}$, $\text{Hg}^{2+}$, and $\text{Ni}^{2+}$ ions.\textsuperscript{21} The authors described that such biosensors demonstrate picomolar sensitivity, unprecedented selectivity, ratiometric fluorescence response, and near diffusion-controlled response times.\textsuperscript{136}

### 2.5 Fluorescence-Based Assays for Carbonic Anhydrase Inhibitors

Several examples of systems utilizing proteins in array-based fluorescence sensing has been reported. Rotello and coworkers demonstrated versatility of fluorescence-based arrays to successfully distinguish a wide range of protein targets using pattern recognition techniques. They investigated fluorescence-based arrays approach using water soluble conjugated polymers,\textsuperscript{137} and nanoparticles.\textsuperscript{138,139} In 1996, Brogan and coworkers suggested to use myoglobin plus carbonic anhydrase (hCA III) assay for early diagnosis of acute myocardial infarction since hCA III is released from tissues upon muscular damage. This assay using $\text{Eu}^{3+}$-labeled hCA III reportedly provided improved specificity compared to then-established assays based on creatine kinase-MB.\textsuperscript{140} To the best of our knowledge there has not been any fluorescence-based array utilizing CAs for the analysis of CAIs.
Established and widely used highly specific sensing protocols (e.g., ELISA) require utilization of expensive and protein-specific high-affinity receptors. In our approach, we decided to exploit the direct correlation between inhibitor affinity toward the Zn(II) center with enzyme inhibition and utilize a supramolecular approach employing an indicator-displacement assay, in which a sulfonamide ligand (L) is attached to a fluorescent dye (F). The function of the fluorophore (F) is to signal the binding event. Such a sensor is bound by the CA with associated change in sensor fluorescence. In the presence of a CA-binding inhibitor (I), the sensor is displaced from the enzyme while the original fluorescence is recovered. The principle of this supramolecular assay is shown in the scheme below (Figure 2.10). Furthermore, we decided to use this simple principle in a high-throughput screening settings in order to investigate a relationship between the change in fluorescence intensity of the sensors and the affinity of various CAI to CAs. We believe that this principle is general, and could be used whenever an enzyme binds a ligand that can be conjugated to a dye. We decided to use a supramolecular approach based on an indicator-displacement assay.

2.6 Carbonic Anhydrase Sensors: Design and Synthesis

We synthesized sensors S1-S7 (Schemes 2.1-2.8) featuring 1,3,4-thiadiazol-2-sulfonamide moiety, known to possess very high binding affinity for zinc ion embedded in the binding pocket.
of carbonic anhydrases. This high-affinity moiety was conjugated with four different fluorophores: coumarin-, dansyl-, pyrene- and sulforhodamine-based dyes. The dyes were selected so that the emission signals provided are distinct, known and well characterized. One type of indicator was prepared by direct coupling of the 2-amino-1,3,4-thiadiazole-5-sulfonamide with a dye while the other type contains a seven or ten atoms long spacer linking the ligand with fluorophore.

Commercially available acetazolamide was first deacetylated by hydrochloric acid to yield 2-amino-1,3,4-thiadiazole-5-sulfonamide obtained as hydrochloride which was subsequently

![Figure 2.11. Fluorescent high-affinity sensors used in this study.](image)
reacted with the acyl- or sulfonylchloride derivatives of the corresponding fluorophore (3-chlorocarbonyl-7-methoxycoumarin, dansyl chloride, and sulforhodamine sulfonyl chloride, respectively) to yield indicators S1, S3, S7 (Figure 2.11). For the synthesis of the longer-spacer indicators S2, S4 and S5, 5-amino-1,3,4-thiadiazole-2-sulfonamide was first reacted with succinic anhydride to provide the extended carboxylic acid terminated chain (Scheme 2.1). The corresponding fluorophore acyl- or sulfonylchloride derivatives were first reacted with tert-butoxycarbonyl mono-protected ethylenediamine, which were after deprotection with trifluoroacetic acid used in the amide coupling reaction with the succinate using EDC/DMAP to yield the extended sensors S2, S4 and S5. Sensor S6 was synthesized by amide coupling of the aminothiadiazole succinylchloride with pyrene methylamine. Overall reaction yields for the sensors S1-S7 were relatively low mainly due to high purity required for fluorescence bioassays.

2.6.1 Materials and Methods

Starting materials and all reagents were obtained from commercial suppliers and used without further purification unless stated otherwise. Organic solvents were routinely dried and/or distilled prior to use and stored over molecular sieves under argon or nitrogen atmosphere. Anhydrous solvents were obtained from HPLC grade dichloromethane, tetrahydrofuran (THF) and acetonitrile purchased from EMD Millipore Corp. (Billerica, MA) by additional purification using solvent purification system (MBraun) under nitrogen atmosphere. Methanol, N,N-dimethylformamide (DMF), diethyl ether, chloroform, hexanes and ethyl acetate were purchased from EMD Millipore Corp. (Billerica, MA) and used as received unless stated otherwise. Anhydrous dimethyl sulfoxide (DMSO), DMF, N,N-diisopropylethyl amine (DIPEA) and triethylamine were prepared from solvents purchased from Alfa Aesar (Ward Hill, MA) by
further treatment with activated 4Å molecular sieves. A reverse osmosis purified water (> 17 MΩ cm) was used throughout the experiments involving proteins. Synthesis yields refer to pure isolated substances. 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC, CAS: 1892-57-5) was purchased from Aapptec (Louisville, KY). Oxalyl chloride and 4-dimethylaminopyridine (DMAP, CAS: 1122-58-3) were purchased from Alfa Aesar (Ward Hill, MA). Dansyl chloride [5-(N,N-dimethylamino)naphthalene-1-sulfonyl chloride, CAS: 605-65-2] and trifluoroacetic acid were purchased from TCI America (Portland, OR). Acetazolamide (5-acetamido-1,3,4-thiadiazole-2-sulfonamide, CAS: 59-66-5), methanesulfonamide (CAS: 3144-09-0), p-nitrobenzene sulfonamide (CAS: 6325-93-5) and 4-phenoxybenzene sulfonamide (CAS: 123045-62-5) were purchased from Alfa Aesar (Ward Hill, MA). Celecoxib {4-[5-(p-tolyl)-3-(trifluoromethyl)-1H-pyrazol-1-yl]benzenesulfonamide, CAS: 169590-42-5} was purchased from Matrix Scientific (Columbia, SC), o-toluenesulfonamide (CAS: 88-19-7) and p-toluenesulfonamide (CAS: 70-55-3), thiophene-2-sulfonamide (CAS: 6339-87-3) and brinzolamide (CAS: 138-890-62-7) were purchased from TCI (Tokyo, Japan), naphthalene-2-sulfonamide (CAS: 1576-47-2), ethoxzolamide (6-ethoxy-2-benzothiazolesulfonamide, CAS: 452-35-7) and 1-pyrenemethylamine hydrochloride (CAS: 93324-65-3) were purchased from Sigma-Aldrich (St. Louis, MO), tert-butylsulfonamide (CAS: 34813-49-5) was purchased from Oakwood Chemical (West Columbia, SC) and 4-(tert-butyl)benzensulfonamide (CAS: 6292-59-7) from Ark Pharm, Inc. (Libertyville, IL). Methazolamide (CAS: 554-57-4) was purchased from Chem-Impex Int’l Inc. (Wood Dale, IL). 3-Carboxy-7-methoxycoumarin (20, 7-methoxy-2-oxo-2H-chromene-3-carboxylic acid, CAS: 20300-59-8) was purchased from Combi-Blocks (San Diego, CA). Lissamine™ rhodamine B sulfonyl chloride, mixed isomers (28, CAS: 62796-29-6) was purchased from Life Technologies (Grand Island, NY). Carbonic anhydrase isozyme II from
bovine erythrocytes (bCA II, cat. no. C2522-5MG, lot no. SLBG6651V) and carbonic anhydrase isozyme II from human erythrocytes (hCA II, cat. no. C6165-1MG, lot no. SLBJ4019V and SLBD9637) were purchased as lyophilisates from Sigma-Aldrich (St. Louis, MO). Recombinant human carbonic anhydrase isozyme III (polyhistide tag at the C-terminus) (hCA III) was purchased from Sino Biological (Beijing, PRC, cat. no. 10503-H08E, lot no. LC05MC2110) and obtained as lyophilisate from sterile 50 mM Tris, 500 mM NaCl and 10% glycerol. Recombinant human carbonic anhydrase isozyme I (E. coli) (hCA I) was purchased from Jena Bioscience (Jena, Germany, cat. no. PR-1001, lot no. 1001001) as PBS and NaN₃ (0.05 M) buffers solution (concentration of protein as delivered 0.85 mg/mL). Human plasma albumin (HSA) (cat. no. 16-16-011202, lot no. AL2010-02) was purchased as a lyophilisate from deionized water from Athens Research & Technology, Inc. (Athens, GA). HEPES buffer aqueous solutions were prepared from HEPES free acid (CAS: 7365-45-9) purchased from Amresco (Solon, OH). pH values of the buffer solutions (pH 6.5, 7.2, and 8.0) were adjusted by addition of sodium hydroxide (6 M) and/or hydrochloric acid (6 M) using SevenMulti pH meter with glass electrode (both by Mettler Toledo, Columbus, OH). The buffer solutions were then used to prepare protein stock solutions (1 mg/mL) and these were kept in a freezer at –20 °C and used to prepare desired aliquots. Column chromatography was carried out using commercially available bulk flash silica gel 32-63u (Dynamic Adsorbents Inc., Norcross, GA). Preparative thin-layer chromatography (TLC) was carried out on the Uniplate preparative silica gel TLC plates 2 mm, 20 × 20 cm with F₂₅⁴ fluorescent dye (Analtech Inc., Newark, DE). Analytical thin-layer chromatography was performed using commercial glass-backed pre-coated silica gel plates (0.25 mm) with F₂₅⁴ fluorescent dye (Sorbent Technologies, Norcross, GA). Tert-butyl (2-aminoethyl)carbamate,¹⁴³ (CAS: 57260-73-8), 5-amino-1,3,4-thiadiazole-2-sulfonamide hydrochloride,¹⁴⁴ (18, CAS: 14949-
tert-butyl (2-[[5-(dimethylamino)naphthalene]-1-sulfonamido]ethyl)carbamate,145 (25, CAS: 160291-44-1) and 2-[[5-(dimethylamino)naphthalene]-1-sulfonamido]ethan-1-aminium 2,2,2-trifluoroacetate,145 (26, CAS: 1266664-72-5) were prepared according to reported protocols.

2.6.2 Synthesis Schemes

Scheme 2.1. Synthesis of intermediate 19: a) aq. HCl (1M), reflux, 3 h, 98%; b) succinic anhydride, DMF, 50 °C, 20 h, 20%.

Scheme 2.2. Synthesis of sensor S1: a) (COCl)₂, DMF (cat.), DCM, 0 °C to r.t., 5 h, quant.; b) 18, Et₃N, THF, 0 °C to r.t., 3 h, 11%.

Scheme 2.3. Synthesis of sensor S2: a) tert-butyl (2-aminoethyl)carbamate, Et₃N (anhydr.), DCM, 0 °C to r.t., 3 h, 34%; b) TFA, DCM, 0 °C to r.t., 20 h, 97%; c) 19, EDC, DMAP, DIPEA, THF/MeCN/DMF, 0 °C to r.t., 12 h, 14%.
Scheme 2.4. Synthesis of sensor S3: a) 18, Na$_2$CO$_3$, acetone / water, 0 °C to r.t., 24 h, 15%.

Scheme 2.5. Synthesis of sensor S4: a) tert-butyl (2-aminoethyl)carbamate, DIPEA (anhydr.), DCM, 0 °C to r.t., 12 h, 90%; b) TFA, DCM, 0 °C to r.t., 21 h, 72%; c) 19, EDC, DMAP, DIPEA, THF/MeCN/DMF, r.t., 20 h, 14%.
Scheme 2.6. Synthesis of sensor S5: a) (COCl)$_2$, DMF (cat.), DCM, 0 °C to r.t., 11 h, quant.; b) tert-butyl (2-aminoethyl)carbamate, Et$_3$N (anhydr.), DCM, 0 °C to r.t., 12 h, 12%; c) TFA, DCM, 0 °C to r.t., 4 h, 78%; d) 19, EDC, DMAP, DIPEA, DMF, 0 °C to r.t., 20 h, 48%.

Scheme 2.7. Synthesis of sensor S6: a) (COCl)$_2$, DMF (cat.), THF, 0 °C to r.t., 2 h, quant.; b) 1-pyrenemethylamine, DIPEA, DCM/THF, 0 °C to r.t., 12 h, 21%.
Scheme 2.8. Synthesis of sensor S7: a) 18, pyridine, r.t., 60 h, 3%.

2.6.3 Spectroscopy

Proton NMR (\textsuperscript{1}H-NMR) and carbon-13 NMR (\textsuperscript{13}C-NMR) spectra were recorded on Bruker Avance III spectrometer at 500 MHz or 125 MHz, respectively at 25 °C. Proton and carbon NMR chemical shifts (\(\delta\)) are reported in parts per million (ppm) relative to residual solvent signals in CDCl\textsubscript{3} (\(\delta = 7.26, 77.16\)) or in DMSO-\(d_6\) (\(\delta = 2.50, 39.52\)). Coupling constants (\(J\)) are reported in hertz (Hz) and refer to apparent multiplicities. The following abbreviations are used for the multiplicities: s (singlet), d (doublet), t (triplet), q (quartet), p (pentet), m (multiplet), br (broad).

High-resolution mass spectra (HRMS) were obtained on Shimadzu AXIMA Performance MALDI TOF mass spectrometer in reflectron mode using \(\alpha\)-cyano-4-hydroxycinnamic acid (CAS: 28166-41-8) as a matrix.\textsuperscript{146} The emission spectra were recorded between 385 nm and 550 nm for S1, 400 nm and 750 nm for S3, and 360 nm and 500 nm for S6, respectively, using appropriate optical cut-off filters placed in an emission channel where necessary. The emission from probes was scanned in 1 nm step with appropriate excitation and emission monochromators band pass settings with dwell time 0.30 sec. Scans were taken under laboratory temperature. Fluorescence guest and competitive titrations were performed in non-degassed aqueous HEPES buffer (50 mM) at pH 6.5,
7.2, and 8.0 at laboratory temperature. Titration isotherms were constructed from changes in the fluorescence maximum at 420 nm for **S1**, 508 nm for **S3**, and 392 nm for **S6**, respectively.

### 2.6.4 Synthesis Protocols

5-Amino-1,3,4-thiadiazole-2-sulfonamide hydrochloride (**18**). Commercially available acetazolamide **1** (25.46 g, 14.2 mmol) was refluxed in 1M hydrochloric acid (73 mL, 74 mmol, 5.2 mol. equiv.) for 6 h. The solvents were then evaporated on a rotary evaporator and the residue was chromatographed on silica gel (chloroform/methanol = 8 : 2 v/v) to yield product **18** as a pale yellow powder (24.82 g, 98%); *R*<sub>f</sub> = 0.24 (chloroform/methanol = 8 : 2 v/v). The spectral data obtained are in an agreement with previously reported data.\(^{144}\)

Triethylammonium 4-oxo-4-[(5-sulfamoyl-1,3,4-thiadiazol-2-yl)amino]butanoate (**19**). Compound **18** (1.03 g, 5.72 mmol) was reacted with succinic anhydride (1.12 g, 11.21 mmol) in anhydrous DMF (20 mL) at 50 °C for 20 h. The solvent was then evaporated on a rotary evaporator and the residue was chromatographed on silica gel (gradient chloroform 100% to chloroform/methanol/triethylamine = 70 : 30 : 0.1 v/v) to yield product **19** as a pale yellow hygroscopic foam (491 mg, 23%); *R*<sub>f</sub> = 0.16 (chloroform/methanol/triethylamine = 70 : 30 : 0.1 v/v). The spectral data obtained are in an agreement with previously reported data.\(^{147}\)

7-Methoxy-2-oxo-2*-H*-chromene-3-carbonyl chloride (**21**). The solution of 3-carboxy-7-methoxycoumarin **20** (206 mg, 0.94 mmol) in anhydrous DCM (15 mL) and anhydrous DMF (20 μL, 0.25 mmol) was cooled down in an ice-water bath to 0 °C and the solution of oxalyl chloride (90 μL, 1.06 mmol) in anhydrous DCM (1 mL) was added drop-wise over 5 min upon stirring.
under nitrogen atmosphere. The mixture was allowed to warm up to room temperature and the solvents were evaporated on rotary evaporator after 5 h and the residual solvents removed in vacuo. Acylchloride 21 was isolated as a pale yellow solid (215 mg, 96%) and was directly used without further purification in next step; \( R_f = 0.37 \) (AcOEt/hexanes = 1 : 1 v/v).

7-Methoxy-2-oxo-N-(5-sulfamoyl-1,3,4-thiadiazol-2-yl)-2H-chromene-3-carboxamide (S1). The solution of compound 18 (434 mg, 2.0 mmol) in anhydrous THF (70 mL) and anhydrous triethylamine (510 μL, 3.66 mmol) was cooled down in an ice-water bath to 0 °C and the solution of crude acyl chloride 21 (437 mg, 1.83 mmol) in anhydrous THF (30 mL) was added drop-wise over 5 min upon stirring under nitrogen atmosphere. The mixture was allowed to warm up to room temperature and after 3 h the solvents were evaporated on rotary evaporator and the residue was chromatographed on silica gel (chloroform/methanol = 9 : 1 v/v). Pure product S1 was obtained by precipitation from chloroform/pentane mixture as a light yellow powder (87 mg, 11%); \( R_f = 0.32 \) (chloroform/methanol = 9 : 1 v/v). Spectral data for S1: \(^1\)H-NMR (DMSO-\(d_6\), 500 MHz): \( \delta = 12.54 \) (br s, 1H, NHCO), 9.06 (s, 1H, ArH), 8.43 (s, 2H, SO\(_2\)NH\(_2\)), 7.99 (d, \( J = 8.8 \) Hz, 1H, ArH), 7.22 (d, \( J = 2.3 \) Hz, 1H, ArH), 7.13 (dd, \( J = 8.8, 2.4 \) Hz, 1H, ArH), 3.94 (s, 3H, ArOC\(_3\)H). \(^{13}\)C-APT (DMSO-\(d_6\), 125 MHz): \( \delta = 165.65, 165.23, 161.13, 160.58, 160.39, 156.82, 150.05, 132.32, 114.36, 112.71, 112.05, 100.67, 56.56 \) ppm. HRMS (MALDI) \( m/z \) found 383.0265 [M+H]; calcd 383.0115 for C\(_{13}\)H\(_{11}\)N\(_4\)O\(_6\)S\(_2\)+.

Tert-butyl [2-(7-methoxy-2-oxo-2H-chromene-3-carboxamido)ethyl]carbamate (22). The solution of compound 21 (547 mg, 2.3 mmol) in anhydrous DCM (30 mL) and anhydrous triethylamine (630 μL, 4.5 mmol) was cooled down in an ice-water bath to 0 °C and the solution of tert-butyl
(2-aminoethyl)carbamate (60 mg, 0.25 mmol) in anhydrous DCM (10 mL) was added drop-wise over 10 min upon stirring under nitrogen atmosphere. The mixture was allowed to warm up and stirred for 3 h at room temperature. The reaction mixture was then diluted with DCM (100 mL) and washed with saturated sodium bicarbonate solution (4 × 50 mL). Organic layer was then dried over sodium sulfate, filtered and organic solvents evaporated on rotary evaporator. The residue was dissolved in DCM and the product 22 was isolated by precipitation from the solution by addition of ethyl acetate as a pale solid (284 mg, 34%); \(R_f = 0.16\) (hexanes/ethyl acetate= 1 : 1 v/v). The spectral data obtained are in an agreement with previously reported data.\(^{148}\)

2-(7-Methoxy-2-oxo-2\(^{H}\)-chromene-3-carboxamido)ethan-1-aminium 2,2,2-trifluoroacetate (23). Trifluoroacetic acid (6 mL, 78 mmol) was added to the solution of 22 (111 mg, 0.24 mmol) in DCM (9 mL) at 0 °C and the mixture was allowed warm up to room temperature over 20 h. Organic solvents were then evaporated on rotary evaporator and the residue dried on vacuum line. The product 23 was isolated as a pale yellow solid (173 mg, 97%); \(R_f = 0.21\) (chloroform/methanol/ammonium hydroxide = 90 : 10 : 1 v/v). The spectral data obtained are in an agreement with previously reported data.\(^{148}\)

\(\text{N}^1\)-(2-(7-Methoxy-2-oxo-2\(^{H}\)-chromene-3-carboxamido)ethyl)-\text{N}^4\)-(5-sulfamoyl-1,3,4-thiadiazol-2-yl)succinamide (S2). 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (84 mg, 0.45 mmol) and 4-(N,N-dimethylamino)pyridine (41 mg, 0.34 mmol) were added to the solution of compound 19 (137 mg, 0.34 mmol) in a mixture of anhydrous THF (10 mL), anhydrous acetonitrile (15 mL) and anhydrous DMF (10 mL) and the resulting solution was stirred for 1 h at room temperature under nitrogen atmosphere. A solution of compound 23 (173 mg, 0.45 mmol)
in anhydrous THF (5 mL) and anhydrous DIPEA (180 μL, 0.93 mmol) was added drop-wise over 10 min upon stirring under nitrogen atmosphere. The mixture was allowed to warm up and stirred for 12 h at room temperature. The organic solvents were then evaporated by rotary evaporator and the residue was chromatographed on silica gel (gradient form chloroform to chloroform/methanol/ammonium hydroxide = 70 : 30 : 3 v/v) and product S2 was isolated by precipitation from methanol/water mixture as a white powder (26 mg, 14%); \( R_f = 0.35 \) (chloroform/methanol/ammonium hydroxide = 70 : 30 : 3 v/v). Spectral data for S2: \(^1\)H-NMR (DMSO-\(d_6\), 500 MHz): \( \delta = 13.02 \) (br s, 1H, N\( \text{H} \)CO), 8.84 (s, 1H, Ar\( \text{H} \)), 8.72 (t, \( J = 5.7 \) Hz, 1H, CON\( \text{H} \)), 8.31 (br s, 2H, SO\(_2\text{NH}_2\)), 8.08 (t, \( J = 5.3 \) Hz, 1H, CON\( \text{H} \)), 7.91 (d, \( J = 8.7 \) Hz, 1H, Ar\( \text{H} \)), 7.12 (d, \( J = 2.2 \) Hz, 1H, Ar\( \text{H} \)), 7.05 (dd, \( J = 8.7, 2.3 \) Hz, 1H, Ar\( \text{H} \)), 3.90 (s, 3H, OC\( \text{H}_3 \)), 3.08-2.90 (m, 2H, C\( \text{H}_2 \)). \(^{13}\)C-NMR (DMSO-\(d_6\), 125 MHz): \( \delta = 171.79, 170.96, 164.44, 164.22, 161.73, 161.12, 160.71, 156.20, 147.88, 131.59, 114.81, 113.69, 112.12, 100.30, 56.28, 38.32, 30.73, 30.26, 29.44 \) ppm. HRMS (MALDI+) \( m/z \) found 547.0508 [M+Na]\(^+\); calcd 547.0682 for C\(_{19}\)H\(_{20}\)N\(_6\)O\(_8\)S\(_2\)Na\(^+\).

5-[[5-(Dimethylamino)naphthalene]-1-sulfonamido]-1,3,4-thiadiazole-2-sulfonamide (S3). The solution of dansyl chloride (875 mg, 3.23 mmol) in acetone (40 mL) was added over 30 min upon stirring to the solution of compound 18 (500 mg, 2.31 mmol) and sodium carbonate (736 mg, 6.92 mmol) in water (40 mL) in a round bottom flask cooled in ice-water bath to 0 °C. The mixture was allowed to warm up to room temperature and then stirred for 24 h. The reaction mixture was then concentrated by rotary evaporator and the residue poured into brine (350 mL). The mixture was then extracted with ethyl-acetate (4 × 150 mL) and combined organic layers washed with brine (3 × 150 mL) and dried with Na\(_2\)SO\(_4\). Na\(_2\)SO\(_4\) was then filtered off, solvents evaporated on rotary
evaporator and the residue chromatographed on silica gel (ethyl acetate → ethyl acetate/methanol = 9 : 1 v/v). The fractions containing the product were combined and the solvents evaporated on rotary evaporator. Pure product S3 was isolated by precipitation from acetone/pentane mixture as a yellow powder (144 mg, 15%); \( R_f = 0.11 \) (ethyl acetate/methanol = 9 : 1 v/v). Spectral data for S3: 

1H-NMR (DMSO-\( d_6 \), 500 MHz): \( \delta = 8.40 \) (d, \( J = 8.7 \) Hz, 1H, ArH), 8.30 (d, \( J = 8.5 \) Hz, 1H, ArH), 7.98 (dd, \( J = 7.2 \), 1.1 Hz, 1H, ArH), 7.48 (ddd, \( J = 13.2 \), 8.6, 7.4 Hz, 2H, ArH), 7.39 (s, 2H, SO\( _2 \)N\( H_2 \)), 7.18 (dd, \( J = 7.5 \), 0.7 Hz, 1H, ArH), 2.81 (s, 6H, N(CH\( 3 \))\( 2 \)). 13C-NMR (DMSO-\( d_6 \), 125 MHz): \( \delta = 170.86, 160.88, 150.75, 141.49, 129.55, 128.89, 127.38, 126.43, 123.33, 121.23, 114.41, 45.17, 30.75 \) ppm. HRMS (MALDI+) \( m/z \) found 436.0233 [M+Na]+; calcd 436.0184 for C\(_{14}\)H\(_{15}\)N\(_5\)O\(_4\)S\(_3\)Na\(^+\).

**Tert-butyl (2-\{[5-(dimethylamino)naphthalene]-1-sulfonamido}ethyl)carbamate (25).** The solution of tert-butyl (2-aminoethyl)carbamate (51 mg, 0.3 mmol) and DIPEA (80 μL, 0.5 mmol) in anhydrous DCM (2 mL) was cooled down to 0 °C in an ice-water bath and the solution of compound 24 (126 mg, 0.5 mmol) in anhydrous DCM (2 mL) was added dropwise over 5 min upon stirring under nitrogen atmosphere and the mixture was allowed to warm up and then stirred overnight. The reaction mixture was then diluted with DCM (30 mL) and extracted with water (3 × 15 mL) and combined organic layers dried with sodium sulfate. Organic solvents were then evaporated by rotary evaporator and the residue was chromatographed on silica gel (hexanes/ethyl acetate = 7 : 3 v/v) to yield product 25 as a pale yellow foam (105 mg, 90%); \( R_f = 0.15 \) (hexanes/ethyl acetate = 7 : 3 v/v). The spectral data obtained are in agreement with previously reported data.\(^{145}\)
2-\{5-(Dimethylamino)naphthalene\}-1-sulfonamido\}ethan-1-aminium 2,2,2-trifluoroacetate (26).

The solution of compound 25 (52 mg, 0.1 mmol) in DCM (1.5 mL) was cooled down to 0 °C in an ice-water bath and the solution of trifluoroacetic acid (0.1 mL, 1.29 mmol) in DCM (0.5 mL) was added dropwise over 30 min upon stirring and the mixture was allowed to warm up and then stirred overnight. The organic solvents were then evaporated on rotary evaporator and the residue dried in vacuo to yield product 26 as a pale brown oil (39 mg, 72 %). The spectral data obtained are in agreement with previously reported data.145

\[\text{N}_{1}\text{-}(2-\{5-(Dimethylamino)naphthalene\}-1-sulfonamido\}\text{ethyl})\text{-N}_{4}\text{-}(5-sulfamoyl-1,3,4-thiadiazol-2-yl)succinamide (S4).}\]

DMAP (40 mg, 0.3 mmol) and EDC (73 mg, 0.4 mmol) were added to the solution of compound 19 (87 mg, 0.2 mmol) in anhydrous THF/MeCN/DMF (1 : 1 : 1 v/v) mixture (10 mL) and the resulting solution was stirred for 30 min at room temperature. The solution of compound 26 (162 mg, 0.4 mmol) and anhydrous DIPEA (170 μL, 1 mmol) in anhydrous THF/MeCN/DMF (1 : 1 : 1 v/v) mixture (10 mL) was added dropwise and the reaction mixture was stirred overnight at room temperature. The reaction mixture was then concentrated on a rotary evaporator and the residue was chromatographed on silica gel (chloroform/methanol = 9 : 1 v/v) to yield product S4 as a white powder (25 mg, 14%); \(R_t = 0.15\) (chloroform/methanol = 9 : 1 v/v). Spectral data for S4: \(^1\)H-NMR (DMSO-\text{d}_6, 500 MHz): \(\delta = 13.00\) (s, 1H, CONH), 8.46 (d, \(J = 8.5\) Hz, 1H, ArH), 8.32 (br s, 2H, SO\text{2NH}_2), 8.26 (d, \(J = 8.7\) Hz, 1H, ArH), 8.09 (dd, \(J = 7.3, 1.1\) Hz, 1H, ArH), 7.98 (t, \(J = 5.9\) Hz, 1H, NH), 7.89 (t, \(J = 5.7\) Hz, 1H, NH), 7.61 (ddd, \(J = 14.5, 8.5, 7.5\) Hz, 2H, ArH), 7.25 (d, \(J = 7.5\) Hz, 1H, ArH), 3.03 (dd, \(J = 13.3, 6.3\) Hz, 2H, CH\text{2}), 2.82 (s, 6H, N(CH\text{3})\text{2}), 2.78 (dd, \(J = 13.4, 6.4\) Hz, 2H, CH\text{2}), 2.67 (t, \(J = 6.9\) Hz, 2H, CH\text{2}), 2.36 (t, \(J = 6.9\) Hz, 2H, CH\text{2}). \(^{13}\)C-NMR (DMSO-\text{d}_6, 125 MHz): \(\delta = 171.74, 170.85, 164.21, 161.10, 151.40,\)
HRMS (MALDI) \( m/z \) found 578.0889 \([\text{M+Na}]^+\); calcd 578.0926 for \( \text{C}_{20}\text{H}_{25}\text{N}_{7}\text{O}_{6}\text{S}_{3}\text{Na}^+ \).

5-(\(N\)-{2-[((\text{Tert}-butoxycarbonyl)amino)ethyl]sulfamoyl})-2-[6-(diethylamino)-3-(diethyliminio)-3\(H\)-xanthen-9-yl]benzenesulfonate (29). The solution of compound 28 (1.105 g, 1.3 mmol) in anhydrous DCM (150 mL) was cooled down in an ice-water bath to 0 °C and the solution of tert-buty1 (2-aminoethyl)carbamate (151 mg, 0.9 mmol) and anhydrous triethylamine (372 μL, 2.68 mmol) in anhydrous DCM (50 mL) were added dropwise to the solution over 1 h upon stirring under nitrogen atmosphere. The resulting mixture was allowed to warm up to room temperature and stirred overnight. The mixture was then diluted with DCM (100 mL) and washed with concentrated sodium carbonate (3 × 50 mL) and the organic layer dried with sodium sulfate. Organic solvents were then evaporated by rotary evaporator and the residue was chromatographed on silica gel (dichloromethane/methanol = 92 : 8 \(v/v\)) to yield product 29 as a dark purple solid (45 mg, 4%); \( R_f = 0.28 \) (dichloromethane/methanol = 92 : 8 \(v/v\)). The spectral data obtained are in agreement with previously reported data.149

5-[\(N\)-(2-Aminoethyl)sulfamoyl]-2-[6-(diethylamino)-3-(diethyliminio)-3\(H\)-xanthen-9-yl]benzenesulfonate (30). The solution of compound 29 (45 mg, 64 μmol) in DCM (7 mL) was cooled down in an ice-water bath to 0 °C and the solution of trifluoroacetic acid (5 mL, 65 mmol) in DCM (3 mL) was added dropwise to the solution. The resulting mixture was allowed to warm up to room temperature and stirred for 4 h. The mixture was then diluted with DCM (50 mL) and washed with concentrated sodium carbonate (3 × 50 mL) and the organic layer dried with sodium sulfate.
sulfate. Organic solvents were then evaporated by rotary evaporator and the residue was chromatographed on silica gel (dichloromethane/methanol/ammonium hydroxide = 90 : 8 : 2 v/v → dichloromethane/methanol/ammonium hydroxide = 83 : 15 : 2 v/v) to yield product 30 as a dark purple solid (30 mg, 78%); $R_f = 0.21$ (dichloromethane/methanol/ammonium hydroxide = 80 : 20 : 0.2 v/v). The spectral data obtained are in agreement with previously reported data.149

2-[6-(diethylamino)-3-(diethyliminio)-3\textit{H}-xanthen-9-yl]-5-\[N-(2-{4-oxo-4-[(5-sulfamoyl-1,3,4-thiadiazol-2-yl)amino]butanamido}ethyl)sulfamoyl]benzenesulfonate (\textbf{S5}). DMAP (6.4 mg, 52 μmol) and EDC (9.4 mg, 48 μmol) were added to the solution of compound 19 (16 mg, 42 μmol) in anhydrous DMF (6 mL) and the resulting solution was cooled down in an ice-water bath to 0 °C and stirred for 1 h. Then the solution of compound 30 (22 mg, 37 μmol) and anhydrous DIPEA (50 μL, 275 μmol) in anhydrous DMF (2 mL) was added dropwise and the reaction mixture was stirred overnight at room temperature. The reaction mixture was then concentrated on a rotary evaporator and the residue was chromatographed on silica gel (chloroform → chloroform/methanol/ammonium hydroxide = 7 : 3 : 0.3 v/v) to yield product \textbf{S5} as a dark purple solid (15 mg, 48%); $R_f = 0.31$ (chloroform/methanol/ammonium hydroxide = 7 : 3 : 0.3 v/v). Spectral data for \textbf{S5}: $^1\text{H}$-NMR (DMSO-$d_6$, 500 MHz): $\delta = 8.41$ (d, $J = 1.9$ Hz, 1H, ArH), 8.03 (br s, 1H, NH), 7.97 (t, $J = 5.7$ Hz, 1H, NHH), 7.93 (dd, $J = 7.9$, 1.9 Hz, 1H, ArH), 7.48 (d, $J = 8.0$ Hz, 1H, ArH), 7.05 (dd, $J = 9.6$, 2.3 Hz, 2H, ArH), 6.98 (d, $J = 9.5$ Hz, 2H, ArH), 6.94 (d, $J = 2.3$ Hz, 2H, ArH), 3.68-3.59 (m, 8H, CH$_2$CH$_3$), 3.13 (dd, $J = 13.0$, 6.4 Hz, 2H, CH$_2$), 2.89 (br s, 2H, CH$_2$), 2.41 (t, $J = 6.9$ Hz, 2H, CH$_2$), 2.30 (t, $J = 7.0$ Hz, 2H, CH$_2$), 1.23-1.19 (m, $J = 13.7$, 6.7 Hz, 12H, CH$_2$CH$_3$). $^{13}$C-NMR (DMSO-$d_6$, 125 MHz): $\delta = 173.88$, 171.28, 157.41, 157.13, 155.04, 148.02, 141.39, 133.15, 132.75, 130.74, 129.67, 126.58, 125.68, 113.68, 113.48, 95.39, 69.80, 55.10,
45.27, 41.97, 38.60, 29.12, 12.48 ppm. MS (ESI-) m/z found 861.30 [(M-H)]−; calcld 861.18 for C_{35}H_{41}N_{8}O_{10}S_{4}−.

4-Oxo-4-[(5-sulfamoyl-1,3,4-thiadiazol-2-yl)amino]butanoyl chloride (31). The solution of compound 19 (53 mg, 0.14 mmol) in anhydrous THF (28 mL) and anhydrous DMF (10 μL, 0.13 mmol) was cooled down in an ice-water bath to 0 °C and the solution of oxalyl chloride (160 μL, 1.84 mmol) in anhydrous THF (2 mL) was added drop-wise over 5 min upon stirring under nitrogen atmosphere. The mixture was allowed to warm up to room temperature and after 2 h the solvents were evaporated by rotary evaporator and the residual solvents removed in vacuo. Acylchloride 31 was isolated as a yellow pale solid (42 mg, quant.) and directly used without further purification; \( R_f = 0.50 \) (dichloromethane/methanol = 85 : 15 v/v).

\( N^1-(\text{Pyren-1-ylmethyl})-N^4-(5\text{-sulfamoyl-1,3,4-thiadiazol-2-yl})\text{succinamide (S6).} \) Compound 31 (119 mg, 0.04 mmol) was dissolved in a mixture of anhydrous DCM/THF (1:1 v/v) (15 mL) upon sonication and cooled down in ice-water bath to 0 °C and upon stirring and the solution of 1-pyrenemethylamine hydrochloride (99 mg, 0.36 mmol) and anhydrous triethylamine (0.56 mL, 0.40 mmol) was added over 30 min under nitrogen atmosphere. The mixture was allowed to warm up to room temperature and stirred overnight. The reaction mixture was then concentrated by rotary evaporator and the residue poured into brine (300 mL). The mixture was then extracted with ethyl acetate (3 × 150 mL) and combined organic layers washed with brine (1 × 150 mL) and dried with Na_{2}SO_{4}. Na_{2}SO_{4} was then filtered off, solvents evaporated by rotary evaporator and the residue chromatographed on silica gel (ethyl acetate → ethyl acetate/methanol = 95 : 5 v/v). The fractions containing the product were combined and the solvents evaporated by rotary evaporator.
Pure product $S_6$ was isolated by precipitation from THF/pentane mixture as a white powder (39 mg, 21%); $R_f = 0.11$ (ethyl acetate/methanol = 9 : 1 $v/v$). Spectral data for $S_6$: $^1$H-NMR (DMSO-$d_6$, 500 MHz): $\delta =$ 13.08 (br s, 1H, NH), 8.68 (t, $J =$ 5.7 Hz, 1H, NH), 8.36-8.34 (m, 3H, ArH), 8.30 (dd, $J =$ 7.7, 2.4 Hz, 2H, ArH), 8.23 (dd, $J =$ 10.6, 8.6 Hz, 2H, ArH), 8.16 (s, 2H, ArH), 8.08 (t, $J =$ 7.6 Hz, 1H, ArH), 8.03 (d, $J =$ 7.8 Hz, 1H, ArH), 5.01 (d, $J =$ 5.7 Hz, 2H, NHCH$_2$), 2.83 (t, $J =$ 6.8 Hz, 2H, COCH$_2$), 2.61 (t, $J =$ 6.8 Hz, 2H, COCH$_2$). $^{13}$C-NMR (DMSO-$d_6$, 125 MHz): $\delta =$ 171.89, 170.69, 164.27, 161.16, 132.89, 130.81, 130.31, 130.11, 128.08, 127.58, 127.41, 127.05, 126.56, 126.30, 125.28, 125.19, 124.70, 124.04, 123.92, 123.22, 40.50, 30.38, 29.52 ppm. HRMS (MALDI) $m/z$ found 516.0721, [M+Na]$^+$; calcd 516.0776 for C$_{23}$H$_{19}$N$_5$O$_4$S$_2$Na$^+$.

2-[6-(Diethylamino)-3-(diethyliminio)-3$H$-xanthen-9-yl]-5-[N-(5-sulfamoyl-1,3,4-thiadiazol-2-yl)sulfamoyl]benzenesulfonate ($S_7$). The solution of compound 18 (47 mg, 0.26 mmol) in anhydrous pyridine (2 mL) was added to the solution of compound 28 (152 mg, 0.26 mmol) in anhydrous pyridine (5 mL) upon stirring under nitrogen atmosphere and the stirring was continued for 60 h. The reaction mixture was then concentrated on rotary evaporator and the residue was chromatographed on silica gel (chloroform/methanol/ammonium hydroxide = 9 : 1 : 0.3 $v/v$ $\rightarrow$ chloroform/methanol/ammonium hydroxide = 7 : 3 : 0.3 $v/v$) to yield product $S_7$ as a dark purple solid (6 mg, 3%); $R_f = 0.13$ (chloroform/methanol/ammonium hydroxide = 7:3:0.3 $v/v$). Spectral data for $S_7$: $^1$H-NMR (MeOH-$d_4$, 500 MHz): $\delta =$ 8.71 (s, 1H, ArH), 8.12 (d, $J =$ 7.9 Hz, 1H, ArH), 7.41 (d, $J =$ 7.9 Hz, 1H, ArH), 7.08 (d, $J =$ 9.5 Hz, 2H, ArH), 6.98 (dd, $J =$ 9.5, 2.3 Hz, 2H, ArH), 6.90 (d, $J =$ 2.2 Hz, 2H, ArH), 3.65 (q, $J =$ 7.1 Hz, 8H, CH$_2$CH$_3$), 1.28 (t, $J =$ 7.1 Hz, 12H, CH$_2$CH$_3$). $^{13}$C-NMR (MeOH-$d_4$, 125 MHz): $\delta =$ 159.38, 158.21, 157.12, 146.61, 146.20, 134.63, 133.79,
132.05, 128.73, 127.08, 115.34, 115.02, 96.88, 79.49, 71.28, 46.77, 12.82 ppm. HRMS (MALDI+)

$m/z$ found 721.1451 [M+Na]$^+$; calcd 721.1237 for C$_{29}$H$_{33}$N$_6$O$_8$S$_4^+$. 
2.7 Carbonic Anhydrase Sensors: NMR Spectra

Figure 2.12. $^1$H-NMR spectrum (500 MHz) of sensor S1 in DMSO-$d_6$. 
Figure 2.13. APT-NMR spectrum (125 MHz) of sensor S1 in DMSO-$d_6$. 
Figure 2.14. $^1$H-NMR spectrum (500 MHz) of sensor S2 in DMSO-$d_6$. 
Figure 2.15. $^{13}$C-NMR spectrum (125 MHz) of sensor S2 in DMSO-$d_6$. 
Figure 2.16. $^1$H-NMR spectrum (500 MHz) of sensor S3 in DMSO-$d_6$. 
Figure 2.17. $^{13}$C-NMR spectrum (125 MHz) of sensor S3 in DMSO-$d_6$. 
Figure 2.18. $^1$H-NMR spectrum (500 MHz) of sensor S4 in DMSO-$d_6$. 
Figure 2.19. $^{13}$C-NMR spectrum (125 MHz) of sensor S4 in DMSO-$d_6$. 
Figure 2.20. $^1$H-NMR spectrum (500 MHz) of sensor S5 in DMSO-$d_6$. 
Figure 2.21. $^{13}$C-NMR spectrum (125 MHz) of sensor S5 in DMSO-$d_6$. 
Figure 2.22. $^1$H-NMR spectrum (500 MHz) of sensor S6 in DMSO-$d_6$. 
Figure 2.23. $^{13}$C-NMR spectrum (125 MHz) of sensor S6 in DMSO-$d_6$. 
Figure 2.24. $^1$H-NMR spectrum (500 MHz) of sensor S7 in MeOH-$d_4$. 
Figure 2.25. $^{13}$C-NMR spectrum (125 MHz) of sensor S7 in MeOH-$d_4$. 
2.8 Carbonic Anhydrase Sensors: Photophysical Properties

Photophysical properties of sensors S1-S7 (*Table 2.2*) were measured in aqueous HEPES buffer (50 mM, pH 7.2) at room temperature and without previous degassing. Absorption spectra were acquired using Hitachi U-3010 double beam UV visible spectrophotometer (Tokyo, Japan). Fluorescence spectra were obtained at laboratory temperature in a quartz cuvette with 1 cm path length and at right angle detection. Emission spectra and fluorescence lifetime measurements were carried out on Edinburgh FLS920-stm combined steady state and lifetime spectrofluorimeter.

*Table 2.2.* Photophysical properties of sensors S1-S7. Absorption and emission maxima (\(\lambda_{A,max}\), \(\lambda_{E,max}\), respectively), absolute fluorescence quantum yields \(\Phi_{FL}\), fluorescence lifetimes \(\tau_{FL}\), and molar extinction coefficients \(\varepsilon_{M}\) were acquired in aqueous HEPES (50 mM pH 7.2) solutions.

<table>
<thead>
<tr>
<th>Sensor</th>
<th>(\lambda_{A,max})</th>
<th>(\lambda_{E,max})</th>
<th>(\Phi_{FL} (\lambda_{EXC}))</th>
<th>(\tau_{FL})</th>
<th>(\varepsilon_{M} (at \lambda_{ABS,max}))</th>
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<tr>
<td>S1</td>
<td>359</td>
<td>419</td>
<td>18.35 (360 nm)</td>
<td>1.62 ± 0.03 (92.31 %)</td>
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<tr>
<td>S2</td>
<td>350</td>
<td>407</td>
<td>51.48 (351 nm)</td>
<td>2.91 ± 0.04 (82.01 %)</td>
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<tr>
<td>S3</td>
<td>302</td>
<td>517</td>
<td>1.43 (300 nm)</td>
<td>8.91 ± 0.14 (17.99 %)</td>
<td>1.09</td>
</tr>
<tr>
<td>S4</td>
<td>327</td>
<td>574</td>
<td>14.27 (330 nm)</td>
<td>13.44 ± 0.02</td>
<td>0.49</td>
</tr>
<tr>
<td>S5</td>
<td>568</td>
<td>589</td>
<td>29.02 (568 nm)</td>
<td>12.87 ± 0.36 (10.66 %)</td>
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</tr>
<tr>
<td>S6</td>
<td>342</td>
<td>376</td>
<td>13.22 (276 nm)</td>
<td>5.50 ± 0.04 (89.34 %)</td>
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</tr>
<tr>
<td>S7</td>
<td>567</td>
<td>588</td>
<td>28.27 (567 nm)</td>
<td>194.0 ± 0.5 (82.87 %)</td>
<td>11.30</td>
</tr>
</tbody>
</table>

\(a\) Only the lowest energy maxima are listed. \(b\) Only the highest energy maxima are listed. \(c\) Absolute quantum yields were determined upon excitation at wavelength indicated for solutions with optical density \(A = 0.1\) (all errors < 4%). All measurements were carried out in non-deoxygenated solutions.

(Edinburgh Instruments Ltd., Livingston, UK). Laser radiation (\(\lambda = 355\) nm) was used as an excitation source for fluorescence lifetime experiments. Optical cut-off filters were used in fluorimeter’s emission channel where appropriate. Absolute quantum yields were obtained upon excitation at absorption maxima using Hamamatsu Quantaurus-QY C11347-11 Absolute PLQY Spectrometer equipped with 150 W Xe lamp and multichannel detector/CCD sensor (Hamamatsu, Japan). The photophysical properties are summarized in *Table 2.2*. Absorption maxima observed...
range from 302 nm (S3) to 568 nm (S6) with the coumarin-based sensors S1 and S2 having maxima at around 350 nm, the dansyl-based sensors S3 and S4 with maxima around 315 nm, the pyrene-based sensor S6 with maximum at 342 nm and the sulforhodamine-based sensors with maxima around 570 nm. The fluorescence maxima span from 419 nm (S1) to 589 nm (S5). The coumarin-based sensors S1 and S2 have emission maxima at 517 nm (green) and 574 nm (yellow green), respectively. Dansyl-based sensors S3 and S4 have emission maxima at 419 nm (purple) and 407 nm (deep purple), respectively. Pyrene-based S6 has emission at 376 nm (deep purple) and sulforhodamines S5 and S7 emit at 589 nm (yellow) and 588 nm (yellow), respectively. Interestingly, the fluorescence quantum yields show that higher values were obtained for extended indicators S2, S4, and S6 (52, 10, and 28%, respectively), compared to their short congeners S1 and S3 (17 and 1%, respectively). The sensors (S1, S2, S4, S5 and S6) showed double-exponential fluorescence lifetime decay. Fluorescence lifetime measurement of pyrene-based sensor S6 did not indicate the formation of excimer.
Figure 2.26. UV/Vis and PL spectra of sensors S1-S7 with prominent peaks labeled obtained in aqueous HEPES 50 mM, pH 7.2 at room temperature.
2.9 Direct Sensor Titrations

First, we decided to investigate affinity of sensors S1-S7 for hCA I, hCA II, hCA III and bCA II using direct fluorescence titrations. The constants obtained show that the length of the linker chain dramatically affects the binding affinity towards the target enzyme. A lower affinity and smaller magnitude of difference between On/Off state was recorded for indicators with longer linker. This suggests that the distance between the fluorescent dye and the binding site might be too large to significantly affect photophysical properties of the reporter. On the other hand, the effect of the short linker affects the response of the indicator more dramatically, perhaps, due to the fact that the indicator is more efficiently accommodated within the binding pocket. Table 2.3 shows the obtained binding affinities are very high \((10^5-10^9 \text{ M}^{-1})\). The affinities obtained for human CA isozyme I (with S1 and S3) are 2 and 3 orders of magnitude lower than those obtained for human or bovine CA isozyme II. This confirms generally observed lower affinity of human CA isozyme I for sulfonamide-based ligands versus human CA isozyme II which is known to exhibit generally very high affinities (Table 2.1). Interestingly, the fluorescence of coumarin-based sensors S1 and S2 was quenched upon the addition of CA while the fluorescence of dansyl-containing sensors S3 and S4 as well as the fluorescence of pyrene-based sensor S6 was amplified. On the other hand, sensors S5 and S7 showed a relatively large fluorescence even in the absence of CA, and thus, no significant change in fluorescence occurred upon addition of the protein. These results indicated that the rather hydrophobic sensors S1-S4 and S6, not the zwitterionic S5 and S7, are suitable as a fluorophore for a clear Off/On response detection. The mechanism of the fluorescence signal transduction in the sensors used upon their interaction with the proteins is not fully understood as we did not perform any investigation to reveal the mechanism. Most likely, the mechanism will involve electron and/or energy transfer from and to CA (more accurately, the
amino acid residues participating in the binding event) and/or internal quenching within the sensor molecules as a result of aggregation/dissociation as described by Hamachi and coworkers.\textsuperscript{127} It is very possible that these mechanisms might be involved simultaneously and to a various extent and lead to the resulting fluorescence signal modulation observed. A molecular docking results presented at the end of this section suggested however, that in the short sensor the fluorescence label interacts directly with the hydrophobic patch of the binding pocket. Perhaps not surprisingly, the response behavior and affinities obtained for hCA II and bCA II were found to be comparable which is supported by their high sequence homology.\textsuperscript{36} Thus, bCA II can be used as a good model for its human homolog. Titration spectra and isotherms are shown in Figure 2.28-2.42.

Additionally, we investigated a selectivity of sensors S1, S3 and S6 for CA II over a common interferent found in human blood plasma (Figure 2.41). Importantly, sensors S1, S3 and S6 showed high selectivity for CA isozyme II over human serum albumin, a type of globular protein found in blood plasma and acting as a molecular carrier via a non-specific binding of hydrophobic molecules. This is an important observation perhaps allowing the use of sensors S1, S3 and S6 to selectively detect CA in human blood serum. Such a possibility, however was not investigated in this project.

The titrations were accomplished as follows. The sensor solutions (2.5 mL, 500 nM) in HEPES 50 mM, pH 7.2 in cuvette were titrated with stock solution of protein (1 mg/mL in HEPES 50 mM, pH 7.2) and the resulting mixture was gently agitated and incubated for 5 min at room temperature after each addition. The fluorescence spectrum was subsequently recorded and titration isotherms were plotted and binding constants calculated using 1:1 binding model by Newton’s iterative method. The affinity constants of S1-S7 for hCA I, hCA II, hCA III and bCA II are summarized in Table 2.3. The fluorescence spectra were corrected by subtraction of Raman
scattering spectrum acquired under the same conditions as the spectra corrected. Figure 2.27 shows normalized emission spectra of human and bovine carbonic anhydrases in aqueous HEPES buffer (50 mM, pH 7.2).

![Fluorescence spectra of human (red) and bovine (blue) carbonic anhydrases isozyme II in aqueous HEPES buffer (50 mM) at pH 7.2. \( \lambda_{EX} = 280 \text{ nm} \)](image)

**Figure 2.27.** Fluorescence spectra of human (red) and bovine (blue) carbonic anhydrases isozyme II in aqueous HEPES buffer (50 mM) at pH 7.2. \( \lambda_{EX} = 280 \text{ nm} \).

**Table 2.3.** Binding affinities \( K (\text{M}^{-1})^{a} \) of sensors S1-S7 (500 nM) for carbonic anhydrase isozymes (human, hCA, and bovine, bCA) obtained from fluorescence titration in aqueous HEPES buffer (50 mM, pH 7.2) at ambient temperature.

<table>
<thead>
<tr>
<th>Sensor</th>
<th>hCA I</th>
<th>hCA II</th>
<th>hCA III</th>
<th>bCA II</th>
</tr>
</thead>
<tbody>
<tr>
<td>S1</td>
<td>( 3.99 \times 10^{6} ) (↓)</td>
<td>( 6.48 \times 10^{8} ) (↓)</td>
<td>( 2.91 \times 10^{8} ) (↓)</td>
<td>( 5.82 \times 10^{8} ) (↓)</td>
</tr>
<tr>
<td>S2</td>
<td>ND</td>
<td>( 4.99 \times 10^{7} ) (↓)</td>
<td>ND</td>
<td>( 9.80 \times 10^{7} ) (↓)</td>
</tr>
<tr>
<td>S3</td>
<td>( 1.22 \times 10^{5} ) (↑)(^b)</td>
<td>( 1.31 \times 10^{8} ) (↑)(^b)</td>
<td>ND</td>
<td>( 1.79 \times 10^{8} ) (↑)(^b)</td>
</tr>
<tr>
<td>S4</td>
<td>ND</td>
<td>( 4.87 \times 10^{7} ) (↑)</td>
<td>ND</td>
<td>( 1.96 \times 10^{8} ) (↑)</td>
</tr>
<tr>
<td>S5</td>
<td>ND</td>
<td>ND(^c)</td>
<td>ND</td>
<td>ND(^c)</td>
</tr>
<tr>
<td>S6</td>
<td>ND</td>
<td>( 5.02 \times 10^{6} ) (↑)(^b)</td>
<td>ND</td>
<td>( 1.91 \times 10^{7} ) (↑)(^b)</td>
</tr>
<tr>
<td>S7</td>
<td>ND</td>
<td>ND(^c)</td>
<td>ND</td>
<td>ND(^c)</td>
</tr>
</tbody>
</table>

\(^{a}\) \( K \)'s were calculated based on the change in fluorescence maxima upon addition of each protein by using 1:1 binding model. \(^{b}\) Fluorescence titration was carried out in aqueous HEPES 50 mM, pH 7.2, 10% (v/v) DMSO solution. \(^{c}\) Binding constant could not be determined accurately due to large error. Symbols “↓” and “↑” indicate fluorescence quenching or amplification, respectively, upon addition of incremental amounts of the protein.
Table 2.4. Binding affinity errors $\Delta K$ ($\text{M}^{-1}$) of the binding affinities $K$ of sensors S1-S7 (500 nM) for carbonic anhydrase isozymes (human, hCA, and bovine, bCA) obtained from fluorescence titration in aqueous HEPES buffer (50 mM, pH 7.2) at ambient temperature.

<table>
<thead>
<tr>
<th>Sensor</th>
<th>hCA I</th>
<th>hCA II</th>
<th>hCA III</th>
<th>bCA II</th>
</tr>
</thead>
<tbody>
<tr>
<td>S1</td>
<td>$0.69 \times 10^6$</td>
<td>$0.84 \times 10^8$</td>
<td>$&gt;10^6$</td>
<td>$1.34 \times 10^8$</td>
</tr>
<tr>
<td>S2</td>
<td>ND</td>
<td>$1.86 \times 10^7$</td>
<td>ND</td>
<td>$3.09 \times 10^7$</td>
</tr>
<tr>
<td>S3</td>
<td>$0.64 \times 10^5$</td>
<td>$0.24 \times 10^8$</td>
<td>ND</td>
<td>$0.59 \times 10^8$</td>
</tr>
<tr>
<td>S4</td>
<td>ND</td>
<td>$&gt;10^6$</td>
<td>ND</td>
<td>$0.52 \times 10^8$</td>
</tr>
<tr>
<td>S5</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>S6</td>
<td>ND</td>
<td>$&gt;10^6$</td>
<td>ND</td>
<td>$0.35 \times 10^7$</td>
</tr>
<tr>
<td>S7</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

$\Delta K$s were calculated based on the change in fluorescence maxima upon addition of each protein by using 1:1 binding model.

2.10 Direct Sensor Titrations: Human Carbonic Anhydrase Isozyme I

Figure 2.28. Fluorescence titration spectra (A) and fluorescence titration isotherm (B) of S1 (500 nM) upon the addition of an incremental amounts of hCA I in aqueous HEPES buffer (50 mM) at pH 7.2. $\lambda_{\text{exc}} = 373$ nm, [hCA I] = 0-32 µg/mL.
Figure 2.29. Fluorescence titration spectra (A) and fluorescence titration isotherm (B) of S3 (500 nM) upon the addition of an incremental amounts of hCA I in aqueous HEPES buffer (50 mM) at pH 7.2. \( \lambda_{\text{EXC}} = 303 \) nm, [hCA I] = 0-122 μg/mL.

2.11 Direct Sensor Titrations: Human Carbonic Anhydrase Isozyme II

Figure 2.30. Fluorescence titration spectra (A) and fluorescence titration isotherm (B) of S1 (500 nM) upon the addition of an incremental amounts of hCA II in aqueous HEPES buffer (50 mM) at pH 7.2. \( \lambda_{\text{EXC}} = 373 \) nm, [hCA II] = 0-9 μg/mL.
Figure 2.31. Fluorescence titration spectra (A) and fluorescence titration isotherm (B) of S2 (500 nM) upon the addition of an incremental amounts of hCA II in aqueous HEPES buffer (50 mM) at pH 7.2. $\lambda_{\text{exc}} = 375$ nm, [hCA II] = 0-16 μg/mL.

Figure 2.32. Fluorescence titration spectra (A) and fluorescence titration isotherm (B) of S3 (500 nM) upon the addition of an incremental amounts of hCA II in aqueous HEPES buffer (50 mM) at pH 7.2. $\lambda_{\text{exc}} = 302$ nm, [hCA II] = 0-20 μg/mL.
Figure 2.33. Fluorescence titration spectra (A) and fluorescence titration isotherm (B) of S4 (500 nM) upon the addition of an incremental amounts of hCA II in aqueous HEPES buffer (50 mM) at pH 7.2. $\lambda_{\text{EXC}} = 296$ nm, $[\text{hCA II}] = 0-13$ μg/mL.

Figure 2.34. Fluorescence titration spectra (A) and fluorescence titration isotherm (B) of S6 (500 nM) upon the addition of an incremental amounts of hCA II in aqueous HEPES buffer (50 mM) at pH 7.2. $\lambda_{\text{EXC}} = 296$ nm, $[\text{hCA II}] = 0-15$ μg/mL.
2.12 Direct Sensor Titrations: Human Carbonic Anhydrase Isozyme III

![Figure 2.35](image1.png)

**Figure 2.35.** Fluorescence titration spectra (A) and fluorescence titration isotherm (B) of S1 (500 nM) upon the addition of an incremental amounts of hCA III in aqueous HEPES buffer (50 mM) at pH 7.2. $\lambda_{\text{EXC}} = 373$ nm, $[\text{hCA III}] = 0$-16 $\mu$g/mL.

2.13 Direct Sensor Titrations: Bovine Carbonic Anhydrase Isozyme II

![Figure 2.36](image2.png)

**Figure 2.36.** Fluorescence titration spectra (A) and fluorescence titration isotherm (B) of S1 (500 nM) upon the addition of an incremental amounts of hCA II in aqueous HEPES buffer (50 mM) at pH 7.2. $\lambda_{\text{EXC}} = 373$ nm, $[\text{bCA II}] = 0$-1.52 $\mu$g/mL.
Figure 2.37. Fluorescence titration spectra (A) and fluorescence titration isotherm (B) of S2 (500 nM) upon the addition of an incremental amounts of hCA II in aqueous HEPES buffer (50 mM) at pH 7.2. $\lambda_{\text{exc}} = 375$ nm, [bCA II] = 0-15 μg/mL.

Figure 2.38. Fluorescence titration spectra (A) and fluorescence titration isotherm (B) of S3 (500 nM) upon the addition of an incremental amounts of hCA II in aqueous HEPES buffer (50 mM) at pH 7.2. $\lambda_{\text{exc}} = 302$ nm, [bCA II] = 0-25 μg/mL.
Figure 2.39. Fluorescence titration spectra (A) and fluorescence titration isotherm (B) of S4 (500 nM) upon the addition of an incremental amounts of hCA II in aqueous HEPES buffer (50 mM) at pH 7.2. \( \lambda_{\text{exc}} = 296 \text{ nm} \), [bCA II] = 0-15 μg/mL.

Figure 2.40. Fluorescence titration spectra (A) and fluorescence titration isotherm (B) of S6 (500 nM) upon the addition of an incremental amounts of hCA II in aqueous HEPES buffer (50 mM) at pH 7.2. \( \lambda_{\text{exc}} = 296 \text{ nm} \), [bCA II] = 0-22 μg/mL.
2.14 Direct Sensor Titrations: Selectivity

Figure 2.41 presents results of selectivity titration experiments showing that the sensors S1, S3 and S6 binds selectively only to CAs and provide a change in fluorescence response. HSA showed only negligible response to the sensors.

Figure 2.41. Fluorescence titration isotherms of S1 (A), S3 (B) and S6 (C) (all 500 nM) upon the addition of incremental amounts of carbonic anhydrase isozymes II and human serum albumin in aqueous HEPES buffer (50 mM) at pH 7.2. S1: $\lambda_{exc} = 373$ nm, [Protein] = 0-32 μg/mL, S3: $\lambda_{exc} = 302$ nm, [Protein] = 0-25 μg/mL, S6: $\lambda_{exc} = 296$ nm, [Protein] = 0-22 μg/mL.
2.15 Competitive Fluorescence Titrations

To further investigate the ability of the CA-sensor complexes to signal the presence and affinity of CA inhibitors, we used a library of non-fluorescent sulfonamide CAIs to set up a competitive assay. The structures were selected so that various electronic and steric effects on the competitive binding could be evaluated (Figure 2.42). The library included two aliphatic (I1, I2), six aromatic (I3-I9) sulfonamides as well as five drugs widely used in clinical practice, acetazolamide (I10), methazolamide (I11), ethoxzolamide (I12), brinzolamide (I13) and celecoxib (I14). In order to determine the affinity constants of the model inhibitors I1-I14 to carbonic anhydrase, competitive fluorescence titrations were carried out and binding affinities were calculated from the titration isotherms using competitive binding model (Part 2.21). We used indicator S1 since this indicator provided relatively high quantum yield and a strong response.

Competitive fluorescence titrations with S1 were carried out as follows. A stock solution of bCA II (concentration 1 mg/mL) in HEPES buffer (50 mM, pH 7.2) was added to the solution

Figure 2.42. Inhibitors I1-I14 used in competitive assays in this study.
of S1 (2.5 mL, 500 nM) in HEPES buffer (50 mM, pH 7.2) in fluorescence cuvette (1 cm) to give a final concentration of bCA II in cuvette 9.6 μg/mL and after a gentle agitation the resulting mixture was incubated for 5 min at room temperature. Afterwards, the resulting solution was titrated with inhibitors I1-I14 (Figure 2.42) in DMSO stock solution (8.62665 mM) and the change in fluorescence intensity at $\lambda_{em} = 420$ nm was recorded after 5-30 min (depending on inhibitor) incubation at room temperature (Scheme 2.9). Table 2.5 summarizes the binding affinities $K_G$ obtained for inhibitors I1-I14.

Scheme 2.9. Scheme depicting the experimental procedure of competitive titrations for sensor S1 (500 nM) upon the addition of bovine carbonic anhydrase isozyme II (bCA II) and incremental amounts of inhibitors I1-I14.
Figure 2.43. Fluorescence titration spectra (A) and fluorescence titration isotherm (B) of S1 (500 nM) upon addition of an incremental amount of inhibitor I3 in the presence of bCA II (9.6 μg/mL) in aqueous HEPES buffer (50 mM) at pH 7.2 in the competitive experiment (λ_{EXC} = 373 nm), [I3] = 0-1874 μM.

Figure 2.44. Fluorescence titration spectra (A) and fluorescence titration isotherm (B) of S1 (500 nM) upon addition of an incremental amount of inhibitor I4 in the presence of bCA II (9.6 μg/mL) in aqueous HEPES buffer (50 mM) at pH 7.2 in the competitive experiment (λ_{EXC} = 373 nm), [I4] = 0-3320 μM.
Figure 2.45. Fluorescence titration spectra (A) and fluorescence titration isotherm (B) of S1 (500 nM) upon addition of an incremental amount of inhibitor I5 in the presence of bCA II (9.6 μg/mL) in aqueous HEPES buffer (50 mM) at pH 7.2 in the competitive experiment (λEXC = 373 nm), [I5] = 0-207 μM.

Figure 2.46. Fluorescence titration spectra (A) and fluorescence titration isotherm (B) of S1 (500 nM) upon addition of an incremental amount of inhibitor I6 in the presence of bCA II (9.6 μg/mL) in aqueous HEPES buffer (50 mM) at pH 7.2 in the competitive experiment (λEXC = 373 nm), [I6] = 0-197 μM.
Figure 2.47. Fluorescence titration spectra (A) and fluorescence titration isotherm (B) of S1 (500 nM) upon addition of an incremental amount of inhibitor I7 in the presence of bCA II (9.6 μg/mL) in aqueous HEPES buffer (50 mM) at pH 7.2 in the competitive experiment ($\lambda_{\text{EXC}} = 373$ nm), [I7] = 0-155 μM.

Figure 2.48. Fluorescence titration spectra (A) and fluorescence titration isotherm (B) of S1 (500 nM) upon addition of an incremental amount of inhibitor I8 in the presence of bCA II (9.6 μg/mL) in aqueous HEPES buffer (50 mM) at pH 7.2 in the competitive experiment ($\lambda_{\text{EXC}} = 373$ nm), [I8] = 0-72 μM.
Figure 2.49. Fluorescence titration spectra (A) and fluorescence titration isotherm (B) of S1 (500 nM) upon addition of an incremental amount of inhibitor I9 in the presence of bCA II (9.6 μg/mL) in aqueous HEPES buffer (50 mM) at pH 7.2 in the competitive experiment ($\lambda_{\text{EXC}} = 373$ nm), $[I9] = 0$-1574 μM.

Figure 2.50. Fluorescence titration spectra (A) and fluorescence titration isotherm (B) of S1 (500 nM) upon addition of an incremental amount of inhibitor I10 in the presence of bCA II (9.6 μg/mL) in aqueous HEPES buffer (50 mM) at pH 7.2 in the competitive experiment ($\lambda_{\text{EXC}} = 373$ nm), $[I10] = 0$-145 μM.
Figure 2.51. Fluorescence titration spectra (A) and fluorescence titration isotherm (B) of S1 (500 nM) upon addition of an incremental amount of inhibitor I11 in the presence of bCA II (9.6 μg/mL) in aqueous HEPES buffer (50 mM) at pH 7.2 in the competitive experiment ($\lambda_{\text{EXC}} = 373$ nm), $[\text{I11}] = 0$-$155$ μM.

Figure 2.52. Fluorescence titration spectra (A) and fluorescence titration isotherm (B) of S1 (500 nM) upon addition of an incremental amount of inhibitor I12 in the presence of bCA II (9.6 μg/mL) in aqueous HEPES buffer (50 mM) at pH 7.2 in the competitive experiment ($\lambda_{\text{EXC}} = 373$ nm), $[\text{I12}] = 0$-$0.75$ μM.
Figure 2.53. Fluorescence titration spectra (A) and fluorescence titration isotherm (B) of S1 (500 nM) upon addition of an incremental amount of inhibitor 113 in the presence of bCA II (9.6 μg/mL) in aqueous HEPES buffer (50 mM) at pH 7.2 in the competitive experiment (λ<sub>EXC</sub> = 373 nm), [113] = 0-1.40 μM.

![Image](image1)

Figure 2.54. Fluorescence titration spectra (A) and fluorescence titration isotherm (B) of S1 (500 nM) upon addition of an incremental amount of inhibitor 114 in the presence of bCA II (9.6 μg/mL) in aqueous HEPES buffer (50 mM) at pH 7.2 in the competitive experiment (λ<sub>EXC</sub> = 373 nm), [114] = 0-114 μM.

![Image](image2)
Table 2.5. Calculated inhibitors affinities $K_G$ (M$^{-1}$) for inhibitors I1-I14 to bovine carbonic anhydrase isozyme II (bCA II) with sensor S1 (500 nM) obtained from fluorescence titration in aqueous HEPES buffer (50 mM), pH 7.2, at ambient temperature.

<table>
<thead>
<tr>
<th>$K_G$ (M$^{-1}$)</th>
<th>bCA II</th>
</tr>
</thead>
<tbody>
<tr>
<td>11</td>
<td>ND (no response up to 1 mM)</td>
</tr>
<tr>
<td>12</td>
<td>ND (no response up to 1 mM)</td>
</tr>
<tr>
<td>13</td>
<td>$7.84 \times 10^5$</td>
</tr>
<tr>
<td>14</td>
<td>$1.96 \times 10^4$</td>
</tr>
<tr>
<td>15</td>
<td>$2.01 \times 10^6$</td>
</tr>
<tr>
<td>16</td>
<td>$1.00 \times 10^8$</td>
</tr>
<tr>
<td>17</td>
<td>$1.11 \times 10^8$</td>
</tr>
<tr>
<td>18</td>
<td>$1.07 \times 10^8$</td>
</tr>
<tr>
<td>19</td>
<td>$2.52 \times 10^6$</td>
</tr>
<tr>
<td>110</td>
<td>$1.22 \times 10^9$</td>
</tr>
<tr>
<td>111</td>
<td>$1.27 \times 10^9$</td>
</tr>
<tr>
<td>112</td>
<td>$3.97 \times 10^{10}$</td>
</tr>
<tr>
<td>113</td>
<td>$8.40 \times 10^{10}$</td>
</tr>
<tr>
<td>114</td>
<td>$9.62 \times 10^7$</td>
</tr>
</tbody>
</table>

Figure 2.55. A: Fluorescence titration isotherms of competitive assay for inhibitors I5-I13 obtained from titration data with bovine carbonic anhydrase isozyme II (9.6 μg/mL) using S1 (500 nM) in HEPES buffer (50 mM, pH 7.2). B: Photograph of sensor S1 (7 μM) in the absence (1) and presence (2) of bCA II (325 μg/mL) and after addition of I10 (1495 μM) to the solution of S1 and bCA II (3).
The addition of inhibitors to the solution of the bCA II-S1 complex leads to fluorescence signal recovery, magnitude of which was found to be proportional to the inhibitor affinities obtained from those competitive fluorescence titrations. As expected, the affinity constant of aliphatic inhibitors I1 and I2 could not be determined since the change in fluorescence response was very small/not measurable up to 1 mM concentration of inhibitor added. Interesting trends can be observed among aromatic inhibitors I3-I9. Generally, aromatic sulfonamides are known to exhibit affinities $10^4$-$10^8$ M$^{-1}$ to high affinity for CA II. Here, the para-substitution of benzene sulfonamide in I3 and I5 yields higher affinity than ortho-substitution as in I4. This is caused by the difference in a steric demand. Interestingly, an introduction of electron donor in benzene sulfonamide in para-position (as in I3, I5 and I8) or expansion of aromatic system (I6) leads to higher affinities (I3 < I5 < I6). The lowest affinity out of all aromatic inhibitors was obtained for I4 and the highest for I6, I7 and I8. The highest affinities overall were obtained for high-affinity inhibitors I11-I14 out of which brinzolamide I13 showed the highest affinity observed ($10^{11}$ M$^{-1}$). Celecoxib I14 was originally designed as a cyclooxygenase-2 inhibitor (coxib) but as an aromatic sulfonamide it also showed inhibition properties for CAs. We observed its affinity to be $10^8$ M$^{-1}$. This can be attributed to sterically demanding substitution of the benzene sulfonamide ring moiety. Perhaps the most important outcome of this study is the observation that the magnitude of the indicator fluorescence response observed upon inhibitor addition directly correlates with the affinity of the inhibitor for the CA. Following the data in graph in Figure 2.55A one can see that, for example, at inhibitor concentrations of 5-20 μM the order of the observed fluorescence response is I10 > I11 > I8 > I7 > I14 > I6 > I5 >> I9. Also, I12 (ethoxzolamide) and I13 (brinzolamide) display a substantially higher response and affinity toward CAs. Thus, any unknown inhibitor candidate displaying a fluorescence response amplification between I12 and
I10 would be good drug candidates suitable for further optimization. Interestingly, the process of fluorescence signal recovery upon the addition of a competitive inhibitor to the solution of the complex of sensor S1 with bovine carbonic anhydrase II can be observed even with the naked eye as is shown in the photograph in *Figure 2.55B*. The fluorescence of sensor S1 (cuvette 1) is first quenched upon the addition of bCA II (cuvette 2) and then partially recovered after the addition of I10 (cuvette 3).

Scheme 2.10. Experimental procedure for competitive titrations to investigate effect of pH value on fluorescence response of sensor S1 (5 μM) upon additions of incremental amount of bovine carbonic anhydrase isozyme II (bCA II) and inhibitors I1-I5.

**2.16 Effect of pH Value of the Medium on the Fluorescence Response**

Finally, as we intended to investigate the feasibility of high-throughput screening approach utilizing the competitive sensing ensemble, a set of conditions (variables) must have been screened...
and optimized for use in fluorescence-based array experiments. We decided to investigated the
effect of medium pH value (Figure 2.56-67), HEPES buffer concentration (Figure 2.68-75) and
the presence of NaCl acting as an inert interferent/ionic strength effect (Figure 2.77-87) on the
fluorescence response of sensor S1 upon titration with selected inhibitors I3-I5. Behavior of probe
S1 upon these various conditions is summarized in the following parts (2.17, 2.18 and 2.19)
including fluorescence titration spectra and titration isotherms as well as calculated apparent
binding affinities (Table 2.6-2.8).

We carried out competitive titrations with S1 at three different values of pH of HEPES
buffer: pH 6.5, 7.2 and 8.0. Stock solution of bCA II (concentration 1 mg/mL) in HEPES, 50 mM,
pH 7.2, was premixed with the solution of inhibitors I3-I5 in DMSO (86.2665 mM) in Eppendorf
tube to give final ratio between protein and inhibitors 1:500 n/n and after a gentle agitation the
solution was used to titrate the solution of S1 (2.5 mL, 5 μM) in HEPES 50 mM, pH 6.5, 7.2, or
8.0, in fluorescence cuvette (1 cm). The change in fluorescence intensity was recorded after 0-
15 min of incubation at room temperature. The solution was always gently agitated to mix the
solutions before the incubation.
Figure 2.56. Fluorescence titration spectra (A) and fluorescence titration isotherm (B) of S1 (5 μM) at aqueous HEPES buffer (50 mM) at pH 6.5 upon the addition of an incremental amounts of bovine carbonic anhydrase isozyme II in the presence of inhibitor 13: $\lambda_{\text{EXC}} = 373$ nm, [bCA II]/[inhibitor] = 1:500.

Figure 2.57. Fluorescence titration spectra (A) and fluorescence titration isotherm (B) of S1 (5 μM) at aqueous HEPES buffer (50 mM) at pH 6.5 upon the addition of an incremental amounts of bovine carbonic anhydrase isozyme II in the presence of inhibitor 14: $\lambda_{\text{EXC}} = 373$ nm, [bCA II]/[inhibitor] = 1:500.
Figure 2.58. Fluorescence titration spectra (A) and fluorescence titration isotherm (B) of S1 (5 μM) at aqueous HEPES buffer (50 mM) at pH 6.5 upon the addition of an incremental amounts of bovine carbonic anhydrase isozyme II in the presence of inhibitor I5: $\lambda_{\text{exc}} = 373$ nm, [bCA II]/[inhibitor] = 1:500.

Figure 2.59. Comparison of fluorescence titration isotherms of S1 (5 μM) at aqueous HEPES buffer (50 mM) at pH 6.5 upon the addition of an incremental amounts of bovine carbonic anhydrase isozyme II in the presence of inhibitor I3-I5: $\lambda_{\text{exc}} = 373$ nm, [bCA II]/[inhibitor] = 1:500.
Figure 2.60. Fluorescence titration spectra (A) and fluorescence titration isotherm (B) of S1 (5 μM) at aqueous HEPES buffer (50 mM) at pH 7.2 upon the addition of an incremental amounts of bovine carbonic anhydrase isozyme II in the presence of inhibitor I3: \( \lambda_{\text{exc}} = 373 \text{ nm} \), \([\text{bCA II}]/[\text{inhibitor}] = 1:500\).

Figure 2.61. Fluorescence titration spectra (A) and fluorescence titration isotherm (B) of S1 (5 μM) at aqueous HEPES buffer (50 mM) at pH 7.2 upon the addition of an incremental amounts of bovine carbonic anhydrase isozyme II in the presence of inhibitor I4: \( \lambda_{\text{exc}} = 373 \text{ nm} \), \([\text{bCA II}]/[\text{inhibitor}] = 1:500\).
Figure 2.62. Fluorescence titration spectra (A) and fluorescence titration isotherm (B) of S1 (5 μM) at aqueous HEPES buffer (50 mM) at pH 7.2 upon the addition of an incremental amounts of bovine carbonic anhydrase isozyme II in the presence of inhibitor I5: λ_{EXC} = 373 nm, [bCA II]/[inhibitor] = 1:500.

Figure 2.63. Comparison of fluorescence titration isotherms of S1 (5 μM) at aqueous HEPES buffer (50 mM) at pH 7.2 upon the addition of an incremental amounts of bovine carbonic anhydrase isozyme II in the presence of inhibitor I3-I5: λ_{EXC} = 373 nm, [bCA II]/[inhibitor] = 1:500.
Figure 2.64. Fluorescence titration spectra (A) and fluorescence titration isotherm (B) of S1 (5 μM) at aqueous HEPES buffer (50 mM) at pH 8.0 upon the addition of an incremental amounts of bovine carbonic anhydrase isozyme II in the presence of inhibitor 13: $\lambda_{\text{EXC}} = 373$ nm, [bCA II]/[inhibitor] = 1:500.

Figure 2.65. Fluorescence titration spectra (A) and fluorescence titration isotherm (B) of S1 (5 μM) at aqueous HEPES buffer (50 mM) at pH 8.0 upon the addition of an incremental amounts of bovine carbonic anhydrase isozyme II in the presence of inhibitor 14: $\lambda_{\text{EXC}} = 373$ nm, [bCA II]/[inhibitor] = 1:500.
Figure 2.66. Fluorescence titration spectra (A) and fluorescence titration isotherm (B) of $S_1$ (5 μM) at aqueous HEPES buffer (50 mM) at pH 8.0 upon the addition of an incremental amounts of bovine carbonic anhydrase isozyme II in the presence of inhibitor $I_5$: $\lambda_{\text{exc}} = 373$ nm, [bCA II]/[inhibitor] = 1:500.

Figure 2.67. Comparison of fluorescence titration isotherms of $S_1$ (5 μM) at aqueous HEPES buffer (50 mM) at pH 8.0 upon the addition of an incremental amounts of bovine carbonic anhydrase isozyme II in the presence of inhibitor $I_{13-15}$: $\lambda_{\text{exc}} = 373$ nm, [bCA II]/[inhibitor] = 1:500.
2.17 Effect of Buffer Concentration on the Fluorescence Response

We also investigated the effect of the buffer concentration on the fluorescence response. We carried out competitive titrations with S1 at three different HEPES buffer concentrations: 20, 50 and 100 mM. Stock solution of bCA II (concentration 1 mg/mL) in HEPES buffer, pH 8.0, was premixed with the solution of inhibitors I3-I5 in DMSO (86.2665 mM) in Eppendorf tube to give final ratio between protein and inhibitors 1:500 \( n/n \) and after a gentle agitation the solution was used to titrate the solution of S1 (2.5 mL, 5 \( \mu \)M) in HEPES buffer (20, 50 or 100 mM, pH 8.0), in fluorescence cuvette (1 cm). The change in fluorescence intensity was recorded after 0-15 min of incubation at room temperature. The solution was always gently agitated to mix the solutions before the incubation.

**Figure 2.68.** Fluorescence titration spectra (A) and fluorescence titration isotherm (B) of S1 (5 \( \mu \)M) at aqueous HEPES buffer (20 mM) at pH 8.0 upon the addition of incremental amounts of bovine carbonic anhydrase isozyme II in the presence of inhibitor I3: \( \lambda_{\text{exc}} = 373 \) nm, [bCA II]/[inhibitor] = 1:500.
Figure 2.69. Fluorescence titration spectra (A) and fluorescence titration isotherm (B) of S1 (5 μM) at aqueous HEPES buffer (20 mM) at pH 8.0 upon the addition of an incremental amounts of bovine carbonic anhydrase isozyme II in the presence of inhibitor I4: $\lambda_{\text{EXC}} = 373$ nm, [bCA II]/[inhibitor] = 1:500.

Figure 2.70. Fluorescence titration spectra (A) of S1 (5 μM) at aqueous HEPES buffer (20 mM) at pH 8.0 upon the addition of an incremental amounts of bovine carbonic anhydrase isozyme II in the presence of inhibitor I5: $\lambda_{\text{EXC}} = 373$ nm, [bCA II]/[inhibitor] = 1:500. The fluorescence titration isotherm could not be determined due to negligible change in fluorescence intensity observed during the titration (A).
Figure 2.71. Comparison of fluorescence titration isotherms of S1 (5 µM) at aqueous HEPES buffer (20 mM) at pH 8.0 upon the addition of an incremental amounts of bovine carbonic anhydrase isozyme II in the presence of inhibitor I3-I5: $\lambda_{EXC} = 373$ nm, [bCA II]/[inhibitor] = 1:500.

Figure 2.72. Fluorescence titration spectra (A) and fluorescence titration isotherm (B) of S1 (5 µM) at aqueous HEPES buffer (100 mM) at pH 8.0 upon the addition of an incremental amounts of bovine carbonic anhydrase isozyme II in the presence of inhibitor I3: $\lambda_{EXC} = 373$ nm, [bCA II]/[inhibitor] = 1:500.
Figure 2.73. Fluorescence titration spectra (A) and fluorescence titration isotherm (B) of $S_1$ (5 μM) at aqueous HEPES buffer (100 mM) at pH 8.0 upon the addition of an incremental amounts of bovine carbonic anhydrase isozyme II in the presence of inhibitor $I_{14}$: $\lambda_{\text{EXC}} = 373$ nm, [bCA II]/[inhibitor] = 1:500.

Figure 2.74. Fluorescence titration spectra (A) and fluorescence titration isotherm (B) of $S_1$ (5 μM) at aqueous HEPES buffer (100 mM) at pH 8.0 upon the addition of an incremental amounts of bovine carbonic anhydrase isozyme II in the presence of inhibitor $I_{15}$: $\lambda_{\text{EXC}} = 373$ nm, [bCA II]/[inhibitor] = 1:500.
Finally, we investigated the effect of the ionic strength (NaCl as an additive component) on the fluorescence response. We carried out competitive titrations with S1 at three different sodium chloride concentrations: 5, 50 and 100 mM. Stock solution of bCA II (concentration 1 mg/mL) in HEPES buffer (50 mM, pH 8.0), was premixed with the solution of inhibitors I3-I5 in DMSO (86.2665 mM) in Eppendorf tube to give final ratio between protein and inhibitors 1:500 n/n and after a gentle agitation the solution was used to titrate the solution of S1 (2.5 mL, 5 μM) in HEPES buffer (50 mM, pH 8.0), in fluorescence cuvette (1 cm) in the presence of various NaCl concentrations. The change in fluorescence intensity was recorded after 0-15 min of incubation at room temperature. The solution was always gently agitated to mix the solutions before the incubation.

![Figure 2.75. Comparison of fluorescence titration isotherms of S1 (5 μM) at aqueous HEPES buffer (100 mM) at pH 8.0 upon the addition of an incremental amounts of bovine carbonic anhydrase isozyme II in the presence of inhibitor I3-I5: $\lambda_{exc} = 373$ nm, [bCA II]/[inhibitor] = 1:500.](image_url)
Figure 2.76. Fluorescence titration spectra (A) and fluorescence titration isotherm (B) of S1 (5 μM) at aqueous HEPES buffer (50 mM, pH 8.0) and NaCl (5 mM) upon the addition of an incremental amounts of bovine carbonic anhydrase isozyme II in the presence of inhibitor 13: λ_{EXC} = 373 nm, [bCA II]/[inhibitor] = 1:500.

Figure 2.77. Fluorescence titration spectra (A) and fluorescence titration isotherm (B) of S1 (5 μM) at aqueous HEPES buffer (50 mM, pH 8.0) and NaCl (5 mM) upon the addition of an incremental amounts of bovine carbonic anhydrase isozyme II in the presence of inhibitor 14: λ_{EXC} = 373 nm, [bCA II]/[inhibitor] = 1:500.
Figure 2.78. Fluorescence titration spectra (A) and fluorescence titration isotherm (B) of S1 (5 μM) at aqueous HEPES buffer (50 mM, pH 8.0) and NaCl (5 mM) upon the addition of an incremental amounts of bovine carbonic anhydrase isozyme II in the presence of inhibitor 15: $\lambda_{\text{exc}} = 373$ nm, [bCA II]/[inhibitor] = 1:500.

Figure 2.79. Comparison of fluorescence titration isotherms of S1 (5 μM) at aqueous HEPES buffer (50 mM, pH 8.0) and NaCl (5 mM) upon the addition of an incremental amounts of bovine carbonic anhydrase isozyme II in the presence of inhibitor 13-15: $\lambda_{\text{exc}} = 373$ nm, [bCA II]/[inhibitor] = 1:500.
Figure 2.80. Fluorescence titration spectra (A) and fluorescence titration isotherm (B) of S1 (5 μM) at aqueous HEPES buffer (50 mM, pH 8.0) and NaCl (50 mM) upon the addition of an incremental amounts of bovine carbonic anhydrase isozyme II in the presence of inhibitor 13: λ<sub>EXC</sub> = 373 nm, [bCA II]/[inhibitor] = 1:500.

Figure 2.81. Fluorescence titration spectra (A) and fluorescence titration isotherm (B) of S1 (5 μM) at aqueous HEPES buffer (50 mM, pH 8.0) and NaCl (50 mM) upon the addition of an incremental amounts of bovine carbonic anhydrase isozyme II in the presence of inhibitor 14: λ<sub>EXC</sub> = 373 nm, [bCA II]/[inhibitor] = 1:500.
Figure 2.82. Fluorescence titration spectra (A) and fluorescence titration isotherm (B) of S1 (5 μM) at aqueous HEPES buffer (50 mM, pH 8.0) and NaCl (50 mM) upon the addition of an incremental amounts of bovine carbonic anhydrase isozyme II in the presence of inhibitor 15: λ_{EXC} = 373 nm, [bCA II]/[inhibitor] = 1:500.

Figure 2.83. Comparison of fluorescence titration isotherms of S1 (5 μM) at aqueous HEPES buffer (50 mM, pH 8.0) and NaCl (50 mM) upon the addition of an incremental amounts of bovine carbonic anhydrase isozyme II in the presence of inhibitor 13-15: λ_{EXC} = 373 nm, [bCA II]/[inhibitor] = 1:500.
Figure 2.84. Fluorescence titration spectra (A) and fluorescence titration isotherm (B) of S1 (5 μM) at aqueous HEPES buffer (50 mM, pH 8.0) and NaCl (100 mM) upon the addition of an incremental amounts of bovine carbonic anhydrase isozyme II in the presence of inhibitor I3: λ_{exc} = 373 nm, [bCA II]/[inhibitor] = 1:500.

Figure 2.85. Fluorescence titration spectra (A) and fluorescence titration isotherm (B) of S1 (5 μM) at aqueous HEPES buffer (50 mM, pH 8.0) and NaCl (100 mM) upon the addition of an incremental amounts of bovine carbonic anhydrase isozyme II in the presence of inhibitor I4: λ_{exc} = 373 nm, [bCA II]/[inhibitor] = 1:500.
Figure 2.86. Fluorescence titration spectra (A) and fluorescence titration isotherm (B) of S1 (5 μM) at aqueous HEPES buffer (50 mM, pH 8.0) and NaCl (100 mM) upon the addition of an incremental amounts of bovine carbonic anhydrase isozyme II in the presence of inhibitor 15: λ<sub>EXC</sub> = 373 nm, [bCA II]/[inhibitor] = 1:500.

Figure 2.87. Comparison of fluorescence titration isotherms of S1 (5 μM) at aqueous HEPES buffer (50 mM, pH 8.0) and NaCl (100 mM) upon the addition of an incremental amounts of bovine carbonic anhydrase isozyme II in the presence of inhibitor 13-15: λ<sub>EXC</sub> = 373 nm, [bCA II]/[inhibitor] = 1:500.
Table 2.6. Apparent binding affinities $K_a$ (M$^{-1}$) and errors of the apparent binding affinities $\Delta K_a$ (M$^{-1}$) for inhibitors I3-I5 to bovine carbonic anhydrase II (bCA II) in competitive experiments with sensor S1 (5 µM) obtained from fluorescence titration in aqueous HEPES buffer (50 mM) at pH 6.5, 7.2, 8.0 and ambient temperature.

<table>
<thead>
<tr>
<th>$K_a$ (M$^{-1}$)</th>
<th>pH 6.5</th>
<th>pH 7.2</th>
<th>pH 8.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>I3</td>
<td>$5.90 \times 10^7$ (↓)</td>
<td>$4.93 \times 10^7$ (↓)</td>
<td>$6.80 \times 10^7$ (↓)</td>
</tr>
<tr>
<td>I4</td>
<td>$1.85 \times 10^8$ (↓)</td>
<td>$7.34 \times 10^7$ (↓)</td>
<td>$4.66 \times 10^7$ (↓)</td>
</tr>
<tr>
<td>I5</td>
<td>$1.20 \times 10^8$ (↓)</td>
<td>$3.11 \times 10^7$ (↓)</td>
<td>NR</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>$\Delta K_a$ (M$^{-1}$)</th>
<th>pH 6.5</th>
<th>pH 7.2</th>
<th>pH 8.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>I3</td>
<td>$8.60 \times 10^5$</td>
<td>$9.37 \times 10^5$</td>
<td>$8.05 \times 10^6$</td>
</tr>
<tr>
<td>I4</td>
<td>$1.78 \times 10^7$</td>
<td>$7.14 \times 10^6$</td>
<td>$3.77 \times 10^6$</td>
</tr>
<tr>
<td>I5</td>
<td>$2.20 \times 10^7$</td>
<td>$3.17 \times 10^6$</td>
<td>NR</td>
</tr>
</tbody>
</table>

*Note: $K_a$ and $\Delta K_a$ were calculated based on the change in fluorescence maxima upon addition of protein by using 1:1 binding model. Symbol “↓” indicates fluorescence quenching upon addition of incremental amounts of the bCAII / inhibitor mixture.*

Table 2.7. Apparent binding affinities $K_a$ (M$^{-1}$) and errors of the apparent binding affinities $\Delta K_a$ (M$^{-1}$) for inhibitors I3-I5 to bovine carbonic anhydrase II (bCA II) in competitive experiments with sensor S1 (5 µM) obtained from fluorescence titration in aqueous HEPES buffer (pH 8.0) at HEPES concentration 20, 50 and 100 mM and ambient temperature.

<table>
<thead>
<tr>
<th>$K_a$ (M$^{-1}$)</th>
<th>20 mM</th>
<th>50 mM</th>
<th>100 mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>I3</td>
<td>$7.97 \times 10^7$ (↓)</td>
<td>$6.80 \times 10^7$ (↓)</td>
<td>$1.81 \times 10^8$ (↓)</td>
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<tr>
<td>I4</td>
<td>$3.43 \times 10^7$ (↓)</td>
<td>$4.66 \times 10^7$ (↓)</td>
<td>$1.64 \times 10^7$ (↓)</td>
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<tr>
<td>I5</td>
<td>$2.46 \times 10^{10}$ (↓)</td>
<td>$8.70 \times 10^7$ (↓)</td>
<td>$1.99 \times 10^7$ (↓)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>$\Delta K_a$ (M$^{-1}$)</th>
<th>20 mM</th>
<th>50 mM</th>
<th>100 mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>I3</td>
<td>$2.11 \times 10^7$</td>
<td>$8.05 \times 10^6$</td>
<td>$0.86 \times 10^8$</td>
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<tr>
<td>I4</td>
<td>$0.26 \times 10^7$</td>
<td>$3.77 \times 10^6$</td>
<td>$0.35 \times 10^7$</td>
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<tr>
<td>I5</td>
<td>$&gt;10^6$</td>
<td>$4.21 \times 10^6$</td>
<td>$0.70 \times 10^7$</td>
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</tbody>
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*Note: $K_a$ and $\Delta K_a$ were calculated based on the change in fluorescence maxima upon addition of protein by using 1:1 binding model. Symbol “↓” indicates fluorescence quenching upon addition of incremental amounts of the bCAII / inhibitor mixture.*
These fluorescence titration experiments revealed interesting dependencies of fluorescence response magnitude on varying pH value, HEPES buffer concentration and ionic strength of the media. The magnitude of fluorescence response was found decreasing with increasing pH value of the buffer. Bovine carbonic anhydrase II is positively charged at pH < pI and negatively charged at pH > pI (pI = 5.9). Increasing a density of negative charges on the protein surface by increasing pH necessarily induces conformational changes of the entire protein structure as well as its binding site leading to the decreased fluorescence response as well as $K_a$. We found that HEPES buffer pH = 7.2 was optimal and provided the largest difference between On/Off states. Increasing ionic strength (NaCl concentration) resulted in decreased fluorescence response.

### Table 2.8. Apparent binding affinities $K_a$ (M$^{-1}$) and errors of the apparent binding affinities $\Delta K_a$ (M$^{-1}$) for inhibitors 13-15 to bovine carbonic anhydrase II (bCA II) in competitive experiments with sensor S1 (5 μM) obtained from fluorescence titration in aqueous HEPES buffer (50 mM, pH 8.0) and NaCl at concentration 5, 50 and 100 mM and ambient temperature.

<table>
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<th>$K_a$ (M$^{-1}$)</th>
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<th>50 mM</th>
<th>100 mM</th>
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<td>$3.10 \times 10^7$ (↓)</td>
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<tr>
<td>14</td>
<td>$3.48 \times 10^7$ (↓)</td>
<td>$6.26 \times 10^7$ (↓)</td>
<td>$3.50 \times 10^7$ (↓)</td>
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<tr>
<td>15</td>
<td>$1.13 \times 10^9$ (↓)</td>
<td>$1.13 \times 10^8$ (↓)</td>
<td>$2.26 \times 10^8$ (↓)</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>$\Delta K_a$ (M$^{-1}$)</th>
<th>5 mM</th>
<th>50 mM</th>
<th>100 mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>13</td>
<td>$0.63 \times 10^6$</td>
<td>$0.45 \times 10^7$</td>
<td>$0.48 \times 10^7$</td>
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<tr>
<td>14</td>
<td>$0.22 \times 10^7$</td>
<td>$0.94 \times 10^7$</td>
<td>$0.50 \times 10^7$</td>
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<tr>
<td>15</td>
<td>$0.97 \times 10^9$</td>
<td>$0.82 \times 10^8$</td>
<td>$0.17 \times 10^8$</td>
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</tbody>
</table>

*Note: $K_a$ and $\Delta K_a$ were calculated based on the change in fluorescence maxima upon addition of protein by using 1:1 binding model. Symbol “↓” indicates fluorescence quenching upon addition of incremental amounts of the bCAII / inhibitor mixture.*
2.19 Determination of Binding Affinities: Direct Sensor Titrations

In order to obtain binding affinities of the sensors S1-S7 to CAs, the fluorescence titration isotherms obtained from the direct probe titrations by plotting normalized fluorescence response versus CA molar concentration were fitted by nonlinear fitting function for 1:1 binding model (Eqns. 3 and 4) using Newton’s iterative method as described by Anslyn and Sessler.151–153

\[
[S] = \frac{[S]_0 K_S - [H], K_S - 1}{2 K_S} \sqrt{([H], K_S - [S], K_S + 1)^2 + 4[S], K_S} 
\]  
(Equation 3)

\[
[H \cdot S] = \frac{K_S [S][H],}{1 + K_S [S]} = \frac{F - F_0}{F_i - F_0} 
\]  
(Equation 4)

[S]₀, [H]₀ are total concentrations of sensor and host (enzyme), respectively. \( K_S \) is a binding constant of the sensor and [S], and [H·S] are unknown concentration of the sensor and the complex, respectively. Eqn. 3 defines value of unknown [S] based on \( K_S \) and experimentally obtained values [H]₀ and [S]₀. \( F, F_0, \) and \( F_i \) are the sensor (S) concentration-dependent fluorescence intensity, fluorescence intensity without guest and fluorescence intensity at an infinite guest concentration, respectively.

2.20 Determination of Binding Affinities: Competitive Titrations

This model and related non-linear fluorescence isotherm data fitting script for Origin was developed and implemented by Dr. Mehmet Gökhan Caglayan, a postdoctoral research fellow in Anzenbacher Research Group in the spring of 2016. To obtain competitive binding affinities for the inhibitors I1-I14 (I) to CAs, the titration isotherms obtained from the competitive titrations by plotting the change in fluorescence intensity versus inhibitors concentration were fitted by nonlinear function for modified dye-displacement assay protocol using Newton’s iterative method as reported by Anslyn and Sessler.153 First, we utilized the direct probe fluorescence titration data
for sensor S1 (S) with bovine carbonic anhydrase II (H) to calculate equilibrium concentration of [H·S] at the saturation point (Eqn. 5). Sensors affinity constant $K_S$ was obtained as described above (part 2.9).

$$[H] + [S] \rightleftharpoons [H \cdot S] \quad K_S = \frac{[H \cdot S]}{[H][S]} \quad \text{(Equation 5)}$$

The equilibrium concentration of [H·S] at the saturation point was calculated from the quadratic equation (Eqn. 6) which can be simplified using a substitution (Eqn. 7) using Equation 8 (only the root $[H\cdot S] < [S]_t$ was considered).

$$K_S [H\cdot S]^2 - [H\cdot S] (K_S [H]_t + K_S [S]_t + 1) + K_S [S]_t [H]_t = 0 \quad \text{(Equation 6)}$$

$$Ax^2 + Bx + C = 0 \quad \text{(Equation 7)}$$

$$A = K_S$$

$$B = K_S [H]_t + K_S [S]_t + 1$$

$$C = K_S [S]_t [H]_t$$

$$[H\cdot S]_{1,2} = \frac{-B \pm \sqrt{B^2 - 4AC}}{2A} \quad \text{(Equation 8)}$$

Parameters $f_{HS}$ and $f_S$ are fluorescence constants of H·S and S, respectively, and were calculated from the direct probe fluorescence titration data for sensor S1 (S) with bovine carbonic anhydrase II (H) (Equations 9 and 10), where $F_0$ and $F_{sat}$ are the fluorescence intensities at the initial and saturation point, respectively.

$$f_s = \frac{F_0}{[S]_t} \quad \text{(Equation 9)}$$

$$f_{HS} = \frac{F_{sat}}{[H\cdot S]} \quad \text{(Equation 10)}$$
Table 2.9. Parameters used in the affinity constants model.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value Obtained</th>
<th>Unit</th>
<th>Obtained from</th>
</tr>
</thead>
<tbody>
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<td>28,980</td>
<td>g/mol</td>
<td>literature(^{154})</td>
</tr>
<tr>
<td>[H](_t)</td>
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<td>mol/L</td>
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</tr>
<tr>
<td>[S](_t)</td>
<td>5.00 \times 10^{-7}</td>
<td>mol/L</td>
<td>exp. data</td>
</tr>
<tr>
<td>(F_0)</td>
<td>256,320</td>
<td>cnts</td>
<td>exp. data</td>
</tr>
<tr>
<td>(F_{sat})</td>
<td>29,224</td>
<td>cnts</td>
<td>exp. data</td>
</tr>
<tr>
<td>(A = K_s) for S1 and bCA II</td>
<td>5.82 \times 10^8</td>
<td>M^{-1}</td>
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<td>(B)</td>
<td>-484.7950</td>
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<td>Eqn. 4, 5</td>
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<tr>
<td>(C)</td>
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<td>L</td>
<td>Eqn. 4, 5</td>
</tr>
<tr>
<td>([H\cdot S]_1)</td>
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<td>mol/L</td>
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<tr>
<td>([H\cdot S]_2)</td>
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<tr>
<td>(f_S)</td>
<td>5.1264 \times 10^{11}</td>
<td>cnts L/mol</td>
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<tr>
<td>(f_{HS}) (from ([H\cdot S]_1))</td>
<td>5.7869 \times 10^{10}</td>
<td>cnts L/mol</td>
<td>Eqn. 8</td>
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<tr>
<td>(f_{HS}) (from ([H\cdot S]_2))</td>
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<td>cnts L/mol</td>
<td>Eqn. 8</td>
</tr>
<tr>
<td>(f_{S})</td>
<td>9.6398 \times 10^{-5}</td>
<td>L</td>
<td>Eqn. 4, 5</td>
</tr>
<tr>
<td>(b)</td>
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<td>exp. settings</td>
</tr>
</tbody>
</table>

Known or calculated parameters \(K_s\), \([H]_t\), \([S]_t\), \(f_S\), \(f_{HS}\) were then used as initial conditions for Newton’s iterative method to calculate inhibitors affinity constants \(K_I\) using Origin curve fitting function (Eqn. 11).

\[
F = \frac{[S]_1(f_S b + f_{HS} b K_s [H])}{1 + K_s [H]}
\]  
(Equation 11)

Origin script:

```plaintext
A=Ki*Kg;
B=Ki+Kg+Ki*Kg*It+Ki*Kg*x-Ki*Kg*Ht;
C=1+Ki*It+Kg*x-(Ki+Kg)*Ht;
D=-Ht;
for (H=Ht, step=1; abs(step)>1E-14; H=H-step){
    step=(A*H*H+H+B*H+H+C*H+D)/(3*A*H+2*B*H+C);
}
y=It*(F1+Phi*Ki*H)/(1+Ki*H)
```
where $K_i, K_g, E_i, E_h, H_t, I_t, x$, and $y$ represent $K_S, K_f, f_S, f_{HS}, [H]_t, [S]_t, [I]_t$, and fluorescence intensity ($F$), respectively. $[I]_t$ is a total inhibitor concentration. Parameters $K_S, f_S$ and $f_{HS}$ were determined by the direct probe titration using 1:1 curve fitting (see above) of the host (bCA II) and indicator alone prior to this analysis. Each of these values, however, was treated as adjustable in accordance with Newton’s fitting method. $[H]_t$ and $[S]_t$ were treated as constants, and the variables $[I]_t$ ($x$-axis) and fluorescence intensity ($y$-axis) were read from the data sheet by the program. Given an initial value for $K_I$ (educated guess), the program iteratively found the value of $K_I$ that best fits the experimental data for each inhibitor.

2.21 Qualitative Fluorescence-Based Assays for Bovine and Human CA II

In the following parts, qualitative and quantitative analysis of the competitive fluorescence assays will be discussed. In order to investigate the utility of the dye-displacement type system and its feasibility in high-throughput settings to discriminate between inhibitors $I_1$-$I_{14}$, we designed a fluorescence microarray. For the design of fluorescence arrays, we decided to utilize indicators $S_1, S_3$, and $S_5$, which provided the best response in competitive titration experiments. The statistical evaluation of the array response to the solutions of inhibitors was explored using linear discriminant analysis (LDA).

The array experiments utilized sensors $S_1, S_3$ and $S_6$ (500 nM) and HEPES buffer (50 mM) at pH values 6.5, 7.2 and 8.0 and both human and bovine carbonic anhydrase isozymes II. The experimental work flow is summarized in Figure 2.88. The qualitative assay experiments including data processing were carried out by Ms. Elena G. Shcherbakova, a graduate student in the Anzenbacher Research Group in the spring of 2016.
2.21.1 General Procedure and Samples Preparation

The array experiments were performed in 1536 chemically resistant microplates MatriPlates MCR111-1-1 (MATRICAL), black and total working volume 6 μL. The liquids were dispensed using a robotic high-precision 16-channel liquid handling system NanoDrop Express (BioNex Solution Inc.) with fixed-tip head 2 × 8 configuration, dispense precision ≤ 5% at 1 μL. Each experiment was performed in 24 repetitions. The stock solutions of S1 (3 mM), S3 (3 mM) and S6 (3 mM) in dimethyl sulfoxide (DMSO) were prepared right before the experiments, and further used to prepare aliquots with a concentration of $5 \times 10^{-7}$ M in all experiments. The stock solution of bovine erythrocyte carbonic anhydrase II (bCA II) (1 mg/mL, $3.45066 \times 10^{-5}$ M) in HEPES buffer (50 mM, pH 7.2) was used to obtain final solution of bCA II for assay (5 μg/mL, $1.72533 \times 10^{-7}$ M). The stock solution of human carbonic anhydrase II (hCA II) (1 mg/mL, $3.43466 \times 10^{-5}$ M) in HEPES buffer (50 mM, pH 7.2) was used to obtain final working solution of hCA II.

![Figure 2.88. A work flow chart depicting high-throughput assay experiments using carbonic anhydrase isozymes and inhibitors I1-I14.](image)
for assay (5 μg/ml, $1.71733 \times 10^{-7}$ M). The stock solutions of inhibitors (I) were prepared at concentration of 8.62665 mM in DMSO; **I1**: methanesulfonamide (MetS), **I2**: *tert*-butyl sulfonamide (*tert*-BS), **I3**: 4-methylbenzene sulfonamide (4-MBS), **I4**: 2-methylbenzene sulfonamide (2-MBS), **I5**: 4-(*tert*-butyl)benzene sulfonamide (4-*tert*-BBS), **I6**: naphthalene 2-sulfonamide (Nph-2-S), **I7**: 4-nitrobenzene sulfonamide (4-NBS), **I8**: 4-phenoxybenzene sulfonamide (4-PBS), **I9**: 2-thiophene sulfonamide (2-ThpS), **I10**: acetazolamide (ACZA), **I11**: methazolamide (MZA), **I12**: ethoxzolamide (EtZA), **I13**: brinzolamide (BZA), **I14**: celecoxib (CeLCX). Final concentration of inhibitors was $1.7173 \times 10^{-5}$ M for hCA II, and $1.725 \times 10^{-5}$ M for bCA II which corresponds to 1/100 ($n/n$) protein to inhibitor ratio. HEPES buffers (50 mM) with three pH values were used to expand informational density of the assay: B1, pH 6.5; B2, pH 7.2 and B3, pH 8.0.

HEPES buffer, CAII, and sensor solution were pre-mixed in 0.5 mL PCR tubes from their stock solutions in order to obtain the final concentration. The resulting mixtures were incubated for 15 min in Eppendorf Thermomixer R (23 °C) to ensure the formation of protein/sensor complex. The incubated protein/sensor mixtures were dispensed under mild conditions (slow ramp and rate) using a high-precision 16-channel pipetting system NanoDrop Express into MatriPlates microtiter plates. Subsequently, inhibitor solution was added with NanoDrop Express in order to obtain different inhibitor/sensor ratios and reach a total volume of 5 μL per well with total amount of DMSO 0.4% (v/v). For the control experiments an equal amount of DMSO was added instead of the inhibitor solution. The printed plate was then centrifuged for 4 min (1400 rpm, T = 294 K) to allow to establish the equilibrium in the competitive binding (*Figure 2.88*).

Plates were immediately read after incubation using BMG CLARIOStar® microplate reader. Simultaneous measurements of fluorescence intensity (FI) changes were carried out, and
responses were recorded at the same optics settings. Each sample was analyzed in a set of 24 repetitions.

### 2.2.1.2 Fluorescence Intensity Data Treatment

The response patterns associated with the sensor array are acquired in a form of multivariate data set. Hence, chemometric pattern recognition techniques such as supervised methods including LDA (reduces the dimensionality of the data into a lower dimensional space), and HCA (does not reduce dimensions of the data sets) were employed to identify the samples and separate the data set into groups of clusters based on similarities in the response data.

First steps prior to the application of multivariate data analysis techniques are variable selection/reduction and data pre-treatment. For this purpose, analysis-of-variance (ANOVA) statistical method can be applied in order to evaluate the effect of different variables on the data set. Additionally, data were subjected to the Student’s $t$-test to exclude 4 data points out of 24 repetitions in order to examine the difference (standard deviation) between data within the set of repetitions for each sample and exclude outlying data points from the set of variables. The coefficient of variability among the data within the class of 20 repetitions did not exceed 4 % and data obtained for qualitative analysis were then analyzed using linear discriminant analysis without any further pretreatment.

**Table 2.10.** Optics settings used for microplate fluorescence reader for S1, S3 and S6.

<table>
<thead>
<tr>
<th>Optics settings for sensor S1</th>
<th>Optics settings for sensor S3</th>
</tr>
</thead>
<tbody>
<tr>
<td>360</td>
<td>420</td>
</tr>
<tr>
<td>330</td>
<td>420</td>
</tr>
<tr>
<td>359</td>
<td>450</td>
</tr>
</tbody>
</table>
The LDA analysis revealed an excellent recognition capability of the indicators as illustrated by the 100% correct classification of all 8,640 trials. *Figure 2.89* and *B* show the LDA results in a 3D response space defined by the first three canonical factors (F1-F3) for bovine (A) and human (B) carbonic anhydrase isozymes II. Here, the sensor array recognized the inhibitors I1-I14 and sorted them into three subgroups according to their structural features: aliphatic, aromatic inhibitors, and high-affinity inhibitors (commercially available drugs). Additionally, canonical scores plots for both assays (*Figure 2.90* and *Figure 2.90*) show very good data clustering in 2D even when the response space is defined by other canonical factors. This result is very important because it shows that the observed responses correlate with the structure of the inhibitors.

### 2.22 Semi-Quantitative Fluorescence-Based Assays for Bovine CA II

In order to carry out semi-quantitative assay for inhibitors I5, I9, I10, I11 and I13 we designed a fluorescence-based microarray experiment. The array experiments utilized sensors S1, S3 and S6 (500 nM) and HEPES buffer (50 mM) at pH values 6.5, 7.2 and 8.0 and bovine carbonic anhydrase isozyme II. The experimental work flow is summarized in *Figure 2.88*. The semi-quantitative array experiments including data processing were carried out by Ms. Elena G. Shcherbakova, a graduate student in Anzenbacher Research Group in the spring of 2016.
2.22.1 General Procedure and Samples Preparation

The array experiments were performed in 1536 chemically resistant microplates MaKO™ 1536, Assay/Storage Plate, Solid Bottom, Evaporation Barrier (Aurora Biotechnologies), black and total working volume 9.03 μL. The liquids were dispensed using a robotic high-precision 16-channel liquid handling system NanoDrop Express (BioNex Solution Inc.) with fixed-tip head 2 × 8 configuration, dispense precision ≤ 5% at 1 μL. Each experiment was performed in 24 repetitions. HEPES buffers (50 mM) with three pH values were used to expand informational density of the assay: B1, pH 6.5; B2, pH 7.2 and B3, pH 8.0.

The stock solutions of \textbf{S1} (3 mM), \textbf{S3} (3 mM) and \textbf{S5} (3 mM) in dimethyl sulfoxide (DMSO) were prepared right before the experiments, and further used to prepare aliquots in HEPES buffer with a concentration of $3 \times 10^{-4}$ M in all experiments. The stock solution of bovine erythrocyte carbonic anhydrase II (bCA II) (1 mg/mL, $3.45066 \times 10^{-5}$ M) in HEPES buffer (50 mM, pH 7.2) was used to obtain the final solution of bCA II for assay (5 μg/mL, $1.72533 \times 10^{-7}$ M). The stock solutions of inhibitors (I) were prepared at concentration of 8.62665 mM in DMSO; \textbf{I5}: 4-(\textit{tert}-butyl)benzene sulfonamide (4-tert-BBS), \textbf{I9}: 2-thiophene sulfonamide (2-ThpS), \textbf{I10}: acetazolamide (ACZA); \textbf{I11}: methazolamide (MZA), \textbf{I13}: brinzolamide (BZA). Final concentrations of the inhibitors were chosen based on fluorescence titration data:

\textbf{4-(\textit{tert}-butyl)benzene sulfonamide (I5)}: Saturation concentration for \textbf{I5} determined from fluorescence titration data was $c_{sat} = 207$ μM. Total DMSO concentration in each analyzed mixture was adjusted by DMSO added (added $V_{DMSO}$) to reach 2.5% ($v/v$).
Table 2.11. Table of solutions composition of inhibitor I5 in the semi-quantitative assay for bovine carbonic anhydrases isozyme II.

<table>
<thead>
<tr>
<th>cI5 (μM)</th>
<th>VI (μL)</th>
<th>Vsensor (μL)</th>
<th>VDMSO (μL)</th>
<th>cDMSO (%)</th>
<th>ΔV (μL)</th>
<th>Added VDMSO (μL)</th>
<th>VbeCA II (μL)</th>
<th>Vbuffer (μL)</th>
<th>Vtot (μL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.0</td>
<td>0.7</td>
<td>0.7</td>
<td>0.17</td>
<td>2.3</td>
<td>9.27</td>
<td>2.00</td>
<td>388.1</td>
<td>400</td>
</tr>
<tr>
<td>20</td>
<td>0.9</td>
<td>0.7</td>
<td>1.6</td>
<td>0.4</td>
<td>2.1</td>
<td>8.3</td>
<td>2.00</td>
<td>388.1</td>
<td>400</td>
</tr>
<tr>
<td>40</td>
<td>1.9</td>
<td>0.7</td>
<td>2.5</td>
<td>0.6</td>
<td>1.9</td>
<td>7.4</td>
<td>2.00</td>
<td>388.1</td>
<td>400</td>
</tr>
<tr>
<td>60</td>
<td>2.8</td>
<td>0.7</td>
<td>3.4</td>
<td>0.9</td>
<td>1.6</td>
<td>6.5</td>
<td>2.00</td>
<td>388.1</td>
<td>400</td>
</tr>
<tr>
<td>80</td>
<td>3.7</td>
<td>0.7</td>
<td>4.4</td>
<td>1.1</td>
<td>1.4</td>
<td>5.6</td>
<td>2.00</td>
<td>388.1</td>
<td>400</td>
</tr>
<tr>
<td>100</td>
<td>4.6</td>
<td>0.7</td>
<td>5.3</td>
<td>1.3</td>
<td>1.2</td>
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<td>2.00</td>
<td>388.1</td>
<td>400</td>
</tr>
<tr>
<td>120</td>
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<td>6.2</td>
<td>1.6</td>
<td>0.9</td>
<td>3.7</td>
<td>2.00</td>
<td>388.1</td>
<td>400</td>
</tr>
<tr>
<td>140</td>
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<td>0.7</td>
<td>7.2</td>
<td>1.8</td>
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<td>2.8</td>
<td>2.00</td>
<td>388.1</td>
<td>400</td>
</tr>
<tr>
<td>160</td>
<td>7.4</td>
<td>0.7</td>
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<td>2.0</td>
<td>0.5</td>
<td>1.9</td>
<td>2.00</td>
<td>388.1</td>
<td>400</td>
</tr>
<tr>
<td>180</td>
<td>8.3</td>
<td>0.7</td>
<td>9.0</td>
<td>2.3</td>
<td>0.2</td>
<td>0.9</td>
<td>2.00</td>
<td>388.1</td>
<td>400</td>
</tr>
<tr>
<td>200</td>
<td>9.3</td>
<td>0.7</td>
<td>9.9</td>
<td>2.5</td>
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<td>0.0</td>
<td>2.00</td>
<td>388.1</td>
<td>400</td>
</tr>
</tbody>
</table>

2-Thiophene sulfonamide (I9): Saturation concentration for I9 determined from fluorescence titration data was $c_{\text{sat}} = 952 \, \mu\text{M}$. Total DMSO concentration in each analyzed mixture was adjusted by DMSO added (added $V_{\text{DMSO}}$) to reach 11.8% (v/v).

Table 2.12. Table of solutions composition of inhibitor I9 in the semi-quantitative assay for bovine carbonic anhydrases isozyme II.

<table>
<thead>
<tr>
<th>cI9 (μM)</th>
<th>VI (μL)</th>
<th>Vsensor (μL)</th>
<th>VDMSO (μL)</th>
<th>cDMSO (%)</th>
<th>ΔV (μL)</th>
<th>Added VDMSO (μL)</th>
<th>VbeCA II (μL)</th>
<th>Vbuffer (μL)</th>
<th>Vtot (μL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.0</td>
<td>0.7</td>
<td>0.7</td>
<td>0.17</td>
<td>11.6</td>
<td>46.37</td>
<td>2.00</td>
<td>351.0</td>
<td>400</td>
</tr>
<tr>
<td>100</td>
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<td>0.7</td>
<td>5.3</td>
<td>1.3</td>
<td>10.4</td>
<td>41.7</td>
<td>2.00</td>
<td>351.0</td>
<td>400</td>
</tr>
<tr>
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<td>9.3</td>
<td>0.7</td>
<td>9.9</td>
<td>2.5</td>
<td>9.3</td>
<td>37.1</td>
<td>2.00</td>
<td>351.0</td>
<td>400</td>
</tr>
<tr>
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<td>400</td>
</tr>
<tr>
<td>400</td>
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<td>19.2</td>
<td>4.8</td>
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<td>351.0</td>
<td>400</td>
</tr>
<tr>
<td>500</td>
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<td>23.9</td>
<td>6.0</td>
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<td>2.00</td>
<td>351.0</td>
<td>400</td>
</tr>
<tr>
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<td>7.1</td>
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<td>18.5</td>
<td>2.00</td>
<td>351.0</td>
<td>400</td>
</tr>
<tr>
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<td>0.7</td>
<td>33.1</td>
<td>8.3</td>
<td>3.5</td>
<td>13.9</td>
<td>2.00</td>
<td>351.0</td>
<td>400</td>
</tr>
<tr>
<td>800</td>
<td>37.1</td>
<td>0.7</td>
<td>37.8</td>
<td>9.4</td>
<td>2.3</td>
<td>9.3</td>
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<td>400</td>
</tr>
<tr>
<td>900</td>
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<td>400</td>
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<tr>
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<td>0.0</td>
<td>2.00</td>
<td>351.0</td>
<td>400</td>
</tr>
</tbody>
</table>

Acetazolamide (I10) and methazolamide (I11): Saturation concentration for I10 and I11, determined from fluorescence titration data was $c_{\text{sat}} = 135 \, \mu\text{M}$, and 155 μM, respectively. Total
DMSO concentration in each analyzed mixture was adjusted by DMSO added (added $V_{\text{DMSO}}$) to reach 1.9% (v/v).

**Table 2.13.** Table of solutions composition of inhibitor I10 and I11 in the semi-quantitative assay for bovine carbonic anhydrases isozyme II.

<table>
<thead>
<tr>
<th>$c_{I10}$ or $c_{I11}$ (μM)</th>
<th>$V_1$ (μL)</th>
<th>$V_{\text{sensor}}$ (μL)</th>
<th>$V_{\text{DMSO}}$ (μL)</th>
<th>$c_{\text{DMSO}}$ (%)</th>
<th>$\Delta V$ (μL)</th>
<th>Added $V_{\text{DMSO}}$ (μL)</th>
<th>$V_{\text{beCA II}}$ (μL)</th>
<th>$V_{\text{buffer}}$ (μL)</th>
<th>$V_{\text{tot}}$ (μL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.0</td>
<td>0.7</td>
<td>0.7</td>
<td>0.17</td>
<td>1.7</td>
<td>6.96</td>
<td>2.00</td>
<td>390.4</td>
<td>400</td>
</tr>
<tr>
<td>10</td>
<td>0.5</td>
<td>0.7</td>
<td>1.1</td>
<td>0.3</td>
<td>1.6</td>
<td>6.5</td>
<td>2.0</td>
<td>390.4</td>
<td>400</td>
</tr>
<tr>
<td>20</td>
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<td>0.7</td>
<td>1.6</td>
<td>0.4</td>
<td>1.5</td>
<td>6.0</td>
<td>2.0</td>
<td>390.4</td>
<td>400</td>
</tr>
<tr>
<td>30</td>
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<td>2.1</td>
<td>0.5</td>
<td>1.4</td>
<td>5.6</td>
<td>2.0</td>
<td>390.4</td>
<td>400</td>
</tr>
<tr>
<td>40</td>
<td>1.9</td>
<td>0.7</td>
<td>2.5</td>
<td>0.6</td>
<td>1.3</td>
<td>5.1</td>
<td>2.0</td>
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<td>400</td>
</tr>
<tr>
<td>50</td>
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<td>3.0</td>
<td>0.7</td>
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<td>60</td>
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<td>3.4</td>
<td>0.9</td>
<td>1.0</td>
<td>4.2</td>
<td>2.0</td>
<td>390.4</td>
<td>400</td>
</tr>
<tr>
<td>80</td>
<td>3.7</td>
<td>0.7</td>
<td>4.4</td>
<td>1.1</td>
<td>0.8</td>
<td>3.2</td>
<td>2.0</td>
<td>390.4</td>
<td>400</td>
</tr>
<tr>
<td>100</td>
<td>4.6</td>
<td>0.7</td>
<td>5.3</td>
<td>1.3</td>
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<td>0.0</td>
<td>2.0</td>
<td>390.4</td>
<td>400</td>
</tr>
<tr>
<td>120</td>
<td>5.6</td>
<td>0.7</td>
<td>6.2</td>
<td>1.6</td>
<td>0.3</td>
<td>1.4</td>
<td>2.0</td>
<td>390.4</td>
<td>400</td>
</tr>
<tr>
<td>150</td>
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<td>0.7</td>
<td>7.6</td>
<td>1.9</td>
<td>0.0</td>
<td>0.0</td>
<td>2.0</td>
<td>390.4</td>
<td>400</td>
</tr>
</tbody>
</table>

**Brinzolamide (I13):** Saturation concentration for I13 determined from fluorescence titration data was $c_{\text{sat}} = 900$ nM. Total DMSO concentration in each analyzed mixture was adjusted by DMSO added (added $V_{\text{DMSO}}$) to reach 1.3% (v/v).

**Table 2.14.** Table of solutions composition of inhibitor I13 in the semi-quantitative assay for bovine carbonic anhydrases isozyme II.

<table>
<thead>
<tr>
<th>$c_{I13}$ (nM)</th>
<th>$V_1$ (μL)</th>
<th>$V_{\text{sensor}}$ (μL)</th>
<th>$V_{\text{DMSO}}$ (μL)</th>
<th>$c_{\text{DMSO}}$ (%)</th>
<th>$\Delta V$ (μL)</th>
<th>Added $V_{\text{DMSO}}$ (μL)</th>
<th>$V_{\text{beCA II}}$ (μL)</th>
<th>$V_{\text{buffer}}$ (μL)</th>
<th>$V_{\text{tot}}$ (μL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.0</td>
<td>0.7</td>
<td>0.7</td>
<td>0.17</td>
<td>1.2</td>
<td>4.64</td>
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<td>392.70</td>
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</tr>
<tr>
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<td>0.7</td>
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<td>2.0</td>
<td>392.7</td>
<td>400</td>
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</table>
HEPES buffer, solution of CA II, and sensor solution were pre-mixed in 500 μL PCR tubes from their stock solutions in order to obtain the final concentration. The resulting mixtures were incubated for 15 min in Eppendorf Thermomixer R (23 °C) to ensure the formation of protein/sensor complex. To prepare standard solutions of desired inhibitor concentrations \((c_I)\), different amounts of inhibitor stock solution \((V_I)\) and DMSO \((V_{DMSO})\) were added in order to reach a total volume \((V_{tot})\) of 400 μL in 500 μL PCR tubes. This allowed to prepare solutions with different inhibitor/sensor ratios. The tube contained a standard solution of beCA II \((V_{beCA II})\), sensor standard solution \(V_{sensor}\) and buffer solution \(V_{buffer}\). For the control experiments, only DMSO was added instead of the inhibitor solution: 
\[
V_{tot} = V_I + V_{DMSO} + V_{sensor} + V_{beCA II} + V_{buffer}
\]

Subsequently, the incubated protein/sensor/inhibitor mixtures were dispensed under mild conditions (slow ramp and rate of the dispensing) using a high-precision 16-channel pipetting system NanoDrop Express MaKO™ 1536, Assay/Storage Plates in order to reach a total volume 5 μL with equal amount of DMSO in each well. The printed plates were centrifuged for 4 min (1400 rpm, \(T = 294\) K). For the control experiments an equal amount of DMSO was added instead of the inhibitor solution (Figure 2.88).

Plates were immediately read after incubation using BMG CLARIOStar® microplate reader. Simultaneous measurements of fluorescence intensity (FI) changes were carried out, and responses were recorded at the same optics settings. Each sample was analyzed in a set of 24 repetitions.

### 2.22.2 Fluorescence Intensity Data Treatment

The response patterns associated with the sensor array are acquired in a form of multivariate data set. Quantitative regression analyses of inhibitors (supervised algorithm) such as
support vector machine (SVM) coupled with principal component analysis (PCA) and partial least squares (PLS) methods were applied. The recognition ability of the model was tested using \( n \)-fold cross-validation approach. The training set was used to calibrate the model producing the root mean square error of calibration (RMSEC). The model was validated using \( n \)-fold cross-validation approach producing the root mean square error of cross-validation (RMSECV). The validity and predictive ability of the developed model was then tested using independent data sets (unknown samples). The predictive accuracy of a model was evaluated by the value of the root mean square error of prediction (RMSEP).

The first steps prior to the application of multivariate data analysis techniques are variable selection/reduction and data pre-treatment. For this purpose, analysis-of-variance (ANOVA) statistical method can be applied in order to evaluate the effect of different variables on the data set. Additionally, data were subjected to the Student’s t-test to exclude 4 data points out of 24 repetitions in order to examine the difference (standard deviation) between data within the set of repetitions for each sample and exclude outlying data points from the set of variables. The coefficient of variability among the data within the class of 20 repetitions did not exceed 4% and data obtained for qualitative analysis were then analyzed using linear discriminant analysis without any further pretreatment. Total amount of analytes: 55 (50 different inhibitor concentrations and 5 controls). Controls for each inhibitor were analyzed as one cluster which contains 100 cases. Total amount of variables: 24. Dimensions of input multivariate data matrix: 1,320 \( \times \) 24. (before \( t \)-test). Dimensions of input multivariate data matrix: 1,100 \( \times \) 23 (after \( t \)-test). Total amount of observations: 31,680. Total amount of observations included in the analysis: 25,300. Classical discriminant analysis processed 24 variables (VAR_1$ is the sample identities) and 1,100 cases.
Table 2.15. Optics settings used for microplate fluorescence reader for S1, S3 and S6.

<table>
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<tr>
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<td>7</td>
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<td></td>
<td>330</td>
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<td>1620-1927</td>
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Figure 2.89. Graphical output of qualitative LDAs for a competitive assay of bovine (A) and human (B) carbonic anhydrase isozyme II (5 μg/mL) with inhibitors I1-I14 ([bCA II]/[inhibitor] = [hCA II]/[inhibitor] = 1/100) in HEPES (50 mM, pH 6.5, 7.2 and 8.0) for sensors S1, S3, and S6 (500 nM) obtained in 1536-well plate (total volume 4 μL per well) provided 100% correct classification.
Figure 2.90. Canonical scores plot for LDA of qualitative assay for bovine carbonic anhydrase isozyme II and inhibitors I1-I14 (and control).
Table 2.16. Table of cumulative proportion of total dispersion for the qualitative assay for bovine carbonic anhydrase isozyme II and inhibitors I1-I14 (plus control).

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Table 2.17. Table of cumulative proportion of total dispersion for the qualitative assay for human carbonic anhydrase isozyme II and inhibitors I1-I14 (plus control).

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Figure 2.91. Canonical scores plot for LDA of qualitative assay for human carbonic anhydrase isozyme II and inhibitors I1-I14 (and control).
Table 2.18. Table of cumulative proportion of total dispersion for the semi-quantitative assay for bovine carbonic anhydrase isozyme II and inhibitors I5, I9, I10, I11, I13 (plus control).

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Figure 2.92. Canonical scores plot for LDA of semi-quantitative assay for bovine carbonic anhydrase isozyme II and inhibitors I5, I9, I10, I11, I13 (plus control).
Figure 2.93. Graphical output of the semi-quantitative LDA for a competitive assay of bovine carbonic anhydrase isozyme II (5 μg/mL) with inhibitors I5, I9, I10, I11, I13 in HEPES (50 mM, pH 6.5, 7.2 and 8.0) for sensors S1, S3, and S6 (500 mM) obtained in 1536-well plate (total volume 4 μL per well) provided 100% correct classification.
Figure 2.94. **A:** Graphical output of the semi-quantitative LDA for a competitive assay of bCA II (5 μg/mL) with inhibitors \( I_5 \) (purple), \( I_9 \) (green), \( I_{10} \) (red), \( I_{11} \) (blue) and \( I_{13} \) (orange) ([hCA II]/[inhibitor] = 1:100) using sensors \( S_1, S_3, \) and \( S_6 \) (500 nM). The response space is defined by the first two factors (F1 and F2) and the third dimension is defined by inhibitor concentration (z-axis). **B:** Results of the semi-quantitative LDA for a competitive assay of bCA II (5 μg/mL) with inhibitors \( I_5 \) (purple), \( I_9 \) (green), \( I_{10} \) (red), \( I_{11} \) (blue) and \( I_{13} \) (orange). The LDA recognition capability is 100% for 50 data-points with 20 repetitions in each cluster, and a control with 100 repetitions within the cluster. The response space is defined by the first two factors (F1 and F2) of LDA, 2D plot comprising 72.5% of total variances. **C, D:** Results of the semi-quantitative LDA for a competitive assay of bCA II (5 μg/mL) with inhibitors \( I_5, I_9, I_{10}, I_{11} \) and \( I_{13} \). The response space is defined by the first factor (F1) of LDA using concentration-dependent normalization (y-axis).
The positive outcome of the qualitative assay suggested that a semi-quantitative assay may also be successful. To test this hypothesis, we set up a semi-quantitative assay using bovine carbonic anhydrase II and the same set of variables to identify varying concentrations of $I_5$, $I_9$, $I_{10}$, $I_{11}$ and $I_{13}$ (Figure 2.93 and 95). All five inhibitors showed smooth isotherm-like trends suggesting direct correlation between the response magnitude and inhibitor’s concentration. 

*Figure 2.94* panel A shows the LDA results in a 3D response space defined by the first two canonical factors (F1 and F2 containing 72.5% of data variance) and concentration of inhibitor in mM (z-axes). Panel B represents a projection of the trends shown in panel A into F1-F2 plane. Panels C and D show the semi-quantitative analysis utilizing F1 largely defined by the fluorescence response and concentration-dependent normalization, which highlights the difference in affinity among different inhibitors. As expected, the most efficient inhibitor: $I_{13}$ ($K_G = 8.4 \times 10^{10} \text{M}^{-1}$) elicits the largest magnitude in the fluorescence response from enzyme-indicator assemblies, while on the other extreme, the low response of the $I_9$ ($K_G = 2.5 \times 10^6 \text{M}^{-1}$) reflects a lower affinity compared to the rest of inhibitors in the array. This is encouraging as it suggests that binding affinity of the inhibitors to the CA II may be evaluated based on the magnitude of their fluorescence responses reflected by the canonical factors of LDA.

### 2.23 Quantitative Fluorescence-Based Assay for Bovine CA II

For the quantitative analysis of inhibitors $I_5$, $I_9$, $I_{10}$, $I_{11}$ and $I_{13}$, we used a support vector machine (SVM) linear regression method, which is suitable for modeling complex responses and nonlinear behavior of the data. Here, we used eight inhibitor concentrations to model the behavior of the data and two inhibitor concentrations as a validation data set for the model.
Figure 2.95. The results of simultaneous quantitative linear regression analysis of competitive binding assay of bovine carbonic anhydrase isozyme II using support vector machine algorithm for inhibitors I5 (A), I9 (B), I10 (C), I11 (D) and I13 (E).
The plots of predicted versus real concentration for inhibitors attest to the predictive power of the model (Figure 2.95). The array experiments also utilized sensors S1, S3 and S6 (500 nM) and HEPES buffer (50 mM) at pH values 6.5, 7.2 and 8.0 and bovine carbonic anhydrase isozyme II. The experimental work flow is summarized in Figure 2.88. The plots (Figure 2.95) show simultaneous quantitative linear regression analysis of competitive binding assay of bovine carbonic anhydrase isozyme II. Using support vector machine algorithm for inhibitors I5, I9, I10, I11 and I13, we obtained the actual vs. predicted concentration with high accuracy of prediction for multiple I5 concentration values. The root-mean-square errors (RMSE) of calibration (RMSEC), cross-validation (RMSECV), and prediction (RMSEP) attest to the quality of the model and prediction. Two unknown samples (red circle ●) were simultaneously correctly analyzed for I5 (60 μM and 160 μM), I9 (300 μM and 700 μM), I10 (30 μM and 80 μM), I11 (30 μM and 120 μM) and I13 (200 nM and 800 nM). The quantitative analysis experiments including data processing were carried out by Ms. Elena G. Shcherbakova, a graduate student in the Anzenbacher Research Group in the spring of 2016.

2.24 Determination of the Limit of Detection

We were able to determine the limit of detection (LOD),156 for inhibitors I5, I7, I8, I10 and I12. The LOD values were obtained using the data set from the quantitative assay: I5, I7, I8, I10 and I12 as three standard deviations of twenty repetitions of the validation data set concentration readings for a given inhibitor using S1 (500 nM, HEPES 50 mM, pH 7.2) with bCA II (0.172 μM). The obtained LODs are as follows: tert-butylbenzene sulfonamide, I5 (LOD = 4.2 μM = 890 ppb), 2-thiophene sulfonamide, I9 (LOD = 19.2 μM = 3 ppm), acetazolamide, I10 (LOD = 2.1 μM = 470 ppb), methazolamide, I11 (LOD = 2.8 μM = 660 ppb) and brinzolamide, I13
Importantly, the values of LOD obtained were found to be well below usual therapeutic concentrations, for these clinically used drugs (Table 2.19). This suggests that the presented assay can be used for quantitation of these compounds in body fluids.

Table 2.19. Table of common therapeutic concentrations for selected CAIs.

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<tr>
<th>Drug/Inhibitor</th>
<th>IC50 (hCA II) [nM]</th>
<th>Common therapeutic concentration [μM]</th>
<th>Limit of detection</th>
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<tr>
<td>Acetazolamide</td>
<td>12</td>
<td>13</td>
<td>2.1 μM (470 ppb)</td>
</tr>
<tr>
<td>Methazolamide</td>
<td>14</td>
<td>13</td>
<td>2.8 μM (660 ppb)</td>
</tr>
<tr>
<td>Ethoxzolamide</td>
<td>0.01</td>
<td>1</td>
<td>ND</td>
</tr>
<tr>
<td>Dorzolamide</td>
<td>0.18</td>
<td>18</td>
<td>ND</td>
</tr>
<tr>
<td>Brinzolamide</td>
<td>3</td>
<td>10</td>
<td>14 nM (6 ppb)</td>
</tr>
<tr>
<td>Celecoxib</td>
<td>21</td>
<td>0.3</td>
<td>ND</td>
</tr>
</tbody>
</table>

2.25 Hierarchical Clustering Analysis: Assays for Bovine and Human CA II

Hierarchical clustering analysis (HCA) is a simple and model-free chemometrical method that forms a hierarchical tree (dendrogram) based on clustering of the array response data. HCA for the 14 different analytes (I1-I14) can be seen in Figure 2.96. For the 3,600 total cases, there were no errors and zero misclassifications giving 100% correct classification for both bovine and human carbonic anhydrases.

Data for bovine carbonic anhydrase II were reduced to 10 repetitions in each class (multivariate matrix 150 × 24) and subjected to HCA using Minitab. The HCA does not reduce dimensions of the data set, but uses the distances (or similarities) between variables when forming the new clusters. The Euclidean distances were computed based on the geometrical distance in the multidimensional space. After clusters have been linked together, the Ward's linkage
(amalgamation) method was used to determine the distances between the new clusters. Clustering analysis of observations processed 24 variables and 150 cases.

Figure 2.96. Hierarchical clustering analysis dendrograms (Euclidean Distance, Ward's Linkage, Amalgamation Steps) for the competitive assay of bovine (A) and human (B) carbonic anhydrase isozyme II (5 μg/mL) with inhibitors I1-I14 ([hCA II]/[inhibitor] = 1/100) in HEPES (50 mM, pH 6.5, 7.2 and 8.0) for sensors S1, S3, and S5 (500 mM) displays 100% correct classification. (Green, aliphatic inhibitors; blue, aromatic inhibitors; red, high-affinity inhibitors.)
Data for human carbonic anhydrase II were pre-processed by LDA (14 score factors), and reduced to 10 repetitions (observations) in each class (analyte) excluding control (multivariate matrix $140 \times 14$) and subjected to the HCA. The HCA utilized 14 dimensions and represents the pattern of the data, but uses the distances (or similarities) between variables when forming the new clusters. The Euclidean distances were computed based on the geometrical distance in the multidimensional space. After clusters have been linked together, the Ward's linkage (amalgamation) method used to determine the distances between the new clusters. Clustering analysis of observations processed 14 score factors and 140 cases.

### 2.26 Molecular Modeling: Sensors and Inhibitors Docking Study

The part involving molecular computational modeling is a contribution from Dr. Samer Gozem, a postdoctoral research fellow at University of Southern California, Los Angeles, in the spring of 2016. In the following part, we will outline the protocol used for the docking analysis. First, geometries for inhibitors $I_{11-I_{14}}$ and fluorescent sensors $S_{1-S_{5}}$ were optimized using density functional theory (DFT) with the B3LYP functional, and $6-31G^*$ basis set. Atomic charges were computed using the RESP model, following the same protocol used to derive charges for the Amber force fields by fitting the electrostatic potential computed at the Hartree-Fock/6-31G* level of theory. Quantum chemical calculations were performed with Gaussian 09. All inhibitors and sensors were assumed to be negatively charged due to the deprotonation of the sulfonamide nitrogen, in line with neutron diffraction studies on acetazolamide-bound human carbonic anhydrase II that have indicated an anionic ligand.

The receptor used for docking is human carbonic anhydrase isozyme II (PDB entry 1OKN). In this crystal structure the carbonic anhydrase is complexed with the 4-sulfonamide-
[1-(4-N-(5-fluorescein thiourea)butane inhibitor, but the inhibitor was removed from the crystal structure for docking purposes. The reason for our choice to use 1OKN is justified by Tuccinardi and coworkers.\textsuperscript{166} The authors performed a cross-docking analysis of 40 different sulfonamide-based inhibitors complexed with carbonic anhydrase isozymes II and found that 1OKN gives the best docking results (lowest RMSD error compared to X-ray structure) out of all crystal structures tested.

Since the inhibitors and sensors bind directly to the zinc center within carbonic anhydrase II, it is important to use docking software that can account for the ligand-Zn\textsuperscript{2+} interaction correctly. Therefore, the AutoDock4\textsubscript{Zn} docking software was used with the Lamarckian Genetic Algorithm (LGA). AutoDock4\textsubscript{Zn} has been carefully calibrated and tested on zinc-containing systems and has yielded promising results.\textsuperscript{167} For the docking analysis of \textbf{I1-I14} and \textbf{S1-S4} and \textbf{S6}, the center of the grid box was placed near the center of the binding site, and the grid size was set to 22.5 Å × 22.5 Å × 22.5 Å. For each of \textbf{I1-I14}, 10 docking runs were performed with the maximum number of energy evaluations set to 2.5 × 10\textsuperscript{6}. Instead, for \textbf{S1-S4} and \textbf{S6}, 100 dockings were run with the maximum number of energy evaluations set to 5.0 × 10\textsuperscript{7}, since \textbf{S1-S4} and \textbf{S6} are significantly larger and have a larger number of flexible torsions.

The structures with the lowest energies produced by the docking in AutoDock4\textsubscript{Zn} are shown in Figure 2.97. Those structures are all bound to the zinc center (shown in magenta) via the sulfonamide moiety, indicating that AutoDock4\textsubscript{Zn} is capable of predicting the correct binding for all 19 ligands (5 sensors + 14 inhibitors). A comparison of the docking geometries with available crystal structures of human carbonic anhydrase II bound with acetazolamide (\textbf{I10}), ethoxzolamide (\textbf{I12}), and brinzolamide (\textbf{I13}) show an excellent agreement. Similarly, a comparison of the docking result of methazolamide (\textbf{I11}) with a crystal structure of methazolamide-bound human carbonic
Figure 2.97. Visualization of sensors S1 (A, B), S2 (C, D), S3 (E, F), S4 (G, H) and S6 (I, J) docking (lowest energy states) produced by AutoDock4Zn docking algorithm within the binding pocket of human carbonic anhydrase II. Panels on the left show interaction of the sensors with solvent accessible surface rendered with hydrophobicity mapping (red, hydrophobic; white, hydrophilic character). Panels on the right show sensors coordinated to zinc ion (magenta) bound to three histidine residues (orange). Prominent amino acid residue interactions with the sensors are also shown (green dash).

anhydrase II with two point mutations in the active site also yields very good agreement of the
binding geometry. The affinity constants computed for the \textbf{I10-I14} were also among the high ones, with the highest computed for celecoxib \textbf{I14} (Table 2.21).

While AutoDock4\textsubscript{Zn} yields very good docking geometries, it significantly underestimates the binding affinity of the ligand to the protein when compared to experimental binding affinities (Table 2.20).

\textbf{Table 2.20.} Experimental and calculated affinities \(K_S\) (M\(^{-1}\)) of sensors \textbf{S1-S5} (500 nM) to isozyme II of human and bovine carbonic anhydrase (human, \textit{hCA}, and bovine, \textit{bCA}) obtained from fluorescence titration in aqueous HEPES buffer (50 mM, pH 7.2) at ambient temperature and AutoDock Vina docking algorithm, respectively.

<table>
<thead>
<tr>
<th>Sensor (M(^{-1}))</th>
<th>\textbf{hCA II}</th>
<th>\textbf{bCA II}</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>\textbf{Experiment}</td>
<td>\textbf{AutoDock Vina}</td>
</tr>
<tr>
<td>\textbf{S1}</td>
<td>(6.5 \times 10^8)</td>
<td>(1.2 \times 10^6)</td>
</tr>
<tr>
<td>\textbf{S2}</td>
<td>(5.0 \times 10^7)</td>
<td>(2.3 \times 10^5)</td>
</tr>
<tr>
<td>\textbf{S3}</td>
<td>(1.3 \times 10^8)</td>
<td>(3.7 \times 10^5)</td>
</tr>
<tr>
<td>\textbf{S4}</td>
<td>(4.9 \times 10^7)</td>
<td>(1.4 \times 10^5)</td>
</tr>
<tr>
<td>\textbf{S5}</td>
<td>&gt; (10^6)</td>
<td>(7.8 \times 10^6)</td>
</tr>
</tbody>
</table>

\textbf{Table 2.21.} Measured and calculated binding affinities \(K_I\) (M\(^{-1}\)) for inhibitors \textbf{I1-I14} to bovine and human carbonic anhydrase isozyme II (\textit{bCA II}, \textit{hCA II}, resp.) in competitive experiments using indicator \textbf{S1} (500 nM) obtained from fluorescence titration in aqueous HEPES buffer (50 mM, pH 7.2) at ambient temperature and AutoDock Vina docking algorithm, respectively.

<table>
<thead>
<tr>
<th>Inhibitors (M(^{-1}))</th>
<th>\textbf{hCA II}</th>
<th>\textbf{hCA II}</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>\textbf{Experiment}</td>
<td>\textbf{AutoDock Vina}</td>
</tr>
<tr>
<td>\textbf{I1}</td>
<td>NR</td>
<td>(2.2 \times 10^2)</td>
</tr>
<tr>
<td>\textbf{I2}</td>
<td>NR</td>
<td>(1.4 \times 10^3)</td>
</tr>
<tr>
<td>\textbf{I3}</td>
<td>(7.8 \times 10^5)</td>
<td>(3.0 \times 10^4)</td>
</tr>
<tr>
<td>\textbf{I4}</td>
<td>(2.0 \times 10^4)</td>
<td>(5.9 \times 10^4)</td>
</tr>
<tr>
<td>\textbf{I5}</td>
<td>(2.0 \times 10^6)</td>
<td>(6.9 \times 10^4)</td>
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<tr>
<td>\textbf{I6}</td>
<td>(1.0 \times 10^8)</td>
<td>(5.3 \times 10^5)</td>
</tr>
<tr>
<td>\textbf{I7}</td>
<td>(1.1 \times 10^8)</td>
<td>(4.2 \times 10^4)</td>
</tr>
<tr>
<td>\textbf{I8}</td>
<td>(1.1 \times 10^8)</td>
<td>(8.2 \times 10^4)</td>
</tr>
<tr>
<td>\textbf{I9}</td>
<td>(2.5 \times 10^6)</td>
<td>(5.5 \times 10^3)</td>
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</tr>
<tr>
<td>I10</td>
<td>$1.2 \times 10^9$</td>
<td>$3.5 \times 10^4$</td>
</tr>
<tr>
<td>I11</td>
<td>$1.3 \times 10^9$</td>
<td>$9.1 \times 10^3$</td>
</tr>
<tr>
<td>I12</td>
<td>$4.0 \times 10^{10}$</td>
<td>$4.2 \times 10^4$</td>
</tr>
<tr>
<td>I13</td>
<td>$8.4 \times 10^{10}$</td>
<td>$8.2 \times 10^4$</td>
</tr>
<tr>
<td>I14</td>
<td>$9.6 \times 10^7$</td>
<td>$1.7 \times 10^6$</td>
</tr>
</tbody>
</table>

This is not unexpected, as docking is done using necessarily approximate models that may neglect some important factors. First, the coordination of the ligands to Zn$^{2+}$ may not be described correctly by the simple molecular mechanics models used here. While AutoDock4Zn specifically takes measures towards resolving this issue, it has been calibrated using ligands that bind with a neutral nitrogen atom. It is not certain that this model will apply equally well to describe binding of the deprotonated amide nitrogen as in the ligands studied here. Secondly, the models here do not explicitly account for water molecules coordinated in the binding site (deep water). Such water molecules may help stabilize the ligand by forming hydrogen-bond networks with nearby cavity residues. Finally, the docking algorithm assumes that the protein residues are rigid. While it is possible to incorporate flexibility of some protein residues (e.g., cavity residues) in the model, this would increase the number of flexible bonds well beyond what AutoDock4Zn can accurately describe with the LGA algorithm. This is because the LGA algorithm is optimized to work with systems that incorporate up to ca. 10 flexible bonds. However, S2 and S4 alone have 11 and 12 flexible bonds, respectively. Therefore, including more flexible protein residues with AutoDock4Zn would result in loss of accuracy and efficiency.

One way to incorporate flexibility in the protein and at the same time to work with large ligands such as S1-S4 and S6 is by using AutoDock Vina. Vina uses a simpler scoring function that allows faster searches, and therefore can handle larger systems with more flexible bonds. Using Vina, we were able to perform the docking with incorporating more flexibility in the protein residues that are near the binding site. More specifically, we made all rotatable bonds in His-64,
Asn-67, Gln-92, Val-121, Phe-131, Val-135, Leu-198, and Thr-200 flexible as well as the ligand. The improved flexibility and scoring function of Vina comes at the cost of the treatment of the zinc center coordination, however, which is not treated in Vina in the same way it is treated in AutoDock4Zn. Despite this drawback, Vina is still able to predict the correct binding geometries for all inhibitors I1-I14 and sensors S1-S4 and S6, although these are not always the lowest energy geometries reported.

2.27 Results Summary

In summary, we have developed a simple yet powerful dye displacement assay to evaluate the efficiency of inhibitors for carbonic anhydrase enzymes that bind sulfonamide inhibitors. The fluorescence based displacement study revealed that the indicators used herein display an Off/On behavior where the fluorescence is largely quenched in the presence of CA, but is recovered in the presence of enzyme inhibitor. Indicators described here enable array-based displacement assay for various potential and standard CA inhibitors. This assay allows for qualitative study of the inhibitors and evaluating their structure for the application as CAIs. The present qualitative study enabled the selection of potential inhibitors and evaluate their CA-binding affinities in a quantitative manner. Finally, the fluorescence assay was compared with a standard tool of drug discovery, the computer generated docking models. Here, docking results validated the results of the displacement approach and confirmed that these tools are useful for recognizing potential inhibitors and classifying them as high or low affinity inhibitors. However, the utility of the standard docking models is limited when it comes to distinguishing between inhibitors that have similar binding affinities, which is the strong point of the displacement assay. The high-throughput
dye-displacement assay tool presented here complements the docking analysis tools, which are mostly qualitative, by providing a valuable quantitative insight.

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CHAPTER III. FIRST SUPRAMOLECULAR SENSORS FOR
PHOSPHONATE ANIONS

3.1 Abstract

Herein, we present tripodal fluorescent sensors with a 1,3,5-triethylbenzene core that display a turn-on fluorescence response in the presence of phosphonate and phosphate anions. We describe solution and solid state properties of the receptors and sensors and their affinity to various classes anions. The turn-on fluorescence response in the presence of phosphate and, most importantly, nerve agent sarin hydrolysis products, isopropyl methylphosphonate and methylphosphonate can be used in optical sensing. Such a fluorescence signal amplification in the presence of the anions was further utilized in a fluorescence-based microarray demonstrating its suitability for high throughput screening.

3.2 Phosphonate: Structure, Properties and Their Significance

Phosphonates are group of organophosphorus compounds of generalized structure $R_1^{-}P=O(OR_2)(OR_3)$ where $R_1$ or $R_2$ and $R_3$ are alkyl and/or aryl group (Figure 3.1). Phosphonates contain phosphorus(V) and are structurally similar to phosphates. Unlike phosphates that contain P–O bonds only, phosphonates contain one P–C bond as well. The structural substitution of one hydroxyl group in phosphate for an electron-donating alkyl group in phosphonates results in a decrease of acidity of the acid function in phosphonates, even though phosphonic acid ($R_1^{-}P=O(OR_2)$...
H, \( pK_{a,1,2} = 1.3, 6.7 \) is more acidic than phosphoric acid (\( pK_{a,1,2,3} = 2.1, 7.2, 12.3 \)). Phosphates and phosphonates are both commercially important oxyphosphorus compounds and have found a broad range of applications. Phosphates are ubiquitous in the Nature and play an important role in biological processes associated with phosphorylation, maintenance of phosphate homeostasis, and nutrition. Historically, phosphonates were thought not to occur naturally in the living matter due to unknown metabolism of carbon-phosphorus bond. The discovery of naturally occurring 2-aminoethane phosphonic acid (1) in rumen protozoa in 1959 and isolated in crystalline form proved such theory wrong. Later on, phosphonates have been identified in bacteria, fungi, and higher organisms such as certain kinds of snails. This indicates that such organisms must have developed efficient biosynthetic pathways to transform the carbon-phosphorus bond. It was found that a rearrangement of phosphoenolpyruvate (2) to phosphonopyruvate (3) serves as an entry into the phosphonate class of naturally occurring organophosphorus compounds like antibiotic fosfomycin (4), and herbicide bialaphos (5) found in strains of Streptomyces (Figure 3.2).

The environmental impact of phosphonates is significant as they are used in an increasing variety of industrial and household applications including cooling water systems, oil drilling and production, metal finishing, ore recovery, pulp and paper production and processing, cleansing and laundry operations, and agriculture as chelating agents, cleaners or detergents, scale and corrosion inhibitors, and bleach stabilizers. One of the most commonly used phosphonates, dimethyl
methylphosphonate (DMMP, 6) has found a wide-spread use primarily as a flame retardant (for example in lithium ion battery electrolytes)\textsuperscript{15} but also as a plasticizer and an additive for solvents and low-temperature hydraulic fluids.\textsuperscript{16} Phosphonates are very important drugs including bone cancer remedies (alendronic and pamidronic acids),\textsuperscript{17} antibiotics (fosfomycin),\textsuperscript{18} drugs related to calcium metabolism and osteoporosis (etidronic and clodronic acids)\textsuperscript{19} and antiretroviral drugs (cidofovir, adefovir, tenofovir, foscarnet).\textsuperscript{20,21} Also, phosphonates are used as alkylation agents in Horner–Wadsworth–Emmons\textsuperscript{22} and Michaelis-Arbuzov reactions.\textsuperscript{23} A large group of phosphonates that are relatively nontoxic to humans are among the most efficient pesticides.\textsuperscript{24} This group includes non-selective systemic herbicide glyphosate (marketed as Roundup\textsuperscript{®} by Monsanto),\textsuperscript{25} insecticide metrifonate\textsuperscript{26} or a widely used plant growth regulator ethephon (Figure 3.3).\textsuperscript{27} Finally, the majority of warfare nerve agents (NA) and the products formed upon their hydrolysis are organophosphonates. These NAs (Figure 3.5) include sarin GB (O-isopropyl methylphoshonofluoridate, \textbf{16}), soman GD (3,3-dimethyl-2-butyl methylphosphonofluoridate,

\textbf{Figure 3.2.} Examples of naturally occurring phosphonates: Phophonolipid precursor 2-aminoethane phosphonate (\textbf{1}). Phosphoenolpyruvate (\textbf{2}) isomerizes to phosphonopyruvate (\textbf{3}), an important metabolic precursor for other naturally occurring phosphonates. Fosfomycin (\textbf{4}) and bialaphos (\textbf{5}) are two examples of phosphonates with an antibiotic and herbicide activity found in certain strains of \textit{Streptomyces}.  

...
Figure 3.3. Examples of important phosphonates: Dimethyl methylphosphonate (DMMP, 6) used as a flame retardant and hexamethylenediamine tetramethylene phosphonic acid (7) is a popular scale inhibitor. Alendronic (8) and etidronic (9) acids are used to treat osteoporosis. Foscarnet (10), cidofovir (11), adefovir (12) are antiviral drugs used to treat CMV, HSV and hepatitis B infections. Pesticides glyphosate (13) and metrifonate (14) and plant growth regulator ethephon (15) are widely used in agriculture.

17), tabun (\(N,N\)-dimethylethyl phosphoroamidocyanidate, 18), cyclosarin GF (\(O\)-cyclohexyl methylphosphonofluoridate, 19), VX (\(O\)-ethyl-\(S\)-diisopropylaminoethyl methylphosphothioate, 20), R-VX (\(O\)-isobutyl-\(S\)-2-diethylaminoethyl methylphosphonothioate, 21). NAs act as irreversible acetylcholine esterase inhibitors and possess carcinogenic properties as well, since they are capable to alkylate DNA, and an exposure of humans or animals to the NAs results almost certainly to immediate death.\(^{28,29}\) Their use is prohibited by the Chemical Weapons Convention of 1997.

Similarly to phosphonate-based pesticides, the nerve agents show similar behavior upon hydrolysis and produce similar hydrolysis products. For example, sarin 16 cleaves fluoride in the first step producing \(O\)-isopropylmethylphosphonic acid (IMPA, 22) which can be further hydrolyzed to methylphosphonic acid (MPA, 23) as the final product. Figure 3.4 shows hydrolysis
Figure 3.4. Structures and names of G- and V-series warfare nerve agents.

of sarin 16 (A), soman 17 (B) and VX 20 (C). The hydrolysis of tabun 18 in the presence of a base gives in the first step hydrogen cyanide and $O$-ethyl dimethylamido phosphoric acid which is further hydrolyzed to $N,N$-dimethylamide of phosphoric acid ($N,N$-dimethylphosphoramidate) and

![Chemical structures](image)

Figure 3.5. Primary hydrolysis products of isopropyl methylphosphonofluoridate (sarin; A), pinacolyl methylphosphonofluoridate (soman, B) and $O$-ethyl-$S$-[2-(diisopropylamino)ethyl]methylphosphonothioate (VX, C) in the environment.
ethanol. The final product of tabun basic hydrolysis is phosphoric acid. Acidic conditions yield ethylphophoryl cyanidate and dimethylamine. Generally, the hydrolysis of sarin, soman, and cyclosarin gives phosphonic acid and alcohol in addition to fluoride.\textsuperscript{30} The hydrolysis rate of NAs in water is apparently temperature and pH dependent. For example, at 20 °C compound 16 has an estimated half-life 461 h at pH 6.5 and 46 h at pH 7.5. At 25 °C, the half-life is estimated to be 237 h at pH 6.5 and 24 hr at pH 7.5 and 8,300 h at 0 °C at pH 6.5. The last value indicates some persistence at low temperatures.\textsuperscript{31}

### 3.3 Optical Sensing of Nerve Agents and Their Surrogates

The detection and identification of NAs is of particular interest in response to the serious threats to national and global security. Various methodologies have recently been investigated to detect organophosphorus compounds. These include potentiometry,\textsuperscript{32,33} colorimetry,\textsuperscript{34} surface acoustic wave (SAW) devices,\textsuperscript{35} enzymatic assays,\textsuperscript{36,37} gas chromatography/mass spectrometry (GC-MS)\textsuperscript{38} and interferometry,\textsuperscript{39} among others. Optical sensing utilizing change in absorption or fluorescence intensity has been, however, the most studied approach to detect organophosphorus NAs for its relative simplicity and high sensitivity. For obvious reasons, many authors used so-called nerve agent mimics or surrogates (e.g., DMMP, diisopropylfluorophosphate, diethylchlorophosphate, etc.) which have similar reactivity as NAs themselves, but they lack the efficacy of typical nerve agents and hence are good model compounds.\textsuperscript{40}

A significant research effort has been recently devoted to finding sensors capable of direct detection of sarin and its vapors.\textsuperscript{41–50} For example, Gale and coworkers demonstrated a formation of complex of soman 17 with diindolylurea-based dosimeter 28 in MeCN/DMSO and investigated its stoichiometry using \textsuperscript{1}H-NMR study and DFT calculations.\textsuperscript{43} Swager and coworkers developed
a highly sensitive ratiometric indicators based on arylpyridyl silylethers for chemical warfare agents and their mimics. The authors observed that indicator produced a bathochromic absorption and fluorescence in response to sarin mimic diisopropylfluorophosphate (DFP, leading to phosphorylation followed by quick cyclization into highly fluorescent pyridinium product (Figure 3.6). Similar principle was utilized by Rebek and Dale who also studied a plethora of sensory systems for nerve agent mimics. For example, they presented a group of fluorescent pyrene-based dosimeters for sarin mimic diethylchlorophosphate (DCP, 36)
derived from Kemp’s acid (Figure 3.7). The fluorescence of the dosimeters was quenched in the resting state in methanol solution due to PET process (from the electron lone pair of amine to the pyrene photo-excited state). Exposure of sensors 32-35 to DCP led up to 22-fold emission enhancement.

A discrimination of various NAs and their surrogates was also successfully accomplished. LeCaptain and coworkers developed an array of polyhedral oligosilsesquioxanes (POSS) conjugated to various fluorescent dyes capable to discriminate several NA mimics. They studied nine different dyes (e.g., 37 and 39) attached to the POOS nanoscaffold and four different NA mimics (DMMP, 6; DFP, 30; acephate, 40; chloroethyl ethyl sulfide, 41). Interestingly, the authors showed that the POSS nanosensors can be aerosolized and used to remotely sense a “threat clouds” or contaminated surfaces.

![Figure 3.8. Polyhedral oligosilsesquioxane-based nanosensors (POSS) presented by LeCaptain and coworkers for discrimination of NA mimics (Fl = fluorescent dye).](image)

Even though, a large variety of sensors for NAs and their mimics has been developed, the NAs can be also detected indirectly through their hydrolysis products: IMP (22) and MP (23) (Figure 3.4A). Unlike phosphates, phosphonates were mostly overlooked so the development of new methods and design of novel sensors for IMP or MP detection is of major importance. We
designed optical sensors capable to detect both phosphates and phosphonates. This capability of the sensors to differentiate various types of phosphorus oxyanions might be of particular interest for the analysis of traces of IMP and MP in soil exposed to VAs. To the best of our knowledge, there is no direct optical sensor capable of detecting phosphonates to date.

3.4 Phosphonates Sensors: Molecular Design and Structure

In the recent decades, a significant effort has been dedicated to the development of chemical sensors and sensor arrays capable to determine the presence of hazardous materials and the research has made an unprecedented headway. In 1993, Stack, Hou and Raymond presented enterobactin siderophore (42) mimic (43) based on 1,3,5-triaminomethyl-2,4,6-triethylbenzene core (Figure 3.9). In this structure, the design rigidly predefines orientation of the 1,3,5-catecholamide groups in 43 preferentially to one side of the aromatic core thus defines the $\text{C}_\text{ArC}_\text{ArCH}_2\text{NH}$ dihedral angles to be near 90°. Since this report, a plethora of tripodal receptors based on Stack’s pinwheel structure decorated with various binding moieties has been reported by various research groups. The receptors decorated with pyrrole, amide, urea,

![Figure 3.9](image_url). Naturally occurring tripodal siderophore 42 and its catecholamide mimic 43 presented by Stack, Hou and Raymond.
guanidinium,\textsuperscript{59} and imidazolium\textsuperscript{66–68} moieties have been reported and possess $C_3$-symmetry especially suitable for binding of anions with spherical or 3-fold symmetry such as phosphate, MP, and IMP.\textsuperscript{69}

In this part of the dissertation, we present the synthesis, properties, and anion sensing studies of tripodal receptors and sensors S1–S7 (Figure 3.10) exhibiting turn-on fluorescence response for anions including the hydrolysis products of the nerve gas sarin, MP and IMP.

\textbf{Figure 3.10.} Tripodal receptors S1-S7 used in this study.
3.5 Phosphonates Sensors: Synthesis

The receptors S1-S7 were synthesized by me and by Dr. Grigory Zyryanov, a postdoctoral research fellow in the Anzenbacher research group in the spring of 2011. 1,3,5-Tris(aminomethyl)-2,4,6-triethylbenzene 44 and sensor S4 were prepared by Dr. Tsuyoshi Minami, a postdoctoral research fellow in the Anzenbacher research group in the spring of 2012 based on reported protocol.58 Receptors S1, S2, S5 and S7 were prepared based on reported procedure and the spectral data obtained were in an agreement with the reported data.60

Disubstituted benzylic amine 45 was prepared utilizing Lieben-like haloform reaction,70,71 during which 2 mol. equivalents of commercially available 2-(trichloroacetyl)pyrrole precursor reacted with compound 44 in THF to produce bis(pyrroleamide) 45 in 63% yield. Similarly, receptor S1 was prepared in a reaction of 44 with 3 mol. equivalents of 2-(trichloroacetylamino)pyrrole in THF. In a similar way, we reacted benzylic triamine 44 in THF directly with 3-5 mol. equivalents of an appropriate isothiocyanate precursor (ArNCS) to obtain thiourea-based receptors S2-S5. Further reaction of the diamide 45 with 1-2 mol. equivalents of ArNCS in THF provided bis(pyrroleamide) thioureas S6 and S7 (Scheme 3.1).

3.5.1 Materials and Methods

All syntheses were carried out under anhydrous argon atmosphere using standard laboratory techniques. Column chromatography was performed on commercially available bulk flash silica gel 32-63u (Dynamic Adsorbents Inc., Norcross, GA). Preparative thin-layer chromatography (TLC) was carried out on Uniplate preparative silica gel TLC plates 2 mm, 20 × 20 cm with UV254 fluorescent dye (Analtech Inc., Newark, DE). 2-(Trichloroacetylamino)pyrrole was purchased from Aldrich, 4-(dimethylamino)-1-naphthyl-isothiocyanate from TCI America,
4-(phenanthro[9,10d]oxazol-2-yl)phenyl-isothiocyanate from Fluka, and the 4-tolylisothiocyanate was purchased from Acros Organics. All other reactants and reagents used in the syntheses were purchased from Aldrich, Fluka, or TCI America. Solvent grade methanol, N,N-dimethylformamide (DMF), diethylether, chloroform, dichloromethane (DCM), and ethylacetate (EtOAc) were purchased from EMD and used as received unless stated otherwise. Tetrahydrofuran (THF) was distilled over Na/K alloy before use. Anhydrous dimethylsulfoxide (DMSO) was obtained from commercially available DMSO upon the action of 4Å molecular sieves to remove residual water.

3.5.2 Spectroscopy

$^1$H- and $^{13}$C-NMR spectra were recorded on Bruker Avance III spectrometer at 500 MHz or 125 MHz, respectively, at 25 °C with or without tetramethylsilane (TMS) as an internal reference standard. Chemical shifts (δ, ppm) are referenced to the respective solvent or TMS and
splitting patterns are designated as s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet), and bs (broad singlet). MALDI-TOF MS spectra were recorded on Bruker Daltonics Omniflex spectrometer using dithranol or α-cyano-4-hydroxycinnamic acid as matrices. Electrospray ionization mass spectrometry (ESI-MS) experiments were carried out on Shimadzu 2010A LCMS instrument. Emission spectra were recorded on Edinburgh single-photon counting spectrofluorimeter FLSP 920 (Edinburgh Instruments Ltd., Livingston, UK) at room temperature using quartz cuvette with a path length of 1 cm and with a right angle detection. The concentration of the samples was adjusted so that the absorbance used for fluorescence measurements was equal to or below 0.1. Absolute quantum yields were measured with Hamamatsu Quantaurus-QY C11347 absolute quantum yield spectrometer (150 W Xe lamp).

3.5.3 Synthesis Protocols

\[ N,N'-[5-(Aminomethyl)-2,4,6-triethyl-1,3-phenylene]bis(methylene)bis(1H-pyrrole-2-carboxamide) \] (45). 1,3,5-Tris(aminomethyl)-2,4,6-triethylbenzene 45 (211 mg, 0.80 mmol) and 2-(trichloroacetyl)pyrrole (344 mg, 1.6 mmol) were dissolved in freshly distilled tetrahydrofurane (THF) in a vial. The vial was flushed with argon and screw-capped and the mixture was stirred at room temperature for 16 hours. The THF was then evaporated/co-distilled with toluene using a rotary evaporator and the white residue was dried in vacuo. Column chromatography (silica, 70 g, DCM/MeOH, 9 : 1 v/v, \( R_f = 0.14 \), isocratic elution) afforded product 45 (231 mg, 63%). \(^1\)H-NMR (500 MHz, DMSO-\( d_6 \)) : 11.45 (s, 2 H, Het\( NH \)), 7.84 (t, \( J = 4.5 \) Hz, 2 H, CON\( H \)), 6.83-6.82 (m, 4 H, Het\( H \)), 6.04-6.03 (m, 2 H, Het\( H \)), 4.48 (d, \( J = 4.5 \) Hz, 4 H, ArCH\(_2\)NH), 3.80 (s, 2 H, CH\(_2\)NH\(_2\)), 2.82-2.77 (m, 6 H, CH\(_2\)CH\(_3\)), 1.15-1.10 (m, 9 H, CH\(_3\)CH\(_3\)). \(^{13}\)C-\(^1\)H NMR (125 MHz, DMSO-
$d_6$: $\delta = 160.3, 143.0, 142.6, 135.6, 132.1, 126.1, 121.2, 110.7, 108.5, 38.6, 37.1, 22.7, 22.3, 16.5, 16.3$. MS (MALDI-TOF): $m/z = 458.28$, $[M+Na]^+$ calcd. for $C_{25}H_{33}N_5NaO_2$ 458.25.

$1,1',1''-(2,4,6$-Triethylbenzene-1,3,5-triyl)tris(methylene)tris(3-(2-benzyl-1,3-dioxo-2,3-dihydro-1H-benzo[de]isoquinolin-6-yl)thiourea) ($S_5$). 2-Benzyl-6-isothiocyanato-1H-benzo[de]isoquinoline-1,3(2H)-dione (300 mg, 0.87 mmol) was added to THF solution (100 mL) of 1,3,5-tris(aminomethyl)-2,4,6-triethylbenzene (48.3 mg, 0.19 mmol) and the reaction mixture was stirred for 2 days at room temperature. The solvent was evaporated, and the residue was chromatographed on a silica using gradient of ethanol (0-10%, v/v) in chloroform as an eluent. Product $S_5$ was isolated as an orange powder (124 mg, 50%). Mp $> 175$ °C (dec.). $^1$H-NMR (DMSO-$d_6$, 500 MHz): $\delta = 1.27$ (t, $J = 7.3$ Hz, 9 H, CH$_2$CH$_3$), 2.88-2.89 (m, 6 H, CH$_2$CH$_3$), 4.87 (s, 6 H, ArCH$_2$NH), 5.25 (s, 6 H, ArCH$_2$), 7.22 (t, $J = 7.3$ Hz, 3 H, Ar$H$), 7.28 (t, $J = 7.5$ Hz, 6 H, Ar$H$), 7.33-7.35 (m, 6 H, Ar$H$), 7.89 (t, $J = 8.0$ Hz, 3 H, Ar$H$), 8.39 (bs, 3 H, CSNH), 8.47-8.54 (m, 12 H, Ar$H$), 10.08 (bs, 3 H, CSNH). $^{13}$C-$^1$H NMR (125 MHz, DMSO-$d_6$): $\delta = 16.5, 23.12, 42.7, 42.9, 117.6, 122.2, 122.3, 125.9, 126.8, 127.1, 127.5, 128.4, 128.5, 128.9, 131.2, 131.4, 131.9, 137.4, 142.0, 144.4, 163.0, 163.6, 180.8$. MS (MALDI-TOF): $m/z =$ 1304.35, $[M+Na]^+$ calcd. for $C_{75}H_{63}N_9NaO_6S_3$ 1304.40.

$1,1',1''-(2,4,6$-Triethylbenzene-1,3,5-triyl)tris(methylene)tris(3-(4-(dimethylamino)naphthalene-1-yl)thiourea) ($S_3$). The solution of 4-(N,N-dimethylamino)naphthalenethiocyanate (257 mg, 1.1 mmol) in THF was added dropwise over 10 minutes to the solution of 1,3,5-tris(aminomethyl)-2,4,6-triethylbenzene 45 (53.8 mg, 0.22 mmol) in 7 ml of anhydrous THF in a vial (20 mL) upon stirring. The vial was flushed with argon, screw-capped and covered with aluminum foil and the mixture was stirred at room temperature for 36 hours. THF was then evaporated and the residue
chromatographed on silica gel (25 g, toluene-acetone, 9 : 1 v/v, isocratic elution, \( R_f = 0.41 \) in toluene-acetone, 8 : 2 v/v). Compound S3 was isolated as a pale yellow solid (115 mg, 57%). Mp > 181 °C (dec.). \( ^1 \)H-NMR (DMSO-\( d_6 \), 500 MHz): \( \delta = 9.32 \) (bs, 3 H, CSNH\( _{\text{Ar}} \)), 8.18-8.16 (m, 3 H, \( \text{ArH} \)), 7.86-7.84 (m, 3 H, \( \text{ArH} \)), 7.56-7.51 (m, 6 H, \( \text{ArH} \)), 7.47 (d, \( J = 7.8 \) Hz, 3 H, \( \text{ArH} \)), 7.43 (bs, 3 H, CSNH\( _{\text{CH}_2} \)), 7.10 (d, \( J = 8.1 \) Hz, 3 H, \( \text{ArH} \)), 4.76 (bs, 6 H, \( \text{ArCH}_2\text{NH} \)), 2.81 (m, 24 H, \( \text{NCH}_3 + \text{CH}_2\text{CH}_3 \)), 1.15 (t, \( J = 7.1 \) Hz, 9 H, \( \text{CH}_2\text{CH}_3 \)). \( ^{13} \)C-{\( ^1 \)H} NMR (125 MHz, DMSO-\( d_6 \)): \( \delta = 182.3, 149.2, 144.1, 132.2, 131.0, 129.6, 126.0, 125.5, 125.3, 124.2, 123.1, 113.6, 44.9, 42.8, 22.8, 16.5. MS (MALDI-TOF): \( m/z = 933.74, [M]^+ \) calcd. for C\(_{54}\)H\(_{63}\)N\(_9\)S\(_3\) 933.44.

\( N,N'-(5-((3-(4-(\text{Dimethylamino})naphthalen-1-yl)thiourea)\text{methyl})-2,4,6\)-triethyl-1,3-phenylene)bis(methylene)bis(1\( H \)-pyrrole-2-carboxamide) (S6).

4-(\( N,N'\)-Dimethylamino)naphthalenothiocyanate (32.1 mg, 0.14 mmol) was added in one portion to the solution of 45 (31.6 mg, 0.069 mmol) in 10 mL of freshly distilled THF upon stirring. The vial was flushed with argon, screw-capped and the mixture was stirred at room temperature for 15 hours. A white precipitate was filtered off using a paper filter, rinsed with THF and dried in vacuo.

Compound S6 was obtained as a white powder (14 mg, 30%). Mp > 245 °C. \( ^1 \)H-NMR (500 MHz, DMSO-\( d_6 \)): \( \delta = 11.46 \) (bs, 2 H, Het\( \text{NH} \)), 9.37 (bs, 1 H, CSNH), 8.15-8.13 (m, 1 H, \( \text{ArH} \)), 7.87 (t, \( J = 4.6 \) Hz, 2 H, Het\( \text{H} \)), 7.82 (d, \( J = 7.3 \) Hz, 1 H, \( \text{ArH} \)), 7.52-7.46 (m, 2 H, \( \text{ArH} \)), 7.44 (d, \( J = 8.0 \) Hz, 1 H, \( \text{ArH} \)), 7.24 (bs, 1 H, CSNH), 7.07 (d, \( J = 8.1 \) Hz, 1 H, \( \text{ArH} \)), 6.86-6.78 (m, 4 H, Het\( \text{H} \)), 6.05-6.04 (m, 2 H, Het\( \text{H} \)), 4.74 (d, \( J = 3.7 \) Hz, 2 H, \( \text{ArCH}_2\text{NH} \)), 4.50 (d, \( J = 4.5 \) Hz, 4 H, \( \text{ArCH}_2\text{NH} \)), 2.86-2.76 (m, 12 H, \( \text{CH}_2\text{CH}_3 \), \( \text{NCH}_3 \)), 1.15-1.09 (m, 9 H, \( \text{CH}_2\text{CH}_3 \)). \( ^{13} \)C-{\( ^1 \)H} NMR (125 MHz, DMSO-\( d_6 \)): \( \delta = 182.1, 160.3, 149.2, 144.1, 143.5, 132.6, 132.0, 131.0, 130.6, 130.6, 128.5, 126.1, 125.4, 125.3, 124.2, 123.1, 121.3, 113.6, 110.6, 108.6, 44.9, 42.8, 37.0, 34.4, 22.7, 16.4, 16.3. MS (MALDI-TOF): \( m/z = 631.36, [M-S]^+ \) calcd. for C\(_{38}\)H\(_{47}\)N\(_7\)O\(_2\) 631.87.
N,N’-(5-((3-(2-benzyl-1,3-dioxo-2,3-dihydro-1H-benzo[de]isoquinolin-6-yl)thioureido)methyl)-2,4,6-triethyl-1,3-phenylene)bis(methylene)bis(1H-pyrrole-2-carboxamide) (S7). The 2-benzyl-6-isothiocyanato-1H-benzo[de]isoquinoline-1,3(2H)-dione (6.6 mg, 0.014 mmol) and compound 45 (5.2 mg, 0.014 mmol) were dissolved in freshly distilled THF in a vial. The vial was flushed with argon, screw-capped and covered with aluminum foil and the mixture stirred at room temperature for 15 minutes. The solvent was then evaporated on a rotary evaporator and the residue was chromatographed on preparative TLC (silica, 2 mm, 20 x 20 cm, DCM/MeOH, 95 : 5 v/v, Rf = 0.26). Compound S7 was isolated by extraction of the scraped silica layer into THF. Evaporation of THF afforded product S7 as a yellow solid (3.2 mg, 24%). Mp > 170 °C (dec.). 1H-NMR (500 MHz, DMSO-d6): δ = 11.51-11.43 (m, 2 H, HetNH), 10.14-10.06 (m, 1 H, CSNH), 8.54-8.46 (m, 4 H, ArH), 8.36 (m, 1 H, CSNH), 7.90-7.87 (m, 3 H, HetH), 7.35-7.21 (m, 5 H, ArH), 6.85-6.82 (m, 4 H, HetH), 6.05-6.03 (m, 2 H, HetH), 5.25 (s, 2 H, PhCH2), 4.79 (d, J = 3.5 Hz, 2 H, ArCH2NH), 4.54 (d, J = 4.4 Hz, 4 H, ArCH2NH), 2.89-2.82 (m, 6 H, CH2CH3), 1.27-1.06 (m, 12 H, CH2CH3). 13C-{1H} NMR (125 MHz, DMSO-d6): δ = 180.6, 163.6, 163.0, 160.3, 144.3, 143.5, 142.0, 137.4, 132.8, 131.6, 131.2, 128.8, 128.5, 128.4, 127.5, 127.1, 126.8, 126.1, 125.7, 122.3, 121.9, 121.3, 117.5, 110.6, 108.6, 42.8, 42.7, 36.9, 22.8, 22.7, 16.4, 16.3. MS (MALDI-TOF): m/z= 802.43, [M+Na]+ calcd. for C45H45N7NaO4S 802.32.
3.6 Phosphonates Sensors: Synthesis: NMR Spectra

Figure 3.11. $^1$H-NMR spectrum (500 MHz) of S4 in DMSO-$d_6$. 
Figure 3.12. $^{13}$C-NMR spectrum (126 MHz) of S4 in DMSO-$d_6$. 
Figure 3.13. $^1$H-NMR spectrum (500 MHz) of S3 in DMSO-$d_6$. 
Figure 3.14. $^{13}$C-NMR spectrum (126 MHz) of S3 in DMSO-$d_6$. 
Figure 3.15. $^1$H-NMR spectrum (500 MHz) of S6 in DMSO-$d_6$. 
Figure 3.16. $^{13}$C-NMR spectrum (126 MHz) of S6 in DMSO-$d_6$. 
Figure 3.17. $^1$H-NMR spectrum (500 MHz) of S7 in DMSO-$d_6$. 
Figure 3.18. $^{13}$C-NMR spectrum (126 MHz) of S7 in DMSO-$d_6$. 
Figure 3.19. $^1$H-NMR spectrum (500 MHz) of compound 45 in DMSO-$d_6$. 
Figure 3.20. $^{13}$C-NMR spectrum (126 MHz) of compound 45 in DMSO-$d_6$. 
3.7 Phosphonates Sensors: Single Crystal X-Ray Structural Analysis

In order to gain more of an insight into binding modes between the receptors and anions, we decided to obtain single crystals of the receptors and their complexed with anions and analyzed them using x-ray diffractometry. The crystals were obtained as follows: \( \textbf{S1} \cdot (\text{C}_4\text{H}_8\text{O}_2)_6 \): A crystal suitable for single-crystal X-ray diffractometric analysis was obtained by slow evaporation from dioxane solution. The unit cell contains two molecules of receptor 2 and 12 molecules of co-crystallized dioxane. One of the dioxane molecules resides on the inversion centre. \( \textbf{S2} \cdot 3 \text{H}_2\text{PO}_4^- \): Crystals of \( \textbf{S2} \) in presence of tetrabutylammonium dihydrogenphosphate suitable for X-ray analysis were grown from acetone solution. The unit cell of the hexagonal crystal contains two molecules of \( \textbf{S2} \), six dihydrogenphosphate anions and 6 tetrabutylammonium cations. The molecule resides around a crystallographic three-fold rotation axis at \( 2/3, 1/3, z \). \( \textbf{S3} \): Crystallizes in the triclinic system and crystals suitable for X-ray diffraction were obtained by slow evaporation of an acetone solution. Crystallographic parameters for the receptors \( \textbf{S1}, \textbf{S2} \) and \( \textbf{S3} \) are summarized in Table 3.1.

The data were collected on a Nonius Kappa CCD diffractometer using a graphite monochromatized Mo-K\( \alpha \) radiation (\( \lambda = 0.71075 \) Å) at 153 K using Oxford Cryostream low temperature device. Data reduction was performed using DENZO-SMN.\(^{72}\) The structure was solved by direct methods using SIR97\(^{73}\) and refined by full-matrix least-squares on \( F^2 \) with anisotropic displacement parameters for the non-H atoms using SHELXL-97.\(^{74}\) The hydrogen atoms on carbon were calculated in ideal positions with isotropic displacement parameters set to 1.2 \( \times \) \( U_{eq} \) of the attached atom (1.5 \( \times \) \( U_{eq} \) for methyl hydrogen atoms). The function, \( \Sigma w(|F_o|^2 - |F_c|^2)^2 \), was minimized, where \( w = 1/\{[\sigma(F_o)]^2 + (0.0499 \times P)^2 + (1.0626 \times P)\} \) and \( P = \{(|F_o|^2 + \ldots\} \).
2|F₀|²/3. Neutral atom scattering factors and values used to calculate the linear absorption coefficient are from the *International Tables for X-ray Crystallography* (1992). All figures were generated using Mercury from CCDC.

### Table 3.1. X-ray structural analysis details for the crystals obtained with sensor S1, S2 and S3.

<table>
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<th>Compound reference</th>
<th>S1 · diox₆</th>
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<th>S3</th>
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<td>0.71075</td>
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<td>c (Å)</td>
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<td>13.2836(2)</td>
<td>19.6654(2)</td>
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<td>γ (°)</td>
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<td>153(2)</td>
<td>153(2)</td>
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<td>P₆₃</td>
<td>P₁₋₁</td>
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<td>0.0751</td>
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Figure 3.21. Axial (A) and apical (B) view molecular complex of [S2 \cdot 3 \text{H}_2\text{PO}_4] showing displacement ellipsoids scaled to the 50% probability level. For the axial view, the 3 \text{H}_2\text{PO}_4 cluster has been removed for clarity. Axial (C) and apical (D) view of the three \text{H}_2\text{PO}_4 hydrogen bonded phosphate anions have been rendered in different colors.
Figure 3.22. The up-up-down (chair-like) conformation of the side arm substituents in receptor S1 (A) is presumably due to the interaction with neighboring molecules via intermolecular hydrogen bonds (B). An axial view of crystal packing of receptor S1 is shown in C. The dash lines represent hydrogen bonds formed between the pyrrole NH and the amide C=O.
Figure 3.23. Crystal packing of receptor S3 shows the closely interdigitated structure of the crystal. The sensor molecules maximize the number of interactions by adopting an up-up-down arrangement of the side arms (A, C). A network of intermolecular hydrogen bonds is indicated with the dash line (B).
The crystal structures of the free receptor $S_1$ (Figure 3.22) show that the receptor adopts a chair-like structure stabilized by a network of intermolecular hydrogen bonds. Similar conformation to that of $S_1$ is also observed in the crystal structure of sensor $S_3$ (Figure 3.23). Fortunately, we succeeded to grow crystals of X-ray quality of sensor $S_2$ from a concentrated phosphate solution in order to investigate an association preference of the sensor with an anion. Interestingly, the crystal structure revealed a $C_3$-symmetrical species with an all-up conformation of the side arms together with three tightly bound phosphate anions (Figure 3.21). This finding suggested that these simple tripodal receptors might have the capability to accommodate a larger species since they are capable of binding up to three phosphate anions.

3.8 Solution Behavior: Variable Temperature NMR and NMR Titrations

The alternate up-and-down arrangement of the ethyl and amino methylene groups is prearranged due to 1,3,5-triaminomethyl-2,4,6-triethylbenzene scaffold 44. We found, however, that the arrangement of the substituents is affected by different solvents and is template dependent. The choice of the solvent and the presence of a suitable template appears to control whether a chair-like or a cup-like conformation is adopted (Figure 3.24). In the cup-like conformation, three hydrogen-bond donor (NH) groups are located on the same side of the plane defined by the central benzene core thus forming a cavity lined with hydrogen-bond donors. This unexpected

![Figure 3.24](image_url) A schematic of possible conformation states in respect to the orientation of the side arms observed in the receptors studied: cup-like (left) and chair-like (right).
conformation behavior is attributed to intermolecular interactions and larger than expected conformational freedom of the substituent of the central benzene. In order to investigate the conformational behavior of the receptor S1 in the solution, we decided to use variable temperature NMR (VT-NMR) experiment.

The VT-NMR of S1 showed broadening of the amide signals while the methylene proton resonance was found to be split into two distinct signals at low temperature (Figure 3.25). This indicated the formation of two discrete conformation states based on two different orientations of the side arm substituents with respect to the 1,3,5-triaminomethyl-2,4,6-triethylbenzene core. This distinct methylene signal differentiation diminished on NMR scale at higher temperature suggesting fast interconversion between the two states at higher temperature.

![Figure 3.25](image)

**Figure 3.25.** A section of VT-NMR (500 MHz) of receptor S1 (B) in CD3CN/acetone-d6/DMSO-d6 (10:1:0.5, v/v) showed splitting of methylene groups signal due to the existence of two conformers (A).

Interestingly, the cup-like conformation of the receptor S1 was found to be enforced by accommodating an anion such as dihydrogenphosphate (Figure 3.26). In such a case, only the cup-like conformation is reinforced by the formation of the hydrogen bonds with the guest anion and gives rise to no temperature-induced changes in the spectra. This suggests that the conformation state adopted by the receptors is controlled by the presence of a suitable analyte acting as a template.
To avoid freezing of the solution, the VT-NMR experiments of an equimolar mixture of S1/Bu4NH2PO4 (1 : 1, n/n) were carried in CD3CN/acetone-d6/DMSO-d6 (10 : 1 : 0.5, v/v). With decreasing temperature only negligible shift and some broadening of the amidopyrrole and methylene signals was observed. No peak splitting however, was observed.

To reveal the nature of the interactions between the receptors and the anions and to obtain qualitative estimate of stoichiometry of such complexation phenomena, we also performed 1H-NMR titration of S1 and S2 with anions (as tetra-n-butylammonium salt) at laboratory temperature (Figure 3.27-Figure 3.35). Importantly, we did not observe a deprotonation of the receptors taking place by the anion at low anion concentration in water/DMSO-d6 (1 : 9, v/v), and the stoichiometry of the complexes formed suggested by the 1H-NMR titration isotherm were 1 : 1 (S1/S2 vs. an anion).
The titrations were carried out as follows. 1.0 ml of the sensor solution ([S1] = [S2] = 10 mM) was placed in a NMR tube and the NMR spectra were recorded in the absence of anion at room temperature then repeatedly after every addition of 1 μL aliquots of the anion solution. The stock solutions of anions contained the same concentration of sensor as the initial solution titrated.

Figure 3.27. The cup-like conformation of receptor S1 (A). Progress of $^1$H-NMR (500 MHz) titration of S1 (10 mM) upon the addition of MP in DMSO-$_d$6 (B). $^1$H-NMR titration isotherm of the pyrrole NH peak (red) corresponding to the MP-induced chemical shift.
**Figure 3.28.** Progress of $^1$H-NMR (500 MHz) titration of S1 (10 mM) upon the addition of phosphate (0-4.0 mol. equiv.) in DMSO-$d_6$ (A). $^1$H-NMR titration isotherm of the pyrrole NH peak corresponding to the Pi-induced chemical shift (B).

**Figure 3.29.** Progress of $^1$H-NMR (500 MHz) titration of S1 (10 mM) upon the addition of acetate (0-4.0 mol. equiv.) in DMSO-$d_6$ (A). $^1$H-NMR titration isotherm of the pyrrole NH peak corresponding to the acetate-induced chemical shift (B).
Figure 3.30. Progress of $^1$H-NMR (500 MHz) titration of S1 (10 mM) upon the addition of pyrophosphate (0-4.0 mol. equiv.) in DMSO-$d_6$ (A). $^1$H-NMR titration isotherm of the pyrrole NH peak corresponding to the PPI-induced chemical shift (B).

Figure 3.31. Progress of $^1$H-NMR (500 MHz) titration of S1 (10 mM) upon the addition of fluoride (0-4.0 mol. equiv.) in DMSO-$d_6$ (A). $^1$H-NMR titration isotherm of the pyrrole NH peak corresponding to the fluoride-induced chemical shift (B).
Figure 3.32. Progress of $^1$H-NMR (500 MHz) titration of S2 (10 mM) upon the addition of phosphate (0–4.0 mol. equiv.) in DMSO-$d_6$ (A). $^1$H-NMR titration isotherm of the pyrrole NH peak corresponding to the phosphate-induced chemical shift (B).

Figure 3.33. Progress of $^1$H-NMR (500 MHz) titration of S2 (10 mM) upon the addition of acetate (0–4.0 mol. equiv.) in DMSO-$d_6$ (A). $^1$H-NMR titration isotherm of the pyrrole NH peak corresponding to the acetate-induced chemical shift (B).
Figure 3.34. Progress of $^1$H-NMR (500 MHz) titration of S2 (10 mM) upon the addition of pyrophosphate (0-4.0 mol. equiv.) in DMSO-$d_6$ (A). $^1$H-NMR titration isotherm of the pyrrole NH peak corresponding to the PPi-induced chemical shift (B).

Figure 3.35. Progress of $^1$H-NMR (500 MHz) titration of S2 (10 mM) upon the addition of fluoride (0-4.0 mol. equiv.) in DMSO-$d_6$ (A). $^1$H-NMR titration isotherm of the pyrrole NH peak corresponding to the fluoride-induced chemical shift (B).
Importantly, the observations obtained from the NMR titrations can be used to determine relative bonding strength of the anions. The relative change in chemical shift of the resonances directly involved in hydrogen bonding with the guest anion can be correlated directly with the hydrogen bond strength. For example, in all the NMR titrations with S1, the signal corresponding to pyrrole NH underwent a dramatic downfield shift from 10.8 to 14.7, 13.6, and 12.9 ppm for fluoride, phosphate, and MP, respectively. And similarly, the pyrrole-amide NH is shifted downfield from 7.8 to 9.8, 8.6, and 8.8 for fluoride, phosphate, and MP, respectively. This gave us relative order of affinities of receptor S1 for anions MP ≈ Pi > fluoride > acetate.

3.9 Complex Formation: Mass Spectrometry

We confirmed the anion-binding capability of receptor S1 and S2 using ESI-MS. Figure 3.36 shows ESI-MS spectra (in negative mode) of the S1·MP and S2·MP complexes measured in acetonitrile. The spectrum suggests the binding stoichiometry of the complex formed to be 1 : 1.

Figure 3.36. ESI-MS spectrum of the complex of S1 (A) and S2 (B) with MP measured in negative mode in acetonitrile suggested 1 : 1 stoichiometry.
The samples for ESI-MS experiment were prepared by mixing 10 μL of the solution of S1 or S2 (100 μM) in acetonitrile and mixed with an excess amount of a corresponding anion. The ESI spectra were recorded in negative detection mode and suggested 1 : 1 binding stoichiometry.

3.10 Complex Stoichiometry Determination: Job’s Plot

Binding stoichiometries of the complex formation in the solution were determined from absorbance or NMR titration data using method of continuous variation (Job’s method).76 We investigated complex formation stoichiometries for receptor S1 with acetate and dihydrogenphosphate (Figure 3.37), S3 with dihydrogenphosphate and receptor S6 with fluoride (Figure 3.38). The Job’s plots obtained indicated formation of complexes with 1 : 1 binding stoichiometry.

Figure 3.37. Job’s plots of UV/vis titrations of S1 with dihydrogenphosphate (A) and acetate (B) both indicating 1 : 1 binding mode.
Finally, we decided to estimate and quantify the sensing ability of the sensors in respect to the anions utilizing fluorescence spectroscopy (fluorescence titrations). The titrations as well as the photophysical characterization of the sensors were carried out jointly by me and Ms. Nina Esipenko, a graduate student at the Anzenbacher research group in the summer and fall of 2012. The photophysical properties of S3-S6 were reported previously.\(^2\) The sensors S3-S7 were titrated with fluoride, chloride, acetate, dihydrogen phosphate, pyrophosphate as well as methyl phosphonate (MP), isopropyl methylphosphonate (IMP) and phenyl phosphonate (PP) in DMSO using respective isosbestic points for the excitation wavelength. Non-fluorescent receptors S1 and S2 were not studied using fluorescence titrations. Surprisingly enough, we found that the solutions of sensors S3-S7 in DMSO were not particularly fluorescent, even though they contain fluorescent moieties. This observation can be explained by the following hypothesis. Relatively low fluorescence of the sensors without guest anions (resting state) is caused by deactivation of the

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**Figure 3.38.** Job’s plots of UV/vis titration of S3 with dihydrogenphosphate (A) and Job’s plots of NMR titration of S6 with fluoride (B) both indicating 1:1 binding mode.

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### 3.11 Photophysical Studies and Fluorescence Titrations

Finally, we decided to estimate and quantify the sensing ability of the sensors in respect to the anions utilizing fluorescence spectroscopy (fluorescence titrations). The titrations as well as the photophysical characterization of the sensors were carried out jointly by me and Ms. Nina Esipenko, a graduate student at the Anzenbacher research group in the summer and fall of 2012. The photophysical properties of S3-S6 were reported previously.\(^2\) The sensors S3-S7 were titrated with fluoride, chloride, acetate, dihydrogen phosphate, pyrophosphate as well as methyl phosphonate (MP), isopropyl methylphosphonate (IMP) and phenyl phosphonate (PP) in DMSO using respective isosbestic points for the excitation wavelength. Non-fluorescent receptors S1 and S2 were not studied using fluorescence titrations. Surprisingly enough, we found that the solutions of sensors S3-S7 in DMSO were not particularly fluorescent, even though they contain fluorescent moieties. This observation can be explained by the following hypothesis. Relatively low fluorescence of the sensors without guest anions (resting state) is caused by deactivation of the
excited state by intramolecular collisions of the neighboring side-arms with partial contribution of rotational and/or vibrational quenching mechanisms. Importantly, the fluorescence intensity is dramatically amplified in the presence of anions. In such situation, the collisional quenching is not anymore possible since the geometry is enforced by hydrogen bonding between the side arm moieties and the anion resulting in a radiative deactivation of the excited state (fluorescence).\textsuperscript{77}

Photophysical properties of the sensors $S_4$ and $S_7$ are summarized in Figure 3.39 and Figure 3.40. The photophysical properties of all other receptors were reported previously.\textsuperscript{60} The fluorescence turn-on responses to various anions were used to estimate the association constants ($Table$ 3.2).

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure3.39.png}
\caption{Photophysical properties of $S_4$ in DMSO.}
\end{figure}
Figure 3.40. Photophysical properties of S7 in DMSO.

Figure 3.41. Fluorescence titration spectra (A) and fluorescence titration isotherm (B) of S4 (10 μM) in DMSO upon the addition of an incremental amounts of tetrabutylammonium dihydrogenphosphate (0-25 μM), λ_{exc} = 400 nm.
Figure 3.42. Fluorescence titration spectra (A) and fluorescence titration isotherm (B) of S4 (10 μM) in DMSO upon the addition of an incremental amounts of tetrabutylammonium fluoride (0-0.30 μM), λ_{EXC} = 400 nm.

Figure 3.43. Fluorescence titration spectra (A) and fluorescence titration isotherm (B) of S4 (10 μM) in DMSO upon the addition of an incremental amounts of tetrabutylammonium methylphosphonate (0-0.20 μM), λ_{EXC} = 400 nm.
**Figure 3.44.** Fluorescence titration spectra (A) and fluorescence titration isotherm (B) of S4 (10 μM) in DMSO upon the addition of an incremental amounts of tetrabutylammonium isopropyl methylphosphonate (0-0.30 μM), λ<sub>EXC</sub> = 400 nm.

**Figure 3.45.** Fluorescence titration spectra (A) and fluorescence titration isotherm (B) of S4 (10 μM) in DMSO upon the addition of an incremental amounts of tetrabutylammonium phenyl phosphonate (0-0.24 μM), λ<sub>EXC</sub> = 400 nm.
Figure 3.46. Fluorescence titration spectra (A) and fluorescence titration isotherm (B) of S3 (10 μM) in DMSO upon the addition of an incremental amounts of tetrabutylammonium methylphosphonate (0-75 μM), λ<sub>exc</sub> = 330 nm.

Figure 3.47. Fluorescence titration spectra (A) and fluorescence titration isotherm (B) of S3 (10 μM) in DMSO upon the addition of an incremental amounts of tetrabutylammonium isopropyl methylphosphonate (0-0.30 μM), λ<sub>exc</sub> = 330 nm.
**Figure 3.48.** Fluorescence titration spectra (A) and fluorescence titration isotherm (B) of S3 (10 μM) in DMSO upon the addition of an incremental amounts of tetrabutylammonium phenylphosphonate (0-0.16 μM), λ_{EXC} = 330 nm.

**Figure 3.49.** Fluorescence titration spectra (A) and fluorescence titration isotherm (B) of S5 (10 μM) in DMSO upon the addition of an incremental amounts of tetrabutylammonium acetate (0-25 μM), λ_{EXC} = 450 nm.
Figure 3.50. Fluorescence titration spectra (A) and fluorescence titration isotherm (B) of S5 (10 μM) in DMSO upon the addition of an incremental amounts of tetrabutylammonium phosphate (0-90 μM), $\lambda_{\text{EXC}} = 450$ nm.

Figure 3.51. Fluorescence titration spectra (A) and fluorescence titration isotherm (B) of S5 (10 μM) in DMSO upon the addition of an incremental amounts of tetrabutylammonium fluoride (0-20 μM), $\lambda_{\text{EXC}} = 450$ nm.
Figure 3.52. Fluorescence titration spectra (A) and fluorescence titration isotherm (B) of S5 (10 μM) in DMSO upon the addition of an incremental amounts of tetrabutylammonium pyrophosphate (0-40 μM), λ_{EXC} = 450 nm.

Figure 3.53. Fluorescence titration spectra (A) and fluorescence titration isotherm (B) of S5 (10 μM) in DMSO upon the addition of an incremental amounts of tetrabutylammonium pyrophosphate (0-0.42 μM), λ_{EXC} = 450 nm.
Figure 3.54. Fluorescence titration spectra (A) and fluorescence titration isotherm (B) of S5 (10 µM) in DMSO upon the addition of an incremental amounts of tetrabutylammonium isopropyl methylphosphonate (0-0.12 µM), $\lambda_{\text{EXC}} = 450$ nm.

Figure 3.55. Fluorescence titration spectra (A) and fluorescence titration isotherm (B) of S5 (10 µM) in DMSO upon the addition of an incremental amounts of tetrabutylammonium phenyl phosphonate (0-0.80 µM), $\lambda_{\text{EXC}} = 450$ nm.
Figure 3.56. Fluorescence titration spectra (A) and fluorescence titration isotherm (B) of S6 (10 \( \mu \text{M} \)) in DMSO upon the addition of an incremental amounts of tetrabutylammonium methylphosphonate (0-0.75 \( \mu \text{M} \)), \( \lambda_{\text{EXC}} = 330 \text{ nm} \).

Figure 3.57. Fluorescence titration spectra (A) and fluorescence titration isotherm (B) of S6 (10 \( \mu \text{M} \)) in DMSO upon the addition of an incremental amounts of tetrabutylammonium isopropyl methylphosphonate (0-0.21 \( \mu \text{M} \)), \( \lambda_{\text{EXC}} = 330 \text{ nm} \).
Figure 3.58. Fluorescence titration spectra (A) and fluorescence titration isotherm (B) of S6 (10 μM) in DMSO upon the addition of an incremental amounts of tetrabutylammonium phenylphosphonate (0-0.45 μM), λ<sub>exc</sub> = 330 nm.

Figure 3.59. Fluorescence titration spectra (A) and fluorescence titration isotherm (B) of S7 (10 μM) in DMSO upon the addition of an incremental amounts of tetrabutylammonium fluoride (0-0.15 μM), λ<sub>exc</sub> = 400 nm.
Fluorescence titrations revealed that sensors S4–S7 exhibit a stronger fluorescence response towards phosphate over other anions. Based on relative magnitude of fluorescence amplification and binding constants obtained for phosphorus related anions one can arrive at the relative series of affinity: $\text{Pi} \geq \text{MP} > \text{IMP} > \text{PP} \approx \text{PPI}$ (Table 3.2). Sensor S7 was found to show specific binding of dihydrogenphosphate and fluoride. Sensor S4 also showed high specificity for fluoride and phosphate. Unlike S7, S4 was also capable to bind phosphonates. We believe, that this observation may be due to the naphthalimide dye used in these two sensors. Perhaps steric requirements of the bulky fluorophore interfere with efficient binding of the substituted phosphonates. However, we did not observe a dramatic difference in binding affinities based on the number of fluorophores attached to the central core.

Importantly, the sensors investigated did not show a very high affinity for small spherical anions (especially chloride and fluoride). Perhaps due to small size of chloride and fluoride anions,
the sensors are not capable to adopt more compact conformation in order to effectively bind with the anions. On the other hand, anions possessing $C_3$-symmetry (especially phosphate and methyl phosphonate) are size-wise complementary to the sensors thus showing the highest binding affinities observed among the anions. Interestingly, phosphonates substituted with larger group (IMP and PP) showed lower binding constants compared with MP. This illustrates the effect of the bulky substitution on the binding constant (e.g., MP vs. PP).

**Table 3.2. Binding affinities (M$^{-1}$)$^a$ for sensors S3-S7 in DMSO.**

<table>
<thead>
<tr>
<th>Sensor</th>
<th>S3</th>
<th>S4</th>
<th>S5</th>
<th>S6</th>
<th>S7</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fluoride</td>
<td>$ND^b$</td>
<td>$5.6 \times 10^3$</td>
<td>$8.4 \times 10^4$</td>
<td>$ND^b$</td>
<td>$1.1 \times 10^4$</td>
</tr>
<tr>
<td>Chloride</td>
<td>$ND^b$</td>
<td>$ND^b$</td>
<td>$ND^b$</td>
<td>$ND^b$</td>
<td>$ND^b$</td>
</tr>
<tr>
<td>Acetate</td>
<td>$1.6 \times 10^4$</td>
<td>$ND^b$</td>
<td>$3.6 \times 10^4$</td>
<td>$2.1 \times 10^3$</td>
<td>$ND^b$</td>
</tr>
<tr>
<td>Pi</td>
<td>$4.3 \times 10^5$</td>
<td>$7.2 \times 10^3$</td>
<td>$3.1 \times 10^4$</td>
<td>$1.4 \times 10^4$</td>
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<td>$ND^b$</td>
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<tr>
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<td>$1.1 \times 10^4$</td>
<td>$3.1 \times 10^4$</td>
<td>$ND^b$</td>
</tr>
<tr>
<td>IMP</td>
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<td>$1.3 \times 10^4$</td>
<td>$2.9 \times 10^4$</td>
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<td>$7.7 \times 10^3$</td>
<td>$ND^b$</td>
</tr>
</tbody>
</table>

$^a$ The 1 : 1 stoichiometry was confirmed by Job's plot. The $K$ were calculated based on the change in fluorescence maxima upon addition of the analyte. All errors < 14%. $^b$ Binding constants couldn't be determined due to insufficient change in fluorescence response.

In a typical titration procedure, 3.0 mL of a sensor solution were placed in a 1 cm fluorescence cell. The fluorescence spectrum was recorded at room temperature in the absence of an anion. Then the emission spectra were repeatedly acquired after addition of 1-2 μL aliquots of anion solution. The stock solutions of anions contained the same concentration of sensor as the initial solution titrated.
3.12 Qualitative Assay

In order to prove a practical feasibility of the high-throughput experimental design we incorporated sensors S3-S7 into a microarray for anion sensing in the presence of water. Even though the sensors S3-S7 are water insoluble, we found that the fluorescence response is not significantly affected by up to 10% of water in DMSO and that this water content doesn’t interfere with the sensing process. The LDA experiment provided an excellent separation of the data clusters showing that the five sensors are able to distinguish between six aqueous anions and a control (total of seven analytes) and showed 100% correct classification (Figure 3.61). The array

![Figure 3.61](image)

**Figure 3.61.** Graphical output of qualitative LDAs for sensors S3-S7 (10 μM) in DMSO/water (9:1, v/v) obtained in 1536-well plate (total volume 7 μL per well) provided 100% correct classification.
experiment was performed in 1536-well plates. DMSO solutions of sensors were dispensed manually using Gilson micropipettes. Aqueous solutions of analytes were contact-free dispensed using BioRAPTR microfluidic robotic dispenser at 200 nL/s as follows. Each experiment was performed in 24 repetitions. Each well received 6.3 μL of the sensor solution (10 μM) in DMSO, followed by 0.7 μL of the analyte solution (3 mM) in water. This way, final amount of water in DMSO is 10% (v/v). For control experiment, 0.7 μL of water was added instead of analyte solution. After the analyte solution was dispensed, the plate was centrifuged (2 min, 2500 rpm, 21 °C) and

**Figure 3.62.** Canonical scores plot for LDA of qualitative assay for sensors S3-S7 (and control).
immediately read by a BMG PheraStar microplate reader using excitation at 450 nm, emission at 520 nm for S4 and S7, excitation at 450 nm, emission at 550 nm for S5, and excitation at 300 nm, emission at 450 nm for S3 and S6.

Table 3.3. Jackknife classification matrix of the qualitative assay for sensors S3-S7.

<table>
<thead>
<tr>
<th></th>
<th>AcO</th>
<th>Ctrl</th>
<th>F</th>
<th>IMP</th>
<th>MP</th>
<th>PPI</th>
<th>Pi</th>
<th>%Correct</th>
</tr>
</thead>
<tbody>
<tr>
<td>AcO</td>
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<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>Ctrl</td>
<td>0</td>
<td>20</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>F</td>
<td>0</td>
<td>0</td>
<td>20</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
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<td>0</td>
<td>0</td>
<td>20</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>MP</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>20</td>
<td>0</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>PPI</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>20</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>Pi</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>20</td>
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<td>20</td>
<td>20</td>
<td>20</td>
<td>20</td>
<td>100</td>
</tr>
</tbody>
</table>

The resulting emission data were subjected to the Student’s t-test to exclude four data-points (out of 24 repetitions). The coefficient of variability among the data within the class of 20 repetitions was lower than 3%. Then obtained data for qualitative analysis were analyzed using LDA without any further pretreatment. The microarray experiment was carried out by Ms. Nina Esipenko, a graduate student at the Anzenbacher research group in the spring of 2013.

3.13 Results Summary

In summary, this work represents the first optical sensors and fluorescence-based assay capable to detect nerve agents hydrolysis products and differentiate them from other classes of anions. We have synthesized and characterized tripodal fluorescent receptors S1-S7 designed to possess a cup-like binding cavity decorated with a band of hydrogen bond donors provided by thiourea and/or pyrrole-2-yl amide moieties. We utilized NMR spectroscopy and X-ray structural analysis to show that the resting state of the receptors adopts chair-like conformation. The presence
of anions was shown to result in a change of conformation state displaying the up-down conformation as evidenced by the combination of NMR and X-ray experiments. We also verified the anion binding event of S1-S7 by mass and NMR spectroscopy. Importantly, we did not observe deprotonation of the receptors and sensors while the MS spectra indicated a formation of stable anion-receptor complexes. The fluorescence titrations of sensors S3-S7 with a variety of anions including the hydrolysis products of nerve agents, aliphatic and aromatic phosphonates, showed a dramatic turn-on response with up to a 10-fold signal amplification. Finally, we showed that the sensors S3-S7 are capable to differentiate between distinct classes of anions which was demonstrated in the qualitative microarray experiment. We believe that this sensing ensemble suitable for sensing of phosphonates, hydrolysis products of nerve agents, could be utilized in identification of a potential nerve gas use.

3.14 References


(31) Clark, D. N. Review of Reactions of Chemical Agents in Water, Final Report to USA Biomedical Research and Development Laboratory; Battelle, Columbus, OH, 1989.


CHAPTER IV. FLUORESCENT EXPANDED CALIXPYRROLE DERIVATIVES FOR SENSING OF POLYANIONIC ANALYTES

4.1 Abstract

In this part of the dissertation, we describe fluorescent calix[4]pyrrole and expanded calix[2]benzo[4]pyrrole-based sensors exhibiting a dramatic change of color and fluorescence properties upon binding of environmentally and physiologically relevant polyanionic analytes. The complex formation between the sensors and the anion was studied using UV/vis and fluorescence spectroscopy which allowed us to quantify the affinity between the sensors and anions. NMR spectroscopy and mass spectroscopy experiments provided more detailed insight into the complex formation. Stoichiometries of the complexes were also investigated by Job’s method using UV/vis confirming 1 : 1 binding. The fluorescent sensors S1-S5 were used in a design of hydrogel-based cross-reactive microarrays and were capable to differentiate the anions of interest.

4.2 Porphyrins and Their Analogs: Structure and Properties

*Porphyrins* (from Greek *πορφύρα*, meaning *purple*) are naturally occurring group of heterocyclic aromatic macrocyclic compounds composed of four pyrrole unit interconnected at pyrrole 2,5-positions via four methine groups.1 Perhaps the most famous examples of naturally occurring porphyrin derivatives are heme (1), a cofactor of red blood cells pigment hemoglobin, chlorophyll (2), a group of pigments found in the chloroplasts of green plants and algae as well as in certain kinds of cyanobacteria, and cobalamine (vitamin B12). A plethora of synthetic porphyrins have been reported and widely studied for their strong absorption and emission properties and capability to coordinate ions and as building blocks in supramolecular assemblies.2-9 The group of
**Figure 4.1.** Examples of naturally occurring porphyrin derivatives: The most common type of red blood cells pigment is heme B (1). Chlorophyll d (2) is a common type of green pigment found in some cyanobacteria.

**porphyrin analogs** (*Figure 4.2*) includes both conjugated and non-conjugated oligopyrrole macrocycles which are derived from naturally occurring porphyrin moiety (3). The preparation of porphyrin congeners has led to a dramatic expansion of structural diversity and afforded substances exhibiting unprecedented photophysical and supramolecular properties. The diverse group of porphyrin congeners includes porphyrin isomers, ring-contracted and ring-expanded porphyrins, and heteroporphyrins (containing oxygen, sulfur, selenium, and tellurium instead of nitrogen as it is in pyrrole). Interestingly, supramolecular (sensing) properties of porphyrin analogs can be modulated by modifying the molecular design in order to achieve desired set of functions. The modulations usually include arrangement or orientation of pyrrole moieties within the macrocycle, introduction of heteroatoms affecting affinity and selectivity (heteroporphyrins), and finally, the expansion or contraction of the macrocycle often leads to derivatives emitting in near-IR region making these derivatives suitable for biomedical applications (photodynamic therapy). Due to their inherent absorption/emission along with their ion-binding properties with the macrocycle core acting simultaneously as the reporter and the recognition unit, porphyrins and porphyrin
analogs have become a popular platform for the design of molecular chemosensors suitable for anions (hydrogen bonding) and metal ions.\textsuperscript{26}

4.3 Calix[4]pyrrole-Based Anion Chemosensors

Perhaps the most prominent group among porphyrin analogs are \textit{calix[n]pyrroles}. Calix[4]pyrroles (\textit{meso}-octaalkyl porphyrinogens) is a class of tetrapyrrole macrocyclic compounds preparation of which was first described in 1886 by A. Baeyer via HCl-catalyzed condensation of pyrrole with acetone.\textsuperscript{27} This family of macrocyclic oligopyrroles exhibiting tunable color and

\textit{Figure 4.3.} Calix[4]pyrroles 9 (OMCP) and 10 were first reported by Sessler, Gale and coworkers. (Labels indicate commonly used locants for various positions on the macrocycle scaffold.)
fluorescence properties, metal coordination, and anion binding have attracted a broad attention in
designing supramolecular ion chemosensors. They have emerged as one of the most versatile
amongst anion sensor materials nowadays representing a distinct and vast area of supramolecular
chemistry. In 1996, Sessler, Gale, and co-workers were the first to describe preparation and
anion-binding properties of *meso*-octamethyl (OMCP) and *meso*-tetraspirocyclohexyl
porphyrinogens 9 and 10, respectively, and proposed to name this new class of porphyrinogen
compounds as “calixpyrroles” (*Figure 4.3*). Using ¹H-NMR titrations carried out in
dichloromethane-\(d_2\), they revealed an intriguing anion-binding ability of the host molecules and
noticed high binding affinity towards fluoride over other investigated anions. Interestingly, the X-
ray diffraction analysis showed that the binding event enforced conformational conversion from
the 1,3-alternate (resting state) to the cone conformation (*Figure 4.4*).

Utilizing modifications at *meso*- and/or \(\beta\)-positions of the macrocyclic core as well as its
expansion and \(N\)-confusion led to the preparation of novel calix[4]pyrrole derivatives with tailored
affinity and selectivity for various ions and electroneutral molecules and their possible alternation.
Importantly, it was found that the introduction of electron-withdrawing groups (EWG) to the
\(\beta\)-position of macrocycle leads to generally higher binding affinities towards anions compared to

![Figure 4.4. OMCP (9) and its four most stable conformers: 1,3- and 1,2-alternates, partial cone and cone.](image-url)
OMCP while the introduction of the electron-donating groups (EDG) to the $\beta$-position of macrocycle leads to generally lower affinities towards anions.$^{30,31}$ This general observation is expected since EWG at the $\beta$-position increases the acidity of the pyrrole NH groups thus increasing their availability for the hydrogen-bonding with the anions resulting in higher stability of the respective complex. Interestingly, modification of the macrocycle of the *meso*-position can be utilized to tune binding mode between the host and guest.$^{32-34}$ As a representative example, Anzenbacher and coworkers presented so called “walled calix[4]pyrroles” and investigated their anion-binding properties (*Figure 4.5*). By introducing 4-hydroxyphenyl or 4-methoxyphenyl group into *meso*-position afforded walled calix[4]pyrroles 11 and 12 with built-in deep cavities and rigid walls. Interestingly, the introduction of *meso*-aryl groups led to intrinsic anion selectivity.$^{35}$ As a result of $^1$H-NMR titrations in acetonitrile-$d_3$ compound 12 showed 10-times higher binding affinity for fluoride compared to compound 11. A similar example of the effect of *meso*-substitution on the anion-binding properties was recently also reported by Ballester and Matile who also investigated electronic effects on the binding affinity.$^{36}$

![Figure 4.5](image.png)

*Figure 4.5.* Examples of “fixed walls” calix[4]pyrrole derivatives reported by Anzenbacher and coworkers showing intrinsic anion selectivity attributed to the suitable *meso*-substitution.
Calix[4]pyrrole derivatives have demonstrated excellent anion sensing properties and due to the tunability of the anion-binding processes they are promising systems in many applications such as in extraction, transport of ions and drugs.37–42


As mentioned above, one possible approach for the development of new porphyrinogen-based anion sensors is the core modification approach.43–50 The area of expanded calix[4]pyrroles has been overlooked and stays mostly unexplored and only few report has been presented on the preparation and more thorough investigation of sensing properties of expanded calix[4]pyrroles.

It was described that calix[4]pyrroles are very well suited for a binding of small anions (fluoride, chloride, etc.). In order to expand the binding capability of calix[4]pyrroles to larger anions, the expanded calix[4]pyrroles possessing a larger central core were designed. The very first report on the preparation of expanded calixpyrroles was published in 1997 by Sessler, Gale and coworkers, who prepared calix[5]pyrrole-calix[5]arene pseudo dimer species using a template method.51 And this report led to the preparation of higher calix[n]pyrrole derivatives.32,44,52–54 Interestingly, many authors utilized spacer units in order to expand the central core. This way calix[4]pyrroles containing benzene (13) and carbazole (14) units were reported48,55 even though in the latter the two carbazole units used to expand the ring make the species to resemble a hexapyrrole rather than calix[4]pyrrole (Figure 4.6). Cafeo and coworkers reported on the preparation of calix[2]benzo[4]pyrroles 15 and 16 using macrocyclization reaction of dipyrrolyl m- or p-phenylene derivatives by the action of trifluoroacetic acid.47 Both 15 and 16 contained two dipyrromethane moieties and two m-phenylene or p-phenylene units interconnected with
additional sp³ carbon atoms and the authors explored their anion-binding properties by means of NMR titration experiments in dichloromethane.

Indeed, the expansion of the central core tends to lead to increased binding affinity for larger anions. However, a number of examples of expanded calix[4]pyrrole has been reported, the authors investigated the binding properties using NMR spectroscopy and only very limited number of examples of calix[4]pyrrole derivatives containing chromophores and/or fluorophores moieties attached to the outer rim of the central core has been reported so far.⁵⁶–⁵⁸

Toward this end, we decided to prepare meso-expanded calix[4]pyrrole derivatives decorated with fluorophore moieties at the periphery and investigate their anion-binding properties and use them for optical sensing of large polyanionic analytes.

**Figure 4.6.** Examples of meso-expanded calix[4]pyrrole derivatives.

The sensors $S_1$-$S_5$ (Figure 4.7) were synthesized by Dr. Kai-Chi Chang and Dr. Pavel Savchenkov, both postdoctoral research fellows in the Anzenbacher research group in 2014. The sensors $S_1$-$S_5$ utilized calix[4]pyrrole ($S_1$, $S_2$, $S_5$) and calix[2]benzo[4]pyrrole ($S_3$ and $S_4$) as a receptor moiety attached to a chromophore via conjugated system capable to establish an intramolecular partial charge transfer (IPCT) with the push-pull chromophore resulting in a strong fluorescence and color change in response to anions. The sensors were prepared using Knoevenagel condensation of 2-formyl-octamethyl calix[4]pyrrole ($S_1$, $S_2$, $S_5$) or

![Calix[4]pyrrole sensors S1-S5](image)

Figure 4.7. Calix[4]pyrrole sensors $S_1$-$S_5$ used in this study contain push-pull chromophores displaying strong intramolecular charge transfer.
2-formyldodecamethyl calix[2]benzo[4]pyrroles (S3 and S4) with tricyanoethylene moiety of respective fluorophores yielding calix[4]pyrrole anion sensors with push-pull chromophores displaying strong intramolecular charge transfer.\(^{56,57,59}\) 2,7-Dicyanofluorenyl-, 4-cyanophenyl- and 4-(4’-dimethylaminophenyl)phenyl benzene-based groups were used as the push-pull fluorophores as these were previously studied by Anzenbacher and coworkers to design fluorescent calix[4]pyrrole sensor for anions.

In this study, we decided to use the fluorescent sensors S1-S5 for optical sensing of small monoanions as well as large polyanionic species of environmental or physiological relevance. For this reason, we chose halogenides (fluoride, chloride, bromide and iodide), oxyphosphorus anions (phosphate and pyrophosphate) monocarboxylates (acetate, benzoate), dicarboxylates (oxalate, malonate, glutamate, phthalate, isophthalate and terephthalate) and NSAIDs (ibuprofen, naproxen and ketoprofen) and investigated binding affinities of sensors S1-S5 for these anions (Figure 4.8).

We succeeded to obtain single crystals of the sensors S2 and S3 using X-ray diffractometry as follows: \( S_2 \cdot 2(C_4H_8O_2) \): Crystals appeared as clusters of yellow prisms and were obtained by hexane vapor diffusion to dioxane solution. The data crystal was cut from a larger crystal and had approximate dimensions \( 0.17 \times 0.13 \times 0.046 \text{ mm} \). One of the dioxane molecules was disordered. The disorder was modeled by assigning the variable X to the site occupancy for one component of the disorder group and \((1 - X)\) to the site occupancy for the alternate component. A common isotropic displacement parameter was refined while refining X. The geometry of the two components was restrained to be equivalent throughout the refinement process. The data were acquired on Agilent Technologies SuperNova Dual Source diffractometer using a \( \mu \)-focus Cu-K\( \alpha \) radiation source \((\lambda = 1.5418 \text{ Å})\) with collimating mirror monochromators. A total of 577 frames of data were collected using \( \omega \)-scans with a scan range of \( 1^\circ \) and a counting time of 6 seconds per frame with a detector offset of \( \pm 40.8^\circ \) and 17 seconds per frame with a detector offset of \( \pm 108.3^\circ \). The data were collected at 100 K using an Oxford Cryostream low temperature device. Data collection, unit cell refinement and data reduction were performed using Agilent Technologies CrysAlisPro V 1.171.37.31. The structure was solved by direct methods using SuperFlip and refined by full-matrix least-squares on \( F^2 \) with anisotropic displacement parameters for the non-H atoms using SHELXL-2013. Structure analysis was aided by use of the programs PLATON98 and WinGX. The hydrogen atoms were calculated in ideal positions with isotropic displacement parameters set to \( 1.2 \times U_{eq} \) of the attached atom \((1.5 \times U_{eq} \text{ for methyl hydrogen atoms})\). The hydrogen atoms bound to nitrogen atoms were located in a \( \Delta F \) map and refined with isotropic displacement parameters. The function, \( \Sigma w(|F_o|^2 - |F_c|^2)^2 \), was minimized, where \( w = 1/[[\sigma(F_o)]^2 + (0.0498 \times P)^2 + (2.8781 \times P)] \) and \( P = (|F_o|^2 + 2|F_c|^2)/3 \). R\(_w\)(F\(^2\)) refined to 0.120, with R(F)
equal to 0.047 and a goodness of fit, S = 1.01. Definitions used for calculating $R(F)$, $R_w(F^2)$ and the goodness of fit, S, are given below. The data were checked for secondary extinction effects but no correction was necessary. Neutral atom scattering factors and values used to calculate the linear absorption coefficient are from the *International Tables for X-ray Crystallography* (1992). All figures were generated using Mercury from CCDC. 

**S3 · 2 (CH$_3$NO$_2$):** Crystals appeared as pale yellow prisms and were obtained by slow evaporation from dioxane. The data crystal was cut from a larger crystal and had approximate dimensions $0.31 \times 0.21 \times 0.13$ mm. The data were collected on a Rigaku AFC12 diffractometer with a Saturn 724+ CCD using a graphite monochromator with MoK$_\alpha$ radiation ($\lambda = 0.71073$ Å). A total of 1430 frames of data were collected using ω-scans with a scan range of $0.5^\circ$ and a counting time of 30 seconds per frame. The data were collected at 100 K using a Rigaku XStream low temperature device. Data reduction were performed using the Rigaku Americas Corporation’s Crystal Clear version 1.40. The structure was solved by direct methods using SIR20042 and refined by full-matrix least-squares on $F^2$ with anisotropic displacement parameters for the non-H atoms using SHELXL-2014/7. Structure analysis was aided by use of the programs PLATON984 and WinGX. The hydrogen atoms on carbon were calculated in ideal positions with isotropic displacement parameters set to 1.2xUeq of the attached atom (1.5xUeq for methyl hydrogen atoms). The hydrogen atoms bound to nitrogen were located in a ΔF map and refined with isotropic displacement parameters. The function, $\Sigma w(|F_o|^2 - |F_c|^2)^2$, was minimized, where $w = 1/[(\sigma(F_o))^2 + (0.0758 \times P)^2 + (2.5288 \times P)]$ and $P = (|F_o|^2 + 2|F_c|^2)/3$. $R_w(F^2)$ refined to 0.178, with R(F) equal to 0.0687 and a goodness of fit, S = 0.977. Definitions used for calculating R(F), $R_w(F^2)$ and the goodness of fit, S, are given below. The data were checked for secondary extinction effects but no correction was necessary. Neutral atom scattering
factors and values used to calculate the linear absorption coefficient are from the International Tables for X-ray Crystallography (1992).\textsuperscript{61}

The crystallographic parameters for the receptors S\textsubscript{2} and S\textsubscript{3} are summarized in \textit{Table 4.1}. The crystal structure of the sensor S\textsubscript{2} (\textit{Figure 4.9}) revealed that the \textit{meso}-octamethyl calix[4]pyrrole core adopts the 1,3-alternate conformation while the molecule of dioxane, located on the same side as the fluorophore moiety, is coordinated to two pyrrole units of the central core via two hydrogen bonds with the pyrrole NH groups (N···H···O\textsubscript{dioxane}). Surprisingly, the core of the expanded calix[2]benzo[4]pyrrole sensor S\textsubscript{3} adopted a truncated geometry which was not found to include any molecule of the nitromethane which was used as the crystallization solvent (\textit{Figure 4.10}). Unfortunately, we couldn’t succeed to grow crystals of X-ray quality of the sensors with anions that would reveal interesting insight into the binding interactions between the sensors and the analytes.
**Table 4.1. Crystal data and structure refinement for sensor S2 and S3.**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>S2 · diox₂</th>
<th>S3 · (CH₃NO₂)₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>Empirical formula</td>
<td>C₆H₅N₆O₄</td>
<td>C₆H₂N₆</td>
</tr>
<tr>
<td>Formula weight</td>
<td>756.96</td>
<td>1013.26</td>
</tr>
<tr>
<td>Temperature</td>
<td>100(2) K</td>
<td>100(2) K</td>
</tr>
<tr>
<td>Wavelength</td>
<td>1.54184 Å</td>
<td>0.71073 Å</td>
</tr>
<tr>
<td>Crystal system</td>
<td>monoclinic</td>
<td>triclinic</td>
</tr>
<tr>
<td>Space group</td>
<td>P 21/c</td>
<td>P -1</td>
</tr>
<tr>
<td>Unit cell dimensions</td>
<td></td>
<td></td>
</tr>
<tr>
<td>a = 9.6180(4) Å</td>
<td>a = 14.216(2) Å</td>
<td></td>
</tr>
<tr>
<td>b = 45.617(2) Å</td>
<td>b = 14.274(2) Å</td>
<td></td>
</tr>
<tr>
<td>c = 10.2073(4) Å</td>
<td>c = 16.395(2) Å</td>
<td></td>
</tr>
<tr>
<td>α = 90°</td>
<td>α = 65.077(9)°</td>
<td></td>
</tr>
<tr>
<td>β = 105.867(4)°</td>
<td>β = 71.467(11)°</td>
<td></td>
</tr>
<tr>
<td>γ = 90°</td>
<td>γ = 72.189(10)°</td>
<td></td>
</tr>
<tr>
<td>Volume</td>
<td>4307.8(3) Å³</td>
<td>2802.8(8) Å³</td>
</tr>
<tr>
<td>Z</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>Density (calculated)</td>
<td>1.167 Mg/m³</td>
<td>1.201 Mg/m³</td>
</tr>
<tr>
<td>Absorption coefficient</td>
<td>0.597 mm⁻¹</td>
<td>0.076 mm⁻¹</td>
</tr>
<tr>
<td>F(000)</td>
<td>1624</td>
<td>1080</td>
</tr>
<tr>
<td>Crystal size (mm)</td>
<td>0.170 × 0.130 × 0.046</td>
<td>0.310 × 0.210 × 0.130</td>
</tr>
<tr>
<td>Theta range for data collection</td>
<td>3.876 to 74.049°</td>
<td>1.404 to 27.497°</td>
</tr>
<tr>
<td>Index ranges</td>
<td></td>
<td></td>
</tr>
<tr>
<td>-11 ≤ h ≤ 11</td>
<td>-18 ≤ h ≤ 18</td>
<td></td>
</tr>
<tr>
<td>-29 ≤ k ≤ 55</td>
<td>-18 ≤ k ≤ 18</td>
<td></td>
</tr>
<tr>
<td>-8 ≤ l ≤ 12</td>
<td>-21 ≤ l ≤ 21</td>
<td></td>
</tr>
<tr>
<td>Reflections collected</td>
<td>15033</td>
<td>44697</td>
</tr>
<tr>
<td>Independent reflections</td>
<td>8441 [R(int) = 0.0206]</td>
<td>12591 [R(int) = 0.0584]</td>
</tr>
<tr>
<td>Completeness to theta 67.684°</td>
<td>99.2%</td>
<td>98.9%</td>
</tr>
<tr>
<td>Absorption correction</td>
<td>Semi-empirical from equivalents</td>
<td>Semi-empirical from equivalents</td>
</tr>
<tr>
<td>Max. / min. transmission</td>
<td>1.00 / 0.955</td>
<td>1.00 / 0.832</td>
</tr>
<tr>
<td>Refinement method</td>
<td>Full-matrix least-squares on F²</td>
<td>Full-matrix least-squares on F²</td>
</tr>
<tr>
<td>Data / restraints / parameters</td>
<td>8441 / 242 / 585</td>
<td>12591 / 0 / 719</td>
</tr>
<tr>
<td>Goodness-of-fit on F²</td>
<td>0.990</td>
<td>0.977</td>
</tr>
<tr>
<td>Final R indices [I &gt; 2sigma(I)]</td>
<td>R1 = 0.0470, wR2 = 0.1148</td>
<td>R1 = 0.0687, wR2 = 0.1640</td>
</tr>
<tr>
<td>R indices (all data)</td>
<td>R1 = 0.0549, wR2 = 0.1196</td>
<td>R1 = 0.0852, wR2 = 0.1775</td>
</tr>
<tr>
<td>Extinction coefficient</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>Largest diff. peak and hole</td>
<td>0.314 and -0.215 e.Å⁻³</td>
<td>0.765 and -0.579 e.Å⁻³</td>
</tr>
</tbody>
</table>
Figure 4.9. View of the molecular structures of sensor S2: A: Thermal ellipsoid model of the complex of S2 with one molecule of dioxane (solvent). B, C: Detail of crystal packing. (Hydrogen atoms omitted for clarity. Displacement ellipsoids are scaled to the 50% probability level.)
Figure 4.10. View of the molecular structures of sensor S3: A, B: Thermal ellipsoid model of sensor S3. C, D: Detail of crystal packing. (Hydrogen atoms omitted for clarity. Displacement ellipsoids are scaled to the 50% probability level.)
4.7 Solution Behavior: NMR Titrations

To understand the nature of the interactions between the receptors and the anions and to obtain qualitative estimate of stoichiometry of such complexation phenomena, we performed $^1$H-NMR titration of the expanded calix[2]benzo[4]pyrrole sensor $S4$ in solution of acetonitrile-$d_3$ with tetra-$n$-butylammonium chloride, oxalate, malonate, glutamate and phthalate at laboratory temperature. Both in the case of chloride and oxalate, we observed a downfield shift of pyrrolic NH proton signals (1.1 and 3.5 ppm units for chloride and oxalate, respectively) indicating the hydrogen bonding with the anions took place (Figure 4.11 and Figure 4.12). The titrations were carried out as follows: 0.4 mL of the sensor solution ([S4] = 10 mM) in CD$_3$CN was placed in the NMR tube and the NMR spectrum was recorded in the absence of anion at room temperature. Subsequently, the spectra were collected after the solution was carefully mixed after every addition.

![Figure 4.11](image)

**Figure 4.11.** $^1$H-NMR titration (500 MHz) of $S4$ (10 mM) upon the addition of an incremental amount of tetra-$n$-butylammonium chloride in CD$_3$CN-$d_3$: (A) A section of $^1$H-NMR spectrum of sensor $S4$ upon the addition of up to 5 mol. equiv. of chloride. (B) $^1$H-NMR titration isotherm of the pyrrole NH signal corresponding to the chloride-induced chemical shift.
of a corresponding aliquot of 1 μL (2.5 mM addition) of the anion solution in CD$_3$CN (1 M) up to 5 molar equivalents of anion was added in respect to the sensor.

We did not observe a deprotonation of the receptor taking place upon the titration of sensor S4 (10 mM) with chloride and oxalate at low anion concentration of the anions in CD$_3$CN, and the stoichiometry of the complexes formed suggested by the $^1$H-NMR titration isotherm were 1 : 1 (S4 vs. anion). Unfortunately, the proton signals corresponding to the pyrrole NH resonances disappeared, however, upon the titration of sensor S4 with malonate, glutamate and phthalate, suggesting the deprotonation of the sensor NH protons took place within the concentration region investigated.
4.8 Complex Formation: Mass Spectrometry

We also investigated the complex formation for the expanded calix[2]benzo[4]pyrrole sensors S3 and S4 with fluoride, chloride and pyrophosphate in acetonitrile using ESI-MS. The experiment confirmed the anion-binding ability of sensors S3 and S4 upon ESI conditions. *Figure 4.13* and *Figure 4.14* show ESI-MS spectra (in negative mode) of the complexes of sensors S3 and S4 with chloride, fluoride and dihydrogen pyrophosphate obtained in acetonitrile. The spectra for both sensors suggested the binding stoichiometry of the complex formed to be 1 : 1. The samples

![Figure 4.13](image1.png)

**Figure 4.13.** ESI-MS spectrum of the complex of S3 with chloride and fluoride (A) and pyrophosphate (B) suggested 1 : 1 binding stoichiometry. The spectra were measured in negative mode and obtained in acetonitrile.

![Figure 4.14](image2.png)

**Figure 4.14.** ESI-MS spectrum of the complex of S4 with chloride and fluoride (A) and pyrophosphate (B) suggested 1 : 1 binding stoichiometry. The spectra were measured in negative mode and obtained in acetonitrile.
for ESI-MS experiment were prepared by mixing 10 μL of the solution of S1 or S2 (100 μM) in acetonitrile as mixed with an excess amount of a corresponding anion. The ESI spectra were recorded in negative detection mode and suggested 1 : 1 binding stoichiometry.

4.9 Complex Stoichiometry Determination: Job’s Plot

We utilized Job’s method (method of continuous variation) in order to exactly determine the binding stoichiometries of the complex formation in the solution of acetonitrile using absorbance data. We investigated complex formation stoichiometries for receptors S1-S5 (20 μM) with tetrabutylammonium fluoride. Interestingly, the Job’s plots obtained confirmed formation of

![Graphs A, B, C, D showing Job's plots for complexes S1+ Fluoride, S2+ Fluoride, S3+ Fluoride, S4+ Fluoride.](image-url)
complexes with 1 : 1 binding stoichiometry for complexes of fluoride with the sensors S1-S5 (Figure 4.15).


Finally, we decided to probe the sensors capability to bind the anions and to quantify it using both the UV/vis and fluorescence spectroscopy (fluorescence titrations). The UV/vis and fluorescence titration experiments as well as the photophysical characterization of the sensors were carried out jointly by me and Ms. Mariia Pushina, a graduate student at the Anzenbacher research group in the summer of 2015.

The photophysical properties of the sensors S1-S5 (20 μM) were measured in acetonitrile at laboratory temperature and are summarized in Table 4.2. The absorption maxima for sensors S1-S5 range between 380 and 430 nm and emission maxima range between 490 and 570 nm. Fluorescence lifetime measurements showed that the lifetimes obtained were between 12 and 15 ns. For sensors S1 and S5 we observed a double-exponential fluorescence lifetime while the second lifetime was 0.3 ns and 6.3 ns for S1 and S5, respectively. Fluorescence quantum yields obtained
were generally very low (< 1%) which is in agreement with similar compounds reported previously. Spectral properties of the sensors S1 through S5 are summarized in Figure 4.16 (absorption spectra) and Figure 4.17 (fluorescence emission spectra). The UV/vis and fluorescence responses to various anion were used to estimate the association constants.

**Table 4.2.** Photophysical properties of sensors S1-S5 obtained in acetonitrile at room temperature. The data were obtained in acetonitrile at laboratory temperature.

<table>
<thead>
<tr>
<th>Sensors</th>
<th>S1</th>
<th>S2</th>
<th>S3</th>
<th>S4</th>
<th>S5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Absorption maximum (nm)</td>
<td>423</td>
<td>382</td>
<td>431</td>
<td>389</td>
<td>380</td>
</tr>
<tr>
<td>Emission maximum (nm)</td>
<td>532</td>
<td>570</td>
<td>525</td>
<td>490</td>
<td>561</td>
</tr>
<tr>
<td>Fluorescence lifetime (ns)</td>
<td>15.3 (53%)</td>
<td>12.1</td>
<td>14.4</td>
<td>12.3</td>
<td>14.4 (20%)</td>
</tr>
<tr>
<td></td>
<td>0.3 (47%)</td>
<td></td>
<td></td>
<td></td>
<td>6.3 (80%)</td>
</tr>
<tr>
<td>Absolute fl. quantum yield (%)</td>
<td>0.48</td>
<td>0.05</td>
<td>0.37</td>
<td>0.36</td>
<td>1.07</td>
</tr>
</tbody>
</table>

UV/vis spectra were recorded on Hitachi U-3010 double-beam spectrophotometer and the emission spectra were acquired on Edinburgh single-photon counting spectrofluorimeter FLSP 920 (Edinburgh Instruments Ltd., Livingston, UK) at room temperature using quartz cuvette with a path length of 1 cm and with a right angle detection. The concentration of the samples was adjusted so that the absorbance used for fluorescence measurements was equal to or below 0.1. Absolute quantum yields were measured with Hamamatsu Quantaurus-QY C11347 absolute quantum yield spectrometer (150 W Xe lamp).
Figure 4.16. Absorbance spectra of sensors S1-S5 (20 μM) obtained in acetonitrile.
Figure 4.17. Fluorescence emission spectra of sensors S1-S5 (20 μM) obtained in acetonitrile. (The sensors were excited at their absorption maxima.)

Next, we decided to accomplish UV/vis titrations in order to estimate binding affinities for multiple anions. A set of titrations was carried out where the sensors S1-S5 were titrated with halogenides (fluoride, chloride, bromide and iodide), oxyphosphorus anions (phosphate and pyrophosphate) monocarboxylates (acetate, benzoate), dicarboxylates (oxalate, malonate, glutamate, phthalate, isophthalate and terephthalate) and NSAIDs (ibuprofen, naproxen and ketoprofen) in acetonitrile. The binding constants obtained along with the errors are summarized in Table 4.3 and Table 4.4.
Figure 4.18. UV/vis titration spectra and UV/vis titration isotherm (inset) of S1 (20 µM) in acetonitrile upon the addition of incremental amounts of various anions.
Figure 4.19. UV/vis titration spectra and UV/vis titration isotherm (inset) of S2 (20 μM) in acetonitrile upon the addition of incremental amounts of various anions.
Figure 4.20. UV/vis titration spectra and UV/vis titration isotherm (inset) of S3 (20 μM) in acetonitrile upon the addition of incremental amounts of various anions.
Figure 4.21. UV/vis titration spectra and UV/vis titration isotherm (inset) of S4 (20 μM) in acetonitrile upon the addition of incremental amounts of various anions.
Figure 4.22. UV/vis titration spectra and UV/vis titration isotherm (inset) of S5 (20 μM) in acetonitrile upon the addition of incremental amounts of various anions.

Table 4.3. Affinity constants of sensors S1-S5 (20 μM) in acetonitrile for various anions (tetrabutylammonium salts) obtained from UV/vis titrations.

<table>
<thead>
<tr>
<th>Analyte</th>
<th>S1</th>
<th>S2</th>
<th>S3</th>
<th>S4</th>
<th>S5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fluoride</td>
<td>ND</td>
<td>6.90 × 10⁵</td>
<td>ND</td>
<td>ND</td>
<td>4.20 × 10⁵</td>
</tr>
<tr>
<td>Chloride</td>
<td>4.40 × 10⁵</td>
<td>1.90 × 10⁵</td>
<td>5.60 × 10⁴</td>
<td>1.00 × 10⁴</td>
<td>1.80 × 10⁵</td>
</tr>
<tr>
<td>Iodine</td>
<td>NR</td>
<td>NR</td>
<td>NR</td>
<td>NR</td>
<td>NR</td>
</tr>
<tr>
<td>Bromide</td>
<td>5.20 × 10⁴</td>
<td>2.70 × 10³</td>
<td>1.80 × 10³</td>
<td>NR</td>
<td>2.60 × 10³</td>
</tr>
<tr>
<td>Acetate</td>
<td>ND</td>
<td>7.50 × 10⁴</td>
<td>1.40 × 10⁴</td>
<td>1.70 × 10⁴</td>
<td>5.30 × 10⁵</td>
</tr>
<tr>
<td>Benzoate</td>
<td>ND</td>
<td>3.90 × 10⁴</td>
<td>1.70 × 10³</td>
<td>1.80 × 10³</td>
<td>4.10 × 10⁵</td>
</tr>
<tr>
<td>Phosphate</td>
<td>1.35 × 10⁵</td>
<td>5.20 × 10⁴</td>
<td>8.00 × 10⁴</td>
<td>1.90 × 10³</td>
<td>5.10 × 10⁴</td>
</tr>
<tr>
<td>Pyrophosphate</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>2.90 × 10⁵</td>
</tr>
<tr>
<td>Oxalate</td>
<td>ND</td>
<td>ND</td>
<td>2.40 × 10³</td>
<td>6.40 × 10³</td>
<td>1.17 × 10⁶</td>
</tr>
<tr>
<td>Malonate</td>
<td>9.02 × 10⁴</td>
<td>6.60 × 10⁴</td>
<td>4.30 × 10³</td>
<td>3.00 × 10³</td>
<td>7.00 × 10⁴</td>
</tr>
<tr>
<td>Glutamate</td>
<td>ND</td>
<td>1.60 × 10⁵</td>
<td>4.70 × 10³</td>
<td>2.70 × 10³</td>
<td>ND</td>
</tr>
<tr>
<td>Phthalate</td>
<td>ND</td>
<td>ND</td>
<td>4.70 × 10³</td>
<td>ND</td>
<td>7.50 × 10⁴</td>
</tr>
<tr>
<td>Isophthalate</td>
<td>ND</td>
<td>ND</td>
<td>NR</td>
<td>NR</td>
<td>ND</td>
</tr>
<tr>
<td>Ketoprofen</td>
<td>5.20 × 10⁴</td>
<td>1.90 × 10⁴</td>
<td>2.80 × 10³</td>
<td>NR</td>
<td>ND</td>
</tr>
<tr>
<td>Naproxen</td>
<td>1.20 × 10⁴</td>
<td>5.20 × 10³</td>
<td>NR</td>
<td>NR</td>
<td>ND</td>
</tr>
<tr>
<td>Ibuprofen</td>
<td>4.20 × 10⁴</td>
<td>1.30 × 10⁴</td>
<td>NR</td>
<td>NR</td>
<td>1.80 × 10⁴</td>
</tr>
</tbody>
</table>

ND The constant couldn’t be determined due to complex equilibria; NR The constant couldn’t be determined due to small change in absorbance upon the addition of the anion (no response).
Finally, we decided to carry out fluorescence titrations in order to quantify the binding affinities of the fluorescent sensors $S_1$-$S_5$ for multiple anions. A set of titrations was carried out where the sensors $S_1$-$S_5$ were titrated with halogenides (fluoride, chloride, bromide and iodide), oxyphosphorus anions (phosphate and pyrophosphate) monocarboxylates (acetate, benzoate), dicarboxylates (oxalate, malonate, glutamate, phthalate, isophthalate and terephthalate) and NSAIDs (ibuprofen, naproxen and ketoprofen) in acetonitrile and at longest-wavelength isosbestic point. The titrations are summarized in the following section.

### Table 4.4. Affinity constant errors for the affinity constants of sensors $S_1$-$S_5$ (20 μM) in acetonitrile for various anions (tetrabutylammonium salts).

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Binding constant error, M$^{-1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$S_1$</td>
</tr>
<tr>
<td>Fluoride</td>
<td>ND</td>
</tr>
<tr>
<td>Chloride</td>
<td>$6.00 \times 10^4$</td>
</tr>
<tr>
<td>Iodine</td>
<td>NR</td>
</tr>
<tr>
<td>Bromide</td>
<td>$4.00 \times 10^3$</td>
</tr>
<tr>
<td>Acetate</td>
<td>ND</td>
</tr>
<tr>
<td>Benzoate</td>
<td>ND</td>
</tr>
<tr>
<td>Phosphate</td>
<td>$8.00 \times 10^3$</td>
</tr>
<tr>
<td>Pyrophosphate</td>
<td>ND</td>
</tr>
<tr>
<td>Oxalate</td>
<td>ND</td>
</tr>
<tr>
<td>Malonate</td>
<td>$8.20 \times 10^3$</td>
</tr>
<tr>
<td>Glutamate</td>
<td>ND</td>
</tr>
<tr>
<td>Phthalate</td>
<td>ND</td>
</tr>
<tr>
<td>Isophthalate</td>
<td>ND</td>
</tr>
<tr>
<td>Ketoprofen</td>
<td>$4.00 \times 10^3$</td>
</tr>
<tr>
<td>Naproxen</td>
<td>$8.00 \times 10^2$</td>
</tr>
<tr>
<td>Ibuprofen</td>
<td>$4.00 \times 10^3$</td>
</tr>
</tbody>
</table>

$ND$ The constant couldn’t be determined due to complex equilibria; $NR$ The constant couldn’t be determined due to small change in absorbance upon the addition of the anion (no response).


Finally, we decided to carry out fluorescence titrations in order to quantify the binding affinities of the fluorescent sensors $S_1$-$S_5$ for multiple anions. A set of titrations was carried out where the sensors $S_1$-$S_5$ were titrated with halogenides (fluoride, chloride, bromide and iodide), oxyphosphorus anions (phosphate and pyrophosphate) monocarboxylates (acetate, benzoate), dicarboxylates (oxalate, malonate, glutamate, phthalate, isophthalate and terephthalate) and NSAIDs (ibuprofen, naproxen and ketoprofen) in acetonitrile and at longest-wavelength isosbestic point. The titrations are summarized in the following section.
$K_a = (1.3 \pm 0.1) \times 10^{3}$ M$^{-1}$  
$[S2] = 20$ μM in MeCN  
$\lambda_{ex} = 408$ nm

$K_a = (1.9 \pm 0.17) \times 10^{3}$ M$^{-1}$  
$[S2] = 20$ μM in MeCN  
$\lambda_{ex} = 405$ nm

$K_a = (1.1 \pm 0.1) \times 10^{3}$ M$^{-1}$  
$[S2] = 20$ μM in MeCN  
$\lambda_{ex} = 404$ nm

$\left(\frac{I_o - I}{I_o}\right)$ vs. [Iodide], μM

$\left(\frac{I_o - I}{I_o}\right)$ vs. [Phosphate], μM

$\left(\frac{I_o - I}{I_o}\right)$ vs. [Hydrogenpyrophosphate], μM
\[
\begin{align*}
K_a &= (1.6 \pm 0.2) \times 10^5 \text{ M}^{-1} \\
[S2] &= 20 \mu\text{M in MeCN} \\
\lambda_{ex} &= 404 \text{ nm}
\end{align*}
\]

\[
\begin{align*}
K_a &= (2.5 \pm 0.6) \times 10^5 \text{ M}^{-1} \\
[S2] &= 20 \mu\text{M in MeCN} \\
\lambda_{ex} &= 408 \text{ nm}
\end{align*}
\]

\[
\begin{align*}
K_a &= (7.5 \pm 0.5) \times 10^5 \text{ M}^{-1} \\
[S2] &= 20 \mu\text{M in MeCN} \\
\lambda_{ex} &= 406 \text{ nm}
\end{align*}
\]
Figure 4.23. Fluorescence titration spectra (left) and fluorescence titration isotherms (right) of sensor S2 (20 μM) in acetonitrile upon the addition of incremental amounts of various anions.
$K_s = (8.0 \pm 1.1) \times 10^5 \text{M}^{-1}$
[S3] = 20 \, \mu\text{M in MeCN}
$\lambda_w = 363 \text{ nm}$

$K_s = (8.0 \pm 1.6) \times 10^7 \text{M}^{-1}$
[S3] = 20 \, \mu\text{M in MeCN}
$\lambda_w = 369 \text{ nm}$

$K_s = (6.8 \pm 0.7) \times 10^7 \text{M}^{-1}$
[S3] = 20 \, \mu\text{M in MeCN}
$\lambda_w = 367 \text{ nm}$
Figure 4.24. Fluorescence titration spectra (left) and fluorescence titration isotherms (right) of sensor S3 (20 µM) in acetonitrile upon the addition of incremental amounts of various anions.
\( \lambda_{ex} = 396 \text{ nm} \)

\( \lambda_{ex} = 400 \text{ nm} \)

\( \lambda_{ex} = 377 \text{ nm} \)
$K' = (3.8 \pm 0.1) \times 10^4 \text{ M}^{-1}$

[S4] = 20 \text{ \mu M in MeCN}

$\lambda_\text{ex} = 360 \text{ nm}$

$K' = (2.9 \pm 0.2) \times 10^5 \text{ M}^{-1}$

[S4] = 20 \text{ \mu M in MeCN}

$\lambda_\text{ex} = 405 \text{ nm}$

$K' = (6.9 \pm 0.5) \times 10^5 \text{ M}^{-1}$

[S4] = 20 \text{ \mu M in MeCN}

$\lambda_\text{ex} = 390 \text{ nm}$
Figure 4.25. Fluorescence titration spectra (left) and fluorescence titration isotherms (right) of sensor S4 (20 μM) in acetonitrile upon the addition of incremental amounts of various anions.
$K_a = (7.5 \pm 0.6) \times 10^6 \text{ M}^{-1}$

$[S5] = 20 \text{ mM in MeCN}$

$\lambda_{ex} = 395 \text{ nm}$

$K_a = (6.0 \pm 0.2) \times 10^6 \text{ M}^{-1}$

$[S5] = 20 \text{ mM in MeCN}$

$\lambda_{ex} = 403 \text{ nm}$

$K_a = (2.8 \pm 0.1) \times 10^3 \text{ M}^{-1}$

$[S5] = 20 \text{ mM in MeCN}$

$\lambda_{ex} = 406 \text{ nm}$

$\frac{(I_0 - I)}{I_0}$ vs [Fluoride], $\mu\text{M}$

$\frac{(I_0 - I)}{I_0}$ vs [Chloride], $\mu\text{M}$

$\frac{(I_0 - I)}{I_0}$ vs [Bromide], $\mu\text{M}$
\[ K_a = (7.3 \pm 0.5) \times 10^4 \text{ M}^{-1} \]

\[ [S5] = 20 \mu\text{M in MeCN} \]

\[ \lambda_{ex} = 404 \text{ nm} \]

\[ (I_0 - I) / (I_0) \]

\[ \text{[Iodide], } \mu\text{M} \]

\[ K_a = (7.1 \pm 0.5) \times 10^4 \text{ M}^{-1} \]

\[ [S5] = 20 \mu\text{M in MeCN} \]

\[ \lambda_{ex} = 399 \text{ nm} \]

\[ (I_0 - I) / (I_0) \]

\[ \text{[PPI], } \mu\text{M} \]

\[ K_a = (1.04 \pm 0.06) \times 10^5 \text{ M}^{-1} \]

\[ [S5] = 20 \mu\text{M in MeCN} \]

\[ \lambda_{ex} = 400 \text{ nm} \]

\[ (I_0 - I) / (I_0) \]

\[ \text{[Acetate], } \mu\text{M} \]
$K_a = (1.4 \pm 0.08) \times 10^5 \text{ M}^{-1}$

[S5] = 20 µM in MeCN

$\lambda_{ex} = 399 \text{ nm}$

$\frac{I_0 - I}{I_0}$

[Phthalate], µM

$K_a = (2.4 \pm 0.2) \times 10^5 \text{ M}^{-1}$

[S5] = 20 µM in MeCN

$\lambda_{ex} = 399 \text{ nm}$

$\frac{I_0 - I}{I_0}$

[Glutamate], µM

$K_a = (6.8 \pm 0.7) \times 10^5 \text{ M}^{-1}$

[S5] = 20 µM in MeCN

$\lambda_{ex} = 412 \text{ nm}$

$\frac{I_0 - I}{I_0}$

[Naproxen], µM
Figure 4.26. Fluorescence titration spectra (left) and fluorescence titration isotherms (right) of sensor S5 (20 μM) in acetonitrile upon the addition of incremental amounts of various anions.
Table 4.5. Affinity constants of sensors S1-S5 (20 μM) in acetonitrile for various anions (tetrabutylammonium salts) obtained from fluorescence titrations.

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Binding constant, M⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>S1</td>
</tr>
<tr>
<td>Fluoride</td>
<td>ND</td>
</tr>
<tr>
<td>Chloride</td>
<td>ND</td>
</tr>
<tr>
<td>Iodine</td>
<td>ND</td>
</tr>
<tr>
<td>Bromide</td>
<td>ND</td>
</tr>
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<td>Acetate</td>
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<td>Benzoate</td>
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<tr>
<td>Phosphate</td>
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<td>Isophthalate</td>
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<tr>
<td>Ketoprofen</td>
<td>ND</td>
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<tr>
<td>Naproxen</td>
<td>ND</td>
</tr>
<tr>
<td>Ibuprofen</td>
<td>ND</td>
</tr>
</tbody>
</table>

ND The constant couldn’t be determined due to complex equilibria; NR The constant couldn’t be determined due to small change in absorbance upon the addition of the anion (no response).

Table 4.6. Affinity constant errors for the affinity constants of sensors S1-S5 (20 μM) in acetonitrile for various anions (tetrabutylammonium salts).

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Binding constant error, M⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>S1</td>
</tr>
<tr>
<td>Fluoride</td>
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</tr>
<tr>
<td>Chloride</td>
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<tr>
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<td>Bromide</td>
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<td>Acetate</td>
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<td>Benzoate</td>
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<td>Isophthalate</td>
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<tr>
<td>Ketoprofen</td>
<td>ND</td>
</tr>
<tr>
<td>Naproxen</td>
<td>ND</td>
</tr>
<tr>
<td>Ibuprofen</td>
<td>ND</td>
</tr>
</tbody>
</table>

ND The constant couldn’t be determined due to complex equilibria; NR The constant couldn’t be determined due to small change in absorbance upon the addition of the anion (no response).
In general, UV/vis and fluorescence titrations revealed that sensors S2–S5 exhibit a strong fluorescence response towards anions. The binding affinities obtained ranged from $1.30 \times 10^3 \text{ M}^{-1}$ (for S2 with iodide) to $4.10 \times 10^6 \text{ M}^{-1}$ (for S3 with fluoride). Based on relative magnitude of fluorescence amplification and binding constants obtained one can arrive to interesting trends in selectivity. The fluorescence titrations with sensor S1 showed complicated equilibria (fluorescence signal fluctuations) due to which the constants could not be determined. We succeeded to obtain the binding constants for sensor S1 with several anions using UV/vis titration data though. Namely, we could calculate the binding constants for the sensor with chloride, bromide, phosphate, malonate, ketoprofen, naproxen and ibuprofen. Based on both UV/vis and fluorescence titration data, sensor S2 was found to be the most cross-reactive sensor used in this study and the binding affinities obtained for all anion investigated were in the range $10^4$–$10^5 \text{ M}^{-1}$. Sensors S3 showed interesting behavior among halogenides. It provided no response to iodide but showed an affinity to fluoride, chloride and bromide with a difference in the affinity of three orders of magnitude. Interestingly, among NSAIDs, the sensor showed selectivity for ketoprofen and did not show any affinity for naproxen and ibuprofen. Also, sensor S3 showed very high sensitivity for fluoride over other anions while sensor S4 showed higher selectivity for fluoride and pyrophosphate. Complicated equilibria prevented us to determine the bind affinities for sensor S3 with pyrophosphate, oxalate, malonate, glutamate, phthalate, isophthalate and naproxene as well as for sensor S4 with malonate and isophthalate and sensors S5 with phosphate and isophthalate the using fluorescent titrations data. Interesting trends can however observed from the data obtained. Sensor S2 and S4 were found to provide more of cross-reactive response pattern across all the anions investigated. We also found that sensor S5 has somewhat higher selectivity for ketoprofen and glutamate. Interestingly, unlike S2, S5, sensors S3 and S4 exhibited no response to ibuprofen
while they showed a response to other NSAID anions. We believed that the fluorescence response pattern provided by sensors $S_2$-$S_5$ could be further utilized in the design of a fluorescence-based assay. Such a fluorescence-based assay is further discussed in the following part.

The fluorescence titration experiments were performed as follows. 2.5 mL of the sensor solution was placed in a 1 cm fluorescence or UV/vis cell and the spectrum was recorded in the absence of an anion at room temperature. Then the absorption/emission spectra were repeatedly acquired after addition of aliquots of anion solution.

4.13 Polymer Microchip-Based Qualitative Assay

To demonstrate the utility of the cross-reactive sensors $S_1$-$S_5$ and their capability to recognize selected environmentally and physiologically relevant anions, we designed a qualitative assay. We decided to employ a polymer-based microchip approach. This method employing a hydrogel-incorporated fluorescence sensors cast into glass microchip array was developed in the Anzenbacher research group and successfully utilized for the analysis of various species.$^{63-68,69}$ Since the sensors are not soluble in water (but are soluble in organic solvents), we doped a polyurethane-based hydrogel with sensors $S_1$-$S_5$ and cast this polymer solution to an array microchip array. The polyurethane swells in water and acts as a stripping agent to de-solvate anion molecules which are then recognized by the receptor embedded in the polymer matrix. This elegant methodology allows an analysis of the anions by non-soluble sensors in hydrogel environment closely resembling water or buffer solutions. The sensor responses could be then recorded as
images using an UV-scanner capable to read the fluorescence images and the responses were evaluated using standard pattern recognition protocol (LDA). The qualitative analysis was accomplished using 5 mM solution of sensors S1-S5 in THF solution (4% m/m) of polyurethane SP-60D-40 (Lubrizol) and 5 mM solution of the anions in water or water/THF mixture. The LDA experiment provided an excellent separation of the data clusters suggesting that the five sensors are able to distinguish between six aqueous anions and a control (total of seven analytes) and showed 100% correct classification (Figure 4.28).

**Figure 4.27.** Composite image of the qualitative polymer microchip array of sensors S1-S5 (5 mM) embedded in a polyurethane matrix in water/THF and their response to anions (5 mM) under a broad-band UV excitation. Signal deconvolution and intensity integration reveals variation in response intensities in green and red color channels. (Blue color channel not shown.)
Figure 4.28. Graphical output of qualitative LDA for sensors S1-S5 (5 μM) in SP-60D-40 polyurethane matrix in water/THF with various anion (total volume 7 μL per well). The array was achieved using 10 excitation/emission channels for each sensor, 8 repetitions and provided 100% correct classification.
The array experiments were performed as follows. Solutions of sensors S1-S5 (5 mM) in THF solution of polyurethane SP-60D-40 (4% m/m) (Lubrizol) were cast (200 nL per well) into clean and dust-free glass microchip slide using 10 μL microsyringe (Hamilton, Reno, NV) and the solvents let dry for 15 min. Subsequently, water (200 nL) was pipetted into each single well in the same manner and let dry at room temperature for 15 min. After that, the solutions of the analytes in water (200 nL, 5 mM) were cast onto the microchip in such a way that intensity data each analyte could be collected in 10 repetitions (10 well per analyte) for one sensor. After 15 min, fluorescence intensities were collected using Kodak 4000MM Pro image station and the resulting emission data were subjected to the Student’s t-test to exclude two data-points (out of 10 repetitions). The

![Figure 4.29. Canonical scores plot for LDA of qualitative assay for sensors S1-S5 (and control).](image)

The array experiments were performed as follows. Solutions of sensors S1-S5 (5 mM) in THF solution of polyurethane SP-60D-40 (4% m/m) (Lubrizol) were cast (200 nL per well) into clean and dust-free glass microchip slide using 10 μL microsyringe (Hamilton, Reno, NV) and the solvents let dry for 15 min. Subsequently, water (200 nL) was pipetted into each single well in the same manner and let dry at room temperature for 15 min. After that, the solutions of the analytes in water (200 nL, 5 mM) were cast onto the microchip in such a way that intensity data each analyte could be collected in 10 repetitions (10 well per analyte) for one sensor. After 15 min, fluorescence intensities were collected using Kodak 4000MM Pro image station and the resulting emission data were subjected to the Student’s t-test to exclude two data-points (out of 10 repetitions).
coefficient of variability among the data within the class of 8 repetitions was lower than 3%. The settings of emission and excitation channels for the fluorescence intensities data read-outs are summarized in Figure 4.30. The obtained data for qualitative analysis were then analyzed using LDA without any further pretreatment. The microarray experiment including the data processing was carried out by me and Ms. Mariia Pushina, a graduate student at the Anzenbacher research group in the spring of 2016.

Table 4.7. Jacknife classification matrix of the qualitative assay for sensors S1-S5.

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<th>Asp</th>
<th>Br</th>
<th>BzO</th>
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Iodide

Relative Intensity, a.u.

Phosphate

Relative Intensity, a.u.

Hydrogenpyrophosphate

Relative Intensity, a.u.

Acetate

Relative Intensity, a.u.
Figure 4.30. Relative fluorescence intensity responses of sensors S1-S5 in hydrogel-based qualitative assay for various anions.

The polyurethane-based qualitative microchip assay using sensors S1-S5 provided 100% correct classification and showed capability to discriminate environmentally and physiologically relevant analytes used in this study.
4.14 Results Summary

In summary, we have presented fluorescent calix[4]pyrrole and expanded calix[2]benzo[4]pyrrole-based sensors and fluorescence-based microchip array capable to discriminate environmentally and physiologically relevant anions with 100% correct classification. We have described a complex formation ability of the fluorescent calix[4]pyrrole and expanded calix[2]benzo[4]pyrrole-based sensors using NMR spectroscopy and mass spectroscopy. Stoichiometries of the complexes were further investigated by Job’s method using UV/vis. X-ray diffraction analysis confirmed structures of the fluorescent sensors. The presence of anions was shown to result in a change of fluorescence intensity. The fluorescence titrations of sensors S1-S5 with a variety of anions including halogenides, aliphatic dicarboxylates, benzene carboxylates and non-steroidal anti-inflammatory drugs ibuprofen, ketoprofen showed a dramatic change of fluorescence signal. Finally, we showed that the sensors S1-S5 can be embedded in polyurethane hydrogel matrix in a cross-reactive microarray experiment and differentiate among the anions of interest.

4.15 References


